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COLD/HEAT SHOCK PRE-TREATMENTS FOR GYNOGENIC HAPLOID EMBRYO INDUCTION IN *Amaryllıdaceae* AND *Cucurbıtaceae*: A REVIEW

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Abstract

Pre-treatments significantly affect frequency of gynogenic embryogenesis. In gynogenesis, a change from gametophytic phase to the sporophytic phase is provided by stress treatments. Heat and/or cold shocks are given alone or in combination with each other to induce stress conditions. Also, pre-treatments can be applied at different types of explant, such as flower buds, ovules/ovaries, or inflorescence. With regard to different explants, the type, level, and duration of pre-treatment are different, and the regeneration efficiencies vary as well. In this review, it was discussed the effects of pre-treatments, cold or heat shock, in haploid induction via gynogenesis in different vegetables species.

Key words*: Amaryllidaceae, Cucurbitaceae, Gynogenesis, pre-treatments.*

INTRODUCTION

Plants with gametophytic chromosome number in their sporophyte whether diploid or polyploid chromosome number are named haploids (Chen et al., 2011). *In vitro* haploid production is very important in plant breeding because of the shortening of the time required for the production of homozygous lines compared with conventional breeding methods (Shalaby, 2007; Chen et al., 2011). Haploid plants originating from gametes carry the genetic information of only one set of chromosomes, so they can be regarded as genetically homozygotic. Male and female gametophytes are used in production of haploid plants. The sporophytic developmental pathway starting from immature female gametes is known as *in vitro* gynogenesis (Juhasz and Jakse, 2005; Yarali and Yanmaz, 2013). *In vitro* haploid production via gynogenesis has been routinely used for inbred production in many *Amaryllidaceae* and *Cucurbitaceae* species (Gupta and Singh, 2016; Yarali and Yanmaz, 2017). Gynogenesis consists of *in vitro* culture of unfertilized ovule, ovary and whole flower buds (Asif, 2013; Yarali and Yanmaz, 2013; Gupta and Singh, 2016). One of the most important points for success in ovule, ovary or flower bud cultures is the size of the flower bud, that is, the period of ovule development (Mukhambetzhanov, 1997; Bohanec, 2009). However, due to the lack of information on what is the molecular mechanism that triggers gynogenetic development, it is not known exactly how the gynogenesis phenomenon occurs at the cellular level. Many researchers have been recommended that female gametes of flower buds used gynogenesis studies should be collected before anthesis (Bohanec, 2009; Palmer and Keller, 2005; Chen et al., 2011; Asif, 2013). Asif (2013) expressed that development stage of microspores is an excellent indicator to identify the exact time for *in vitro* culture. The most responsive ovaries/ovules had nearly mature or fully mature embryo sacs (Gemes-Juhasz et al., 2002).

The most common factor affecting embryogenesis is stress treatments. Cold or heat shock are commonly used for induce stress conditions. The floral organs are pre-treated to stimulate the process of sporophytic development in female gametophytes. The essential benefits of pre-treatment for stock plants were to eliminate variation arising from outer factors. Without imposing stress, a change from gametophytic to the sporophytic phase is very difficult. Pre-treatments can be applied at different types of explant, such as flower buds, isolated ovules/ovaries, or inflorescence. With regard to different explants, the type, level, and duration of pre-treatment are different, and the regeneration efficiencies

vary as well. However, duration, time, type, and level of pre-treatment vary considerably from one species to another (Asif, 2013; Chen et al., 2011; Dhatt and Thakur, 2014). As a result of these stress factors, leading to the formation of gynogenetic embryos or morphogenic callus, the development of the gametes is diverted from the gametophytic path of development to the sporophytic path (Keller and Korzun, 1996; Chen et al., 2011). However, there are not enough researches on the effect of pre-treatments on embryo induction *in vitro* gynogenesis. In this review, it was discussed the effects cold or heat shock pre-treatments in different vegetables species in *Amaryllidaceae* and *Cucurbitaceae* that gynogenesis has been the most successful method used for haploid plants production.

***Amaryllidaceae* - species:**

The family *Amaryllidaceae* has a large number of species such as onion, garlic and leek. Doubled haploids were obtained successfully from these species using dihaploidization techniques (Yaralı and Yanmaz, 2016). However, more research has been conducted out in onion (*Allium cepa* L.) via gynogenesis. For this reason, researchs on onion are mainly evaluated in this part.

Puddephat et al. (1999) investigated that the effect of temperature pre-treatments of stock ovaries on gynogenic embryogenesis induction. Flower buds of onion excised from stock plants maintained at 15ºC with natural daylight to flower under glasshouse conditions were ten times more responsive than those taken from plants raised under glasshouse conditions, or held at 10ºC. In addition, they found that decreasing donor plant growth temperature in the final phases of flower development increased the efficiency of gynogenesis. Similarly, Alan et al. (2004) reported that flower buds from onion plants stored at 10ºC for 4-23 days in beakers of water had more responsive to induction of gynogenesis and were comparable to fresh flower-buds. In another study, Hanna (1994), reported that cold pre-treatment at 4ºC for 4 days enhanced gynogenic embryo induction in onion. In contrast to these findings, Keller and Korzun (1996) stated that the 30°C temperature pre-treatment was suppressed embryo regeneration in onion. Cold treatments either had no effect or had negative effects in gynogenic embryo induction. In another study used different *Allium* species and varieties, was found that the low temperature applied to flower buds before culture was particularly inhibiting effect on the leek, and there was no effect on the hybrid cultivars (Keller, 1990). Schum et al. (1993) used a dark preculture at 10°C for up to 12 days but did not obtain consistent results for all genotypes. A heat pre-treatment led to suppression of regeneration. No or even negative effects of cold pre-treatment were reported by Muren (1989) (Keller and Korzun, 1996). Hassandokht and Campion (2002), investigated the effect of cold treatment at 17°C applied to flowers in culture was evaluated in six Iranian and two Italian onions. The results showed that an inhibitory effect of cold on haploid formation.

***Cucurbitaceae* - species:**

Diao et al. (2009) investigated the effect of thermal shock pre-treatment at 35^{o}C for different period of time on embryo formation via gynogenesis (ovary culture) in *Cucumis sativus* L. The results showed that heat shock treatment for 3 days at 35^{o}C at the beginning of the culture had higher embryo formation rate than 2 or 4 days. Gemes-Juhasz et al. (2002), aimed produced gynogenic plants of pickling cucumber via gynogenesis. They extracted cucumber ovaries and placed on induction media and cultured in the dark conditions for 2–5 days, at 24^{o}C, 28°C or 35^{o}C. They found that the heat treatment increased the efficiency of gynogenesis. The highest number of embryos occurred following the 35^{o}C induction treatment. The flow cytometry analysis showed that 87.7% of the regenerants were haploid. In another study by conducted out by Wang et al. (2008) was stated that 36°C pre-treatment could induced high frequency embryoids than the 4ºC pre-treatment in cucumber. Shalaby, (2007) investigated that influence of temperature (4ºC and 32°C) for 0, 4, 7 and 12 days on the ovule culture the *in vitro* gynogenesis induction of squash. According to research datas, ovules incubated at 4 or 32°C for 4 days produced a better embryogenic response than others treatments. Contrary to positive results in hot shock temperature

treatments, some researchers suggested that cold treatments were more efficient for induction of gynogenic embryogenesis. For example, Kwack and Fujieda (1988), found that the cold treatment of *Cucurbita moschata* ovaries at 5°C for 2 days was efficient for embryogenesis.

Despite the cited examples of a positive influence of cold or heat treatment, Bohanec (1998) suggested that *in vitro* gynogenesis is generally not stimulated by shock treatment such as low or high temperatures. For example, in *Cucurbita pepo* Metwally et al. (1998), found no beneficial effect of cold pre-treatment on gynogenesis. They picked ovaries from squash plants and exposed to cold temperature (4°C) for 0, 2, 4 and 8 days and ovules were cultured. Then the dishes were incubated at 25 ± 1°C under 16 h photoperiod for 4 weeks. Data from the research indicated that cold treatment at 4°C for 2, 4 or 8 days suppressed of embryogenesis compared with the control. The control ovules gave the highest embryogenic ovules per 100 cultured ovules. Similarly, Yang and Zhou (1982) reported that cold temperature pre-treatment was ineffective in ovary and ovule culture of most species.

CONCLUSIONS

In this review, it was evaluated the effects of cold or heat shock pre-treatments in different vegetables species in *Amaryllidaceae* and *Cucurbitaceae* that gynogenesis has been the most successful method used for haploid plants production. When the researches are evaluated, it can be said that cold and hot pre-treatments have positive effects on the haploid embryo induction. But the numbers of researches on pre-treatments are not enough. Therefore, more research is needed to make a clearer determination about effects of temperature pre-treatments.

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STRATEGIES TO INCREASE CROP YIELDS IN A CLIMATE CHANGE SCENARIO

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Abstract

The forecasted effects of climate change – higher average temperatures, more intense and frequent droughts, increasing scarcity of water for irrigation – will worsen the problem of stress-induced reduction of crop yields, especially in arid regions. Development of new crop cultivars with enhanced tolerance to drought and salinity is probably the most promising strategy to improve agricultural productivity and food production. Some recent examples predict that this goal will be achieved in the near future using both, traditional breeding (with the help of new biotechnological tools) and genetic engineering. A complementary strategy could be based on the domestication of wild species naturally tolerant to stress. Optimizing plant nutrition with new and improved fertilizers will also contribute to stress tolerance of our present crops, as more resources will be available to maintain growth while activating defense mechanisms. There is also an increasing interest in the so-called 'biostimulants', a disparate group of unrelated substances that enhance crop quality traits, nutrition efficiency and/or abiotic stress tolerance, also contributing to increased yields under stress conditions. Examples of all these strategies are presented and discussed.

Key words*: biostimulants, drought, plant breeding, plant nutrition, salinity.*

INTRODUCTION

World population continues growing, albeit at a lower rate than some decades ago, and will reach ~9.3 x 10^9 people by 2050. According to FAO estimates, global agricultural production should increase about 60% over the 2005-2007 levels to meet the expected food demand (Alexandratos and Bruinsma, 2012). This goal, *a priori*, should not be too difficult to achieve if we consider that between 1960 and 2009 the world population more than doubled but, still, we were able to increase the average amount of food available to each human being on the planet, from 2200 Kcal/person/day to >2800 Kcal/person/day. This was possible due to huge increases in crop yields, as a consequence of scientific and technical advances in agriculture, including the development of new, more productive varieties of our major crops – which were the basis of the so-called 'green revolution' (GR) of the 1960s and 1970s (Borlaug and Dowswell, 2005) – together with the massive use of agrochemicals (pesticides, herbicides, chemical fertilizers), the mechanisation of labour and a large increase in the area of irrigated cropland. Yet, the GR had also negative effects, since the high yields of the modern crop cultivars are dependent on high-input, intensive production practices that are not sustainable. In addition, the GR has caused a large loss of genetic diversity: modern agriculture is base on a narrow range of crop species and cultivars and thousand of landraces, minor cultivars and local varieties have been lost forever (or, in some cases, are stored in seed banks). This decreases the opportunities to find new sources of variation to fight future challenges, for example, changes in environmental conditions.

From the mid 1990s, the large-scale cultivation of biotech (GM) crops provided an additional boost (much smaller) to food production, as the transgenic varieties of herbicide-tolerant (HT) and insect-resistant (IR) soybean, maize and rapeseed (and other minor GM crops) have higher average productivity than the corresponding conventional crops. However, GM plants do not solve the drawbacks and limitations of our present agricultural systems

regarding their low biodiversity, high inputs requirements or sustainability issues, since genetic transformation is carried out on previously improved 'GR' varieties.

In any case, today there is enough food to feed everybody on earth although, obviously, this food is not well distributed (FAOSTAT, 2015). Food production, both total and *per capita*, is still growing but the growth rates have been decreasing during the last 30 years, so that, even with an even global distribution of the available food, this increase in food production will not be sufficient to cope with population growth.

CROP YIELDS AND CLIMATE CHANGE

The effects of global climate change, including an increase in average temperatures worldwide, and more frequent, longer and more intense extreme weather phenomena (droughts, 'heat waves', floods...) will further reduce crop yields by contributing to the spreading of desertification and increasing the level of environmental stress conditions affecting the plants growing in the fields.

Good quality water for irrigation will be an increasingly scarce resource, due in part to climate change (lower rainfall) but also to its use for human consumption or for the industry.

There is as well a growing demand for biofuels, which compete with food as they are obtained at present from food crops: oilseeds for biodiesel and cereals (mostly maize) for bioethanol.

Furthermore, the global area of arable land is continuously decreasing, mostly by a change in land use due for urban development, industry or tourism. Yet there are other factors, which are also dependent on climate change effects, contributing to the reduction in the land surface available for agriculture: the loss of rainfed cropland due to prolonged droughts and the loss of irrigated arable land due to secondary salinization of the soil. The latter is an increasing problem in areas cultivated under irrigation in arid and semiarid regions, which happen to be the most productive agricultural lands in the world, where more than 40% of the global food is produced although they represent less than 20% of the total cultivated land.

In addition, it is necessary to develop sustainable agricultural systems that will allow increasing food production without depletion of natural resources and further degradation of the environment (Fita et al., 2015).

WHAT CAN WE DO (AND NOT DO) TO INCREASE FOOD PRODUCTION?

It is obvious that the present circumstances do not allow using the strategies that were successful in the past to improve crop yields. We cannot significantly increase the area of arable land, since marginal soils are not cultivable with the present crop varieties and we should not destroy lands of high ecological value, such as rainforest. We cannot increase the area cultivated under irrigation since not enough water will be available. Furthermore, improving the productivity of our conventional crops by a large increase in the use of (toxic and contaminating) agrochemicals, such as chemical fertilisers, will not be sustainable.

Since transgenic crops provide higher yields than conventional crops, we could extend the relative area of cultivation of the present 'biotech' (GM) crop... except in those countries where they already represent a very high proportion (> 90%) of the corresponding crop; for example, for soybean, maize, rapeseed (and cotton) in the USA. Cultivation of already established, minor transgenic crops can be scaled-up, and new biotech crops can be introduced, some of which have been already approved by the corresponding regulatory bodies or are starting commercial production (e.g., Bt eggplant in Bangladesh, China's Bt rice and phytase-containing maize, or Brazil's virus-resistant beans) (ISAAA, 2016). GM crops will contribute to solve the problem of limited food availability in the near future, but on their own, they will not provide the solution to the problem, as their contribution to increased global yields will not be significant enough.

ABIOTIC STRESS AND CROP YIELDS

For all major crops, average yields are only a fraction of record yields, and these losses – with can vary between 50% and >80% of the record yield, depending on the species – are mostly due to environmental abiotic stress conditions affecting the plants in the field, especially to drought and soil salinity (Buchanan et al., 2000). Therefore, the most

promising strategy to improve crop yields and to increase food production will be based on the development of drought and salt-tolerant varieties of our major crops. For this, all available methods should be applied: traditional breeding techniques, genetic engineering (and soon genome editing) to generate 'biotech' tolerant crops, and even domestication and breeding of wild species tolerant to harsh stress conditions in their natural habitats.

TRADITIONAL BREEDING OF ABIOTIC STRESS TOLERANCE

Conventional breeding to obtain plant varieties with enhanced tolerance to abiotic stresses, such as drought and salinity, is far more complicated than breeding for other traits – for example, for resistance to fungal or bacterial pathogens, a character often dependent on a single resistance gene. The major reason is that, in contrast to the example mentioned above, abiotic stress tolerance is a multigenic trait, controlled by many different genes that generate a continuous variation (QTL, 'quantitative trait loci'). In addition, it is not easy to select the characters that precisely define stress tolerance, as the mechanisms involved are different for different stresses and the phenotypic responses vary with the developmental stage of the plant within the same species. Moreover, in many cases, it is difficult to identify appropriate sources of genetic variability for specific breeding programs. Therefore, it seems logical that this approach has been generally unsuccessful in the past, except for a few specific examples.

Nowadays, the breeder has a wide array of available biotechnological tools which make the breeding process faster and much more efficient. These tools include, for example, 'marked assisted selection' (MAS), or 'next generation sequencing' (NGS) technologies, which allow the identification of very large numbers of molecular markers, the establishment of extremely high-density genetic maps, and facilitate the precise location and cloning of the QTLs. Moreover, microsatellites or 'simple sequence repeats' (SSR) and 'single nucleotide polymorphism', (SNP) markers, generated by NGS, are used for the simultaneous analysis of a large number of individual plants in high-throughput genotyping platforms.

In the last years, with help of these technological advances, several successful examples of crops cultivars with enhanced tolerance to abiotic stress, obtained by 'classical' breeding, have been reported. To give only a few examples, we can mention several maize hybrids with improved water stress tolerance obtained at CIMMYT, in Mexico (Ribaut and Ragot, 2006); the generation of a highly drought tolerant rice derived from Kalinga III (an indica rice variety), using Azucena (a drought-resistant japonica rice variety) as donor parent to improve root morphology (Steele, 2009); or a salt-tolerant durum wheat variety in which the Na^+ transporter *nax2* has been introgressed from the wheat ancestral relative *Triticum monococcum* (Munns et al., 2012).

GENETICALLY MODIFIED STRESS - TOLERANT CROPS

There are thousands of reported experiments in which the expression of different genes in transgenic plants resulted in the enhancement of the tolerance of the transgenic to different stress conditions, in a higher or lower degree. The selection of those genes has been generally based on their known participation in basic, conserved mechanisms of response to abiotic stress in plants, and including, for example, genes encoding ion transporters, enzymes of osmolyte biosynthesis pathways, antioxidant enzymes, splicing proteins, signal transduction proteins or transcription factors (see specific examples in Fita et al., 2015).

Despite the enormous amount of information accumulated over the last 30 years on this topic, and referring specifically to salt tolerance, the fact is that at present there is no commercial, salt-tolerant crop variety growing in the field, so that the usefulness of the aforementioned (and other) genes as biotechnological tools to improve the stress tolerance of transgenic plants has been questioned. The major problem is that the vast majority of those experiments have been carried out in the laboratory or the greenhouse, using model species – *Arabidopsis thaliana*, in most cases – and it is not clear if these results can be extended to crop species. Moreover,

stress tolerance is seldom evaluated from an agronomic point of view, not considering that any improvement of tolerance is useless if the quality of the harvested product or the crop yield is significantly reduced. Nevertheless, more recent experiments (still with *Arabidopsis*) suggest that the controlled expression of some genes, only in the presence of the salt stress conditions and in particular cell types, may be the key to obtain a significant improvement of salinity tolerance in the transgenic (Møller et al., 2009). It is to be expected, therefore, that in the coming years commercial salt-tolerant GM crops will be available.

This approach has been more successful in the case of drought-tolerant biotech crops, and a GM maize variety with enhanced resistance to water deficit has been grown commercially since 2012. This variety was developed in collaboration by Monsanto and BASF and has been transformed with bacterial genes encoding RNA chaperon proteins (Castiglioni et al., 2008). Although the expected increments in yield were modest, the resistant crop performed quite well in summer 2012 in some US States affected by a strong drought that year. A lot of work is being invested to develop drought-resistant varieties of other major crops.

In any case, it is to be expected that commercial biotech crops tolerant to drought and high soil salinity will be available in the near future, significantly contributing to the much-needed increase in crop yield and food production.

DOMESTICATION OF WILD PLANTS NATURALLY TOLERANT TO STRESS

Although the vast majority of wild plants and all major crops are relatively sensitive to abiotic stress, a small percentage of wild species are adapted in nature to extremely harsh environmental conditions, growing in arid (xerophytes) or highly saline (halophytes) habitats. An alternative to the genetic improvement of salt and drought tolerance of conventional crops would be the domestications of some of these wild species: since they already possess the trait that is most important and most difficult to introduce, the stress tolerance, it should be relatively simple to improve other agronomic and commercial characteristics, such as selection of the best genotypes, uniformity of the harvested product, or elimination of anti-nutrients.

Special attention has been given to the possibility of developing a 'saline agriculture' based of highly salt-tolerant plants, the halophytes. This would allow growing food and feed crops (and also crops for fibre, for biofuels, other industrial uses, or as ornamentals) in saline soils where conventional crops cannot be cultivated; this would include both, naturally saline marginal land and salinized arable land. These crops could be irrigated with saline or brackish water, even with seawater. Therefore, 'saline agriculture' will not compete with our present conventional crop for these limited resources: fertile farmland and good-quality fresh water for irrigation.

Among the most promising halophytic species for saline agriculture, we can include species of the related genera *Salicornia* and *Sarcocornia*, traditionally used as vegetables (in salads, for example) in coastal regions, collected from natural populations to be self-consumed or sold in local markets. In addition, they are very rich in minerals, unsaturated fatty acids and antioxidant. These species have a great potential for commercial cultivation, as well as other taxa that can be developed as vegetable crops: *Aster tripolium* (also used as ornamental), *Plantago coronopus*, *Inula crithmoides*... and many others.

Other halophytes can be used as oilseed crops. A good example is *Sarcocornia bigelovii*, which can be grown with seawater irrigation and produce seed yields similar to conventional oilseed crops such as soybean. The seeds are very rich in oil and proteins, and the oil contains a high content of 'healthy' polyunsaturated fatty acids, especially linoleic acid (over 70%). In addition, the seed meal can be used as a protein supplement in fish and ruminant diets.

There are many other examples of halophytes representing potential crops for food, feed or industrial uses, but we should mention the specific case of quinoa, up to now the most successful (and known) example of this approach. Quinoa is not really a 'new' crop, but in fact a very old one, cultivated in the Andean region for thousands of years. However, since it

has not been cultivated at a large scale, it has never been subjected to 'modern' breeding programs. The species shown several remarkable properties: different ecotypes are able to grow from sea level to almost 4000 m, it is extremely tolerant to several types of abiotic stress: frost, drought or salinity, withstanding even irrigation with sea water. The species is considered as a 'pseudocereal', with gluten-free seeds, rich in starch and high-quality protein containing all essential amino acids. The straw is at least as nutritious as the seeds and could be an excellent source for animal feed. Although large-scale cultivation of quinoa is still limited to South America, the crop has extended to many countries in the last years, with FAO support in many cases (see Fita et al., 2015, for more information on 'saline agriculture' and additional examples of potential 'halophytic crops').

COMPLEMENTARY STRATEGIES

In addition to the general approaches described in the previous sections, additional strategies can also contribute, even if only modesty, to the goal of improving crop yields. We could, for example, recover traditional crops and local varieties, now abandoned or cultivated at a small scale, which may be more stress-resistant that our present major crops, and could provide reasonable yields under conditions unfavourable for conventional crops.

It is also possible, and an important trend at present, to improve the productivity of our present crop varieties, in the frame of a more sustainable agriculture, by using a 'new generation' of chemical fertilisers the so-called slow-release and controlled-release fertilisers. Although they can increase crop yields when used at the same doses than conventional fertilisers (or maintain the same production at lower doses), their effect is not very big. Nevertheless, their use is very positive for the quality of the soil, as these fertilisers cause a lower contamination of soil and water.

'BIOSTIMULANTS'

A plant 'biostimulant' can be defined as any substance or microorganism which, when applied to plants, can enhance nutrition efficiency, crop quality and/or abiotic stress tolerance; these effects are observed at low concentrations of the biostimulant and are not dependent on its possible nutrients content. By extension, plant biostimulants also refer to commercial products containing mixtures of those substances and/or microorganisms. In any case, the use of biostimulants in agriculture is continuously increasing and represents a growing business for agrochemical companies.

Biostimulants can be considered to include the so-called 'biofertilizers' – bacterial or fungal biostimulants increasing the availability of nutrients and their utilisation. Yet, biostimulants should be distinguished from fertilisers, pesticides or biocontrol agents.

The nature of biostimulants is diverse, including 'substances' and microorganisms, single compounds or mixtures of compounds (of known composition... or not), organic compounds and inorganic molecules that are produced in nature or synthetic.

The main groups of biostimulants are the following:

a) Humic substances, including humins, humic acids, fulvic acids and supramolecular complexes of them. They are constituents of the soil organic matter and are extracted from that natural organic matter, composts or mineral deposits.

b) Protein hydrolysates (and additional N-containing compounds), which are generally amino acid and peptide mixtures (purified amino acids and derivatives can also be included in this group), produced by chemical or enzymatic protein hydrolysis of agro-industrial by-products (e.g., animal wastes or crop residues).

c) Seaweed extracts, either crude extracts or polysaccharides purified from them. The use of these extracts as biostimulant should not be confused with the traditional use of fresh seaweeds as organic fertilisers in agriculture.

d) Chitosan (and other biopolymers), derived from chitin, a polymer that can be obtained both from natural sources and industrially.

e) Inorganic compounds, 'beneficial elements' that promote plant growth in some species (but not in all plants), and include, for example, Al, Co, Na, Se or Si.

f) Beneficial fungi, both mycorrhizal fungi such as arbuscle-forming-mycorrhiza (AFM) and non-mycorrhizal fungi

g) Beneficial bacteria, such as *Rhizobium* and related taxa (endosymbiotic bacteria) or plant-growth-promoting rhizobacteria (PGPRs), which are present in the rhizosphere of the plants.

Considering this extreme diversity, it is clear that their physiological functions and mechanisms of action must be also diverse, and are in most cases unknown. A general idea is that biostimulants, somehow, can divert a higher proportion of resources (nutrients, water and light) to growth, increased yield and crop quality, reducing the proportion allotted to activation of stress responses. The elucidation of the specific mechanisms of action of the different groups of biostimulants represents an interesting challenge for basic research in the coming years.

CONCLUSIONS

At the present rates, the increase of agricultural production will not be enough to feed the growing world population. Since drought and soil salinity are the major cause of reduction of crop yields, a problem that will worsen in the coming decades due to the effects of climate change, the most promising strategy to increase crop yields and food production in the near future is to develop stress-tolerant crops, using all available approaches: traditional breeding (with the help of modern biotechnological tools), genetic engineering for the generation of tolerant transgenic crops, or even domestication of wild plant species highly tolerant to salt stress (halophytes) or to water deficit (xerophytes) in their natural habitats.

We can also improve the productivity of our present crop varieties but in the frame of a more sustainable agriculture. This could be achieved by using 'new generation' slow-release and controlled-release fertilisers, or by application of low concentrations of the so-called 'biostimulants', a disparate group of biological extracts, more or less characterised substances, defined compounds and microorganisms that, regardless of their nutrient content, enhance plant nutrition efficiency, crop quality traits and/or abiotic stress tolerance.

We should be confident that, despite all unfavourable conditions, application of the strategies reviewed above will allow increasing crop yields and food production to the level required to feed the human population in the foreseeable future.

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RESULTS OF SUGAR ALCOHOLS INFLUENCE OVER DIFFERENT ROMANIAN POTATO VARIETIES

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Abstract

Plantlets of three cultivars Sarmis, Christian and Roclas were induced to microtuberized under dark conditions and at temperature of 17°C. In medium of tuberisation were applied two different sugar alcohols (sorbitol ans mannitol) for evaluate the influence of this under the number of microtubers obtained/plantlet and the average weight of a microtuber. It was used three concentrations of sugar alcohols (0.05; 0.11; 0.17 mol/l) which were compared with controlled medium in which was not added any type sugar alcohol.

Key words*: potato, plantlets, microtubers, hydric stress, manitol, sorbitol.*

INTRODUCTION

Increasing crop production in drought environment may be achieved through breeding crops that are more tolerant to drought (Rao S. and FTZ J., 2013). According to one of the stress concepts, stress is defined as an environmental factor, which can be potentially unfavorable to living organisms (Levitt, J., 1980, quote by Hassanpanah, 2009). Fresh water resources are limited and their use in agricultural production is expected to come under increasing constraints (Albiski K. et al., 2012). How plants cope with drought stress is a topic of an intense debate (Kacem N. S et al., 2017). Biotechnology like tissue culture technology offers rapid alternative in crop improvement. In recent years, tissue culture based *in vitro* selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants (Rao S. and FTZ J., 2013).

Potato is highly amenable to tissue culture (Espinoza et al., 1986, quote by Gopal J. and Iwama K., 2007) and micropropagation and microtuberization have become established methods of rapidly multiplying cultivars for seed production as well as for germplasm conservation and exchange (Roca et al., 1979; Ranalli et al., 1994; Gopal et al., 1998, 2002, 2005; Donnelly et al., 2003, quote by Gopal J. and Iwama K., 2007). *Solanum tuberosum* L. is sensitive to drought due to its shallow root system (Iwama and Yamaguchi, 2006, quote by Bundig C. et al., 2016). Mannitol or sorbitol have been used by several workers as osmotic stress agents for *in vitro* selection (Hassan N.M. et al., 2004; Mohamed M.A.H., 2000). A polyol is an alcohol containing multiple hydroxyl groups. Sugar alcohols include: sorbitol, glycerol, erythritol, maltitol, isomalt, mannitol, lactitol, threitol, arabitol, ribitol and xylitol (Acton A.Q., 2013).

MATERIALS AND METHODS

The starting point for obtaining a free material of potato viruses is the culture of meristems. The meristem is inoculated on test tubes with medium Murashige and Skoog (MS), 1962. After 6-8 months, after more subculture, in function of genotypes from meristems plantlets are developing. To evaluate the phytosanitary quality of the plantlets, ELISA test was made. The infected clones are eliminated. Biological material free of virus is *in vitro* multiplied. While the temperature of 20 ± 20°C is the necessary for plants micropropagation, the temperature required to obtain the microtubers is generally lower (17°C). Sucrose is the most decisive factor for *in vitro* tuber formation. Sucrose is a source of energy and at higher concentrations, is favoring the formation of microtubers. For the production of microtubers, sucrose concentrations are increased from 2% used for plant micropropagation to 8%. On culture recipients with developed plantlets (Figure 1) is put a liquid medium for

microtubersitation. The recipients are kept in dark conditions and after 3 month the microtubers (Figure 2) are harvested.
To studying the effect of sugar alcohol over microtuberisation 6 variants were analyzed in a bifactorial experience, 3 x 2, in 3 repetitions. The graduations of the studied factors were: experimental factor B, the variety, with three graduations: a_1 – Sarmis, a_2 – Christian, a_3 – Roclas; Experimental factor B, sugar alcohol, with two graduations: b_1 – sorbitol, b_2 – mannitol.
Microtuber production is an important rapid multiplication method for prebase stock formation as well as germplasm exchange.

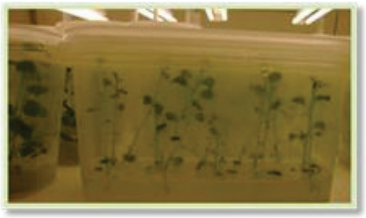

Figure 1. Developed plantlets

Figure 2. Microtubers

RESULTS AND DISCUSSIONS

From the Table 1 we can observe that mannitol determined obtaining a higher number of microtubers/ plantlets (1.07), even if the difference between the two agents is not significant.
From the Table 1 we can see that with the increase in concentration of agent water stress inducing, the number of microtubers/plantlets decreased.
At concentration of 0.05 mol/l sugar alcohol (Table 2), the difference is distinctly significant (-0.23 microtubers/pl), negative, to control, statistically assured. For the other concentrations (0.11; 0.17 mol/l) very significant, negative differences are obtained (-0.33; -0.49 microtubers/pl).
From the analysis of the average values of the number of microtubers/plantlet/variety (Table 3), it is observed that the differences are small, not significant, by 0.16 and 0.17 statistically assured (for Christian and Roclas), distinctly significant, positive.
The statistical interpretation of the combined influence of the two factors (Table 4), respectively the variety and the inducer of hydric stress *in vitro* shows that sorbitol concentrations of 0.05 and 0.011 mol/l present distinctly significant differences (-0.305 and -0.358), negative. Sorbitol concentration of 0.17 presents a very significant difference -0.604. When it is compared the two inductors of hydric stress, mannitol presents better results for 0.17 mol/l concentration with a positive difference, 0.23. Sorbitol presented a stronger osmotic pressure, causing a lower number of microtubers.
Another parameter studied was average weight of a microtuber.
Statistical analysis of the influence of the variety on weight (Table 5) of a microtuber shows that the difference (0.02 g) for mannitol comparative with sorbitol is not significant.
The statistical analysis of the influence of the concentrations of sugar alcohols shows us that 0.05, 0.11 and 0.17 mol/l determined an average weight of a microtuber with very significant (Table 6), negative differences (-0.17, -0.23, -0.28 g).
From the statistical analysis of the influence of the variety and of the sugar alcohols (Table 8) we may have observed that on concentration of 0,05 mol/l the differences are distinctly significant for both sugar alcohols, but negative (-0.17 g for sorbitol and -0.18 for mannitol) comparative with control (nutritive medium to which was not added sugar alcohols).
To next concentrations 0.11 and 0.17 mol/l the differences are very significant for both sugar alcohols, negative, statistically assured (-0.22 g and -0.26 g for sorbitol and -0.25 and -0.31 g for mannitol). It can be seen with increasing of sugar alcohols concentration in nutritive medium, this has as effect decreasing the average weight of a microtubers/plantlet. Regarding the differences between the two

sugar alcohols used with different concentrations, it can be noticed that there are no significant differences for the weight of a microtuber. The average weight of a microtuber is higher when mannitol it is used, so sorbitol has a higher osmotic potential.

Table 1. Influence of sugar alcohols on the average number of microtubers obtained/plantlet

Nutritive medium Murashige Skoog supplemented with sugar alcohols	Average number of microtubers obtained/plantlet		Dif.	Sign.
	Nr.	%		
sorbitol (Ct)	0.96	100.00	-	-
mannitol	1.07	111.58	0.11	ns

Table 2. Influence of sugar alcohols concentrations on the average number of microtubers obtained/plantlet

Concentrations of sugar alcohols (mol/l)	Average number of microtubers obtained/plantlet		Dif.	Sign.
	Nr.	%		
0.00 (Ct)	1.27	100.00	-	-
0.05	1.04	81.92	-0.23	oo
0.11	0.95	74.36	-0.33	ooo
0.17	0.79	61.62	-0.49	ooo

DL 5% = 0.13 DL 1% = 0.18 DL 0.1% = 0.26

Table 3. Influence of variety on the average number of microtubers obtained/plantlet

Variety	Average number of microtubers obtained/plantlet		Dif.	Sign.
	Nr.	%		
Sarmis (Ct)	0.90	100.00	-	-
Christian	1.06	117.55	0.16	Ns
Roclas	1.07	118.95	0.17	Ns

DL 5% = 0.59 DL 1% = 0.79 DL 0.1% = 1.05

Table 4. Combined influence of sugar alcohols and their concentrations on the average number of microtubers obtained/plantlet

Concentrations of sugar alcohols (mol/l)	Sorbitol		Dif.	Sign.	Mannitol		Dif.	Sign.	a2-a1	Sign.
	Nr.	%			Nr.	%			Nr.	
0.00 (Ct)	1.274	100.00	-	-	1.274	100.00	-	-	0.000	ns
0.05	0.969	76.05	-0.305	oo	1.119	87.80	-0.155	ns	0.150	ns
0.11	0.916	71.88	-0.358	oo	0.979	76.85	-0.295	oo	0.063	ns
0.17	0.670	52.58	-0.604	ooo	0.900	70.66	-0.374	ooo	0.230	*

DL 5% = 0.182
DL 1% = 0.256
DL 0.1% = 0.361

DL 5% =0.229
DL 1% = 0.405
DL 0.1% =0.940

Table 5. Influence of sugar alcohols on the average weight of a microtuber

Nutritive medium Murashige Skoog supplemented with sugar alcohols	Average weightof a microtuber		Dif.	Sign.
	(g)	%		
sorbitol (Ct)	0.25	100.00	-	-
mannitol	0.27	110.00	0.02	ns

DL 5% = 0.14 g DL 1% = 0.33 g DL 0.1% = 1.04 g

Table 6. Influence of sugar alcohols concentrations on the average weight of a microtuber

Concentrations of sugar alcohols (mol/l)	Average weight of a microtuber		Dif.	Sign.
	(g)	%		
0.00 (Ct)	0.43	100.00	-	-
0.05	0.26	59.59	-0.17	Ooo
0.11	0.20	45.64	-0.23	Ooo
0.17	0.15	34.63	-0.28	Ooo

DL 5% = 0.07 g, DL 1% = 0.09 g, DL 0.1% = 0.13 g

Statistical analysis of variety influence indicates differences not significant between varieties (Table 7).

Table 7. Influence of variety on the average weight of a microtuber

Variety	Average number of microtubers obtained/plantlet		Dif.	Sign.
	(g)	%		
Sarmis (Ct)	0.29	100.00	-	
Christian	0.26	88.11	-0.03	Ns
Roclas	0.23	78.32	-0.06	Ns

DL 5% = 0.59 g, DL 1% = 0.79g, DL 0.1% = 1.05g

Table 8. Combined influence of sugar alcohols and their concentrations on the average weight of a microtuber

Concentrations of sugar alcohols (mol/l)	Sorbitol		Dif.	Sign.	Mannitol		Dif.	Sign.	a2-a1	Sign.
	(g)	%			g	%			(g).	
0.00 (Ct)	0.43	100.00	-		0.43	100.00	-	-	0.000	ns
0.05	0.25	61.58	-0.17	oo	0.27	57.61	-0.18	oo	0.017	ns
0.11	0.18	49.15	-0.22	ooo	0.21	42.12	-0.25	ooo	0.030	ns
0.17	0.12	40.55	-0.26	ooo	0.17	28.70	-0.31	ooo	0.051	ns

DL 5% = 0.10 g
DL 1% = 0.14 g
DL 0.1% = 0.19 g

DL 5% = 0.16 g
DL 1% = 0.30 g
DL 0.1% = 0.79 g

CONCLUSIONS

Medium with different concentrations in which was added sugar alcohols very significantly reduced the number of microtubers/plantlets and weight of a microtuber compared with the medium to with no these osmotic agents (with 0.00 mol /l sugar alcohols).

Even though there are no significant differences for numbers/plantlets and weight of microtubers between the two sugars, lower values are obtained to sorbitol (0.96 microtubers/pl and 0.25 g) meaning that this is an inducer of *in vitro* drought more powerful than mannitol.

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SUNFLOWER BREEDING FOR WELL DEVELOPING IN CONDITIONS OF THE CLIMATE CHANGE

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Abstract

There are many problems due to the climat changes, in the Romanian agriculture. The water deficit and high or low temperatures, reduce the yield level. The adaptability of sunflower to the environmental conditions, with the purpose to obtain hybrids with high seed yield stability, in all ecological cultivated areas involves a good resistance to drought and low temperatures, specially in germination time.
In our research work we have used different sources from our sunflower germplasm collection.
Some of our best elite lines have been introduced in a process of improvement of resistance to drought. Each generation of selection was planted in drought natural conditions (missing water in soil and high air temperature). All generations of selection were tested for resistance to low temperatures in germination and emergence time. There have been selected the more tolerant ones.

Key words*: sunflower, drought, elite lines, low temperatures, resistance.*

INTRODUCTION

Sunflower is considered to be moderately resistant to drought, but in hot conditions, the plants suffer reduction in fertility, yield performance and quality of products (Vrânceanu, 2000; Popa et al., 2013; Popa et al., 2017). In literature there are mentioned some adoptive mechanisms of plants to drought: escape, avoidance and tolerance, as well as their genetic variability (Skoric, 2012).
Singh (2000) considered it difficult to define the parameters that affect the expresion of drought. Miller (1997) stated that it is important to identify and incorporate into breeding material characteristics that contribute to physiological drought resistance. Different morphological and physiological characteristics were used in study of sunflower resistance to drought (Baldini et al., 1991; Griveau et al., 1996; Chimenti et al., 2004; Sauca et al., 2018). Sunflower breeders believe that drought avoidance can be achieved by developing very early hybrids or by moving the sowing date, in order to avoid the dry period (Skoric, 2012).
Practical results in sunflower breeding for drought resistance have been achieved by using the stay-green phenomenom (Vrânceanu, 2000). For sunflower it is very important to increase the cold resistance in early development stages, at stage of germination, emergence and the stage of 2-3 leaves, in order to facilitate an early sowing.
Wild *Helianthus* species are a very valuable source of resistance in increasing drought resistance as well as resistance to low temperatures in sunflower.

MATERIALS AND METHODS

In our research work we have used different sources from our sunflower germplasm collection, some of them coming from the interspecific hybrids between wild *Helianthus argopyllus* and cultivated sunflower.
Some of our best elite lines have been introduced in a process of improvement of resistance to drought, using recurrent selection. We have used several parameters or characteristics in selection of the tolerant plants: deeper rooting depth and more efficient root uptake of water, area of lives and number of lives, plant ability to recover after wilting under heat stress. Each generation of selection was planted in drought natural conditions (missing water in soil and high air temperature).
For breeding we have used a scheme which has helped us to select the best genotypes, regarding the tolerance to drought (Figure 1).
All generations of selection were tested for resistance to low temperatures in germination and emergence time. There have been selected the more tolerant ones.

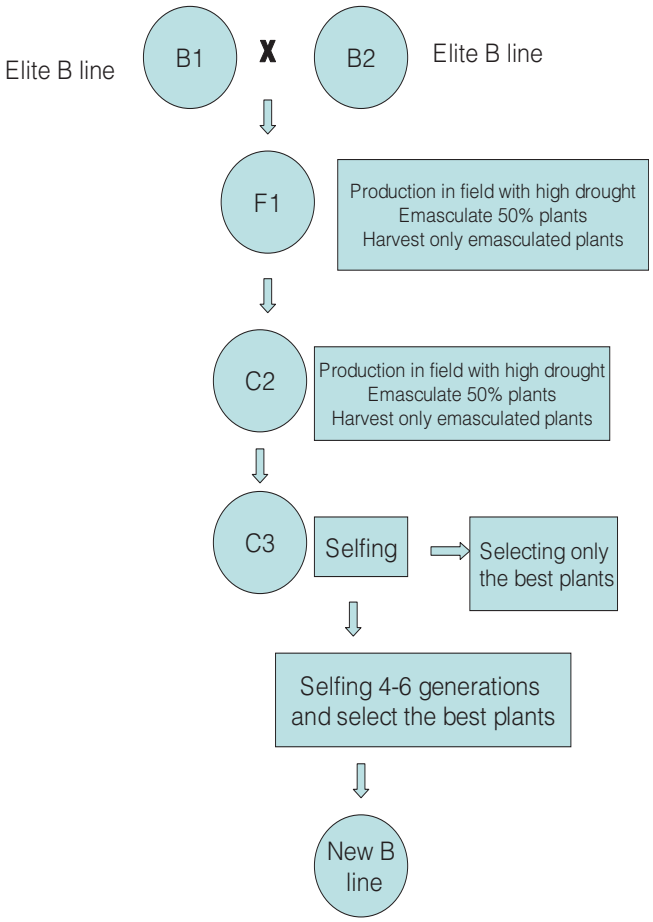

Figure 1. Scheme for breeding to tolerance to drought

RESULTS AND DISCUSSIONS

Using the scheme presented in Figure 1, we have improved the resistance to drought and to cold, in some elite lines from our institute germplasm collection. In table 1 there are presented the results regarding the resistance/tolerance to drought, for some lines (CMS and restorer) this testing being done in natural conditions of drought in area Constanţa in year 2017. Some of these lines have very good tolerance to drought (LCS 253, RS 114).

Table 1. Sunflower genotypes in different generation of selection, tested for resistance to drought and high air temperatures, in field, area Constanţa, 2017 (Resistant=1; Sensitive=9)

Genotype	Generation	Total plants	Resistance to drought
LCS 234	(C3)4	56	2
LCS 241	(C3)4	44	3
LCS 244	(C3)5	52	2
LCS 253	(C3)5	41	1
LCS 259	(C4)2	58	5
LCS 272	(C3)4	62	2
LCS 279	(C4)3	55	2
RS 102	(C3)2	47	2
RS 108	(C3)3	42	2
RS 114	(C3)4	51	1
RS 122	(C3)4	59	2
Check sensitive	-	61	9
Check resistant	-	58	1

In Table 2 we are presenting the results regarding the resistance/tolerance to low temperatures, in germination and emergence time, of the obtained lines (CMS and restorer) in year 2017 in Fundulea. Most of them have very good behavior regarding this aspect (LCS 234, LCS 244, LCS 253, LCS 272, RS 108, RS 114).

Table 2. The inbred lines in different generations of selection for drought, tested for resistance to cold, Fundulea, 2017 (Resistant=1; Sensitive=5)

Genotype	Generation	Total plants	Resistance to cold
LCS 234	(C3)4	49	1
LCS 241	(C3)4	51	2
LCS 244	(C3)5	48	1
LCS 253	(C3)5	50	1
LCS 259	(C4)2	50	3
LCS 272	(C3)4	47	1
LCS 279	(C4)3	45	3
RS 102	(C3)2	50	3
RS 108	(C3)3	51	1
RS 114	(C3)4	49	1
RS 122	(C3)4	47	2
Check sensitive	-	56	5
Check resistant	-	52	1

Some of lines which for it has been improved the resistance/tolerance to drought as well as to low temperatures, have been used for obtaining the hybrid combinations. Some of these have been tested for resistance to drought as well as for resistance to cold.

In Figures 2 and 3 there are presented the climatic conditions in Fundulea area, in 2016 and 2017 years. It could be seen that in 2016 year the air temperature is lower in March, comparing with year 2017, also, the rainfall was higher in 2016 comparing with 2017 year. This is important, because the planting of the hybrids tested for resistance to cold, it has done at the first half of March.

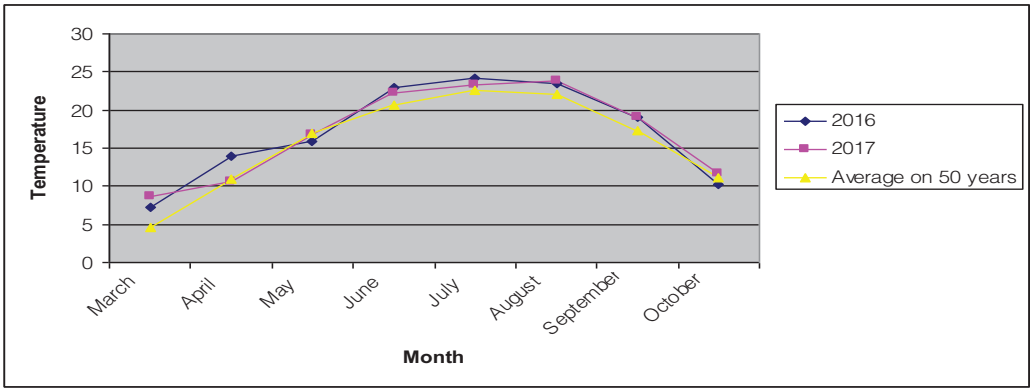

Figure 2. Temperature on two years 2016 and 2017, in Fundulea

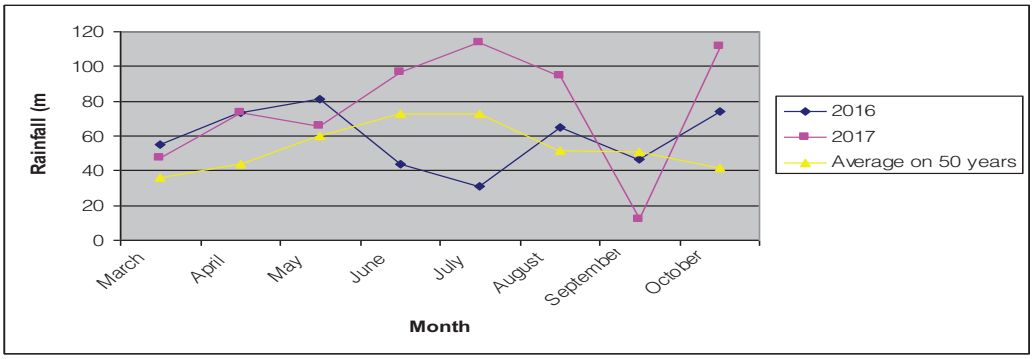

Figure 3. Rainfall on two years, 2016 and 2017, in Fundulea

In Figure 4 there are presented results regarding the behavior to cold, of the hybrids in these two years, 2016 and 2017 in Fundulea. Some of hybrids have good tolerance to cold in both years (HS1020, HS1110, HS1113), some others (HS1020, HS 1025, HS1027, HS1110, HS1113) have been more tolerant in 2017 year.

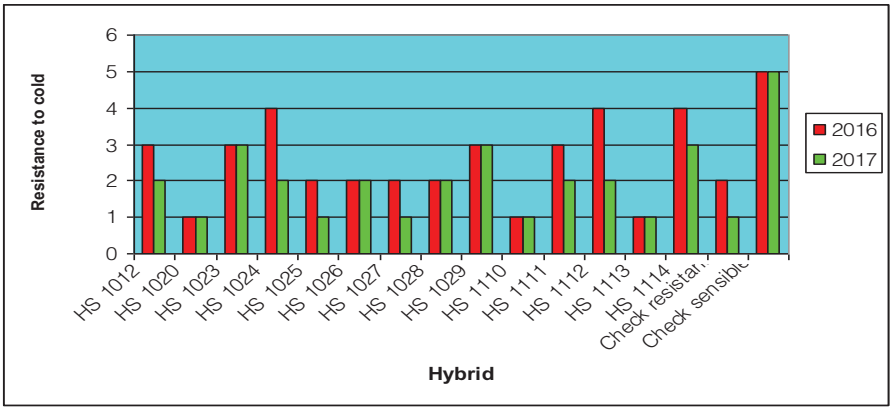

Figure 4. New sunflower hybrids tested for resistance to cold, Fundulea, 2016 and 2017 (Resistant=1; Sensitive=5)

In Figure 5 there are presented the results regarding the behaviour of the hybrids for resistance/tolerance to drought tested in area Constanţa in year 2017. Some hybrids have very good tolerance (HS 1020, HS 1025, HS 1027, HS 1028, HS 1110, HS 1113).

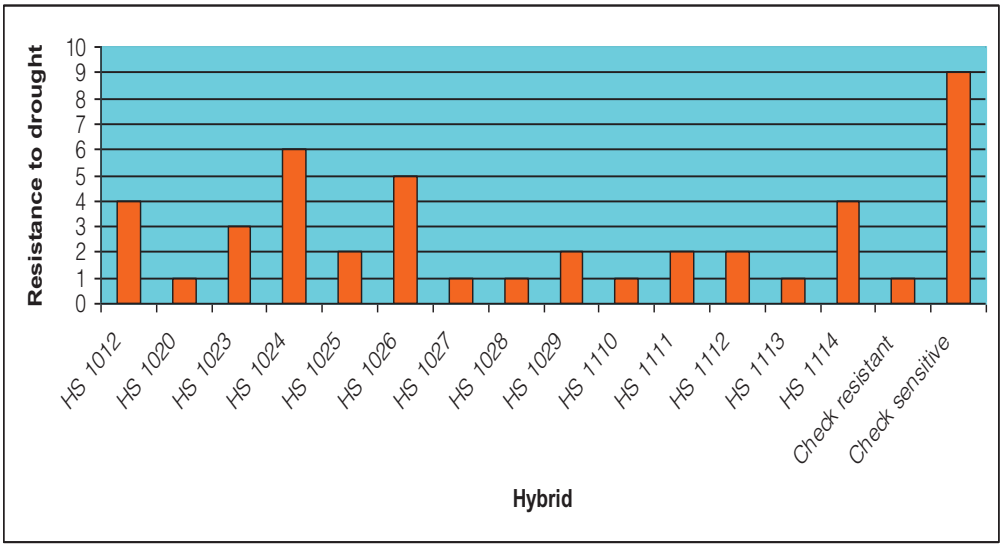

Figure 5. New sunflower hybrids, tested for resistance to drought and high air temperatures, area Constanţa, year 2017 (Resistant=1; Sensitive=9)

CONCLUSIONS

In our sunflower breeding program, at Fundulea Institute, an important objective is resistance/tolerance to drought and to low temperatures in germination and emergence time. In the last three years, there have been obtained good sunflower genotypes (lines LCS 253, RS 114 and hybrids HS 1020, HS 1110, HS 1113) which have a good tolerance to drought as well as to cold.

The released hybrids with this behavior can be cultivated and obtained good results regarding the seed yield and oil content in the climate change conditions.

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NEW GERMPLASM REALISED TO WINTER PEA WITH SUPERIOR AGRONOMIC TRAITS

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Abstract

The developments of the winter pea crop represent a major challenge to expand plant protein production in temperate areas. Breeding winter cultivars requires the combination of freezing tolerance as well as with high seed yield and quality.
In this paper we present data obtained from the F3, F4 and F5 lines of winter peas selected from the four hybrid populations (Specter/F95-927; F98-492/Windham; F95-927/CHECO; Specter/CHECO) and tested at NARDI Fundulea in the year 2017. At these lines were determined winter hardiness, earliness, yield and plant height in comparation with the three winter peas controls (Specter, Checo and Windham).
The results shown that several news F5 lines of winter peasrealized till 6.5 t / ha, exceeding the control varieties and had similar to or better earliness and winter hardiness than that of the parental forms.

Key words*: breeding, winter pea, winter hardiness.*

INTRODUCTION

Pea *(Pisum sativum* L.) is an important annual legume crop grown in temperate regions, can be considered an environmentally friendly crop providing furthermore a high quality source of proteins for animal feeding. By climate change, heat stress and drought are very determinal to the yield, especially for spring pea. Breeders are now developing winter pea varieties, more likely to avoid these stresses occurring at the end of the crop cycle, because they flower earlier. However, the high level of frost risk in winter could limit the extent of peas, even in a warming climate (Castel et al., 2017). One way to improve the yield of the pea crop is through the development of autumn-sown varieties, with a longer development cycle than spring peas, provided that plants are able to cope with low temperatures during fall and winter (Grimaud et al., 2013).
High levels of both tolerance and acclimation rate seem required to best benefit to the winter crops (Rammig et al., 2010). To this aim breeding for better frost tolerance isrequired. For these latter if frost level resistance is of primary importance, the rate of acclimation matters too. Frost stress level decreases systematically with lower rate of acclimation whatever the frost resistance level. The range of the acclimation rate effect is clearly frost resistance dependent. A relevant modelling at high spatial resolution of daily climate change and more robust mimics of theacclimation/de-acclimation processes for crop are needed to account for both warming patterns (abrupt fluctuations, variance, geography) and pea traits (frost resistance level, acclimation rate, date of sowing) (Pagter and Arora, 2013).
The frost stress intensity changes clearly drive the whole decreasing trend. While subtleincrease in the frost events support the „paradoxical" increase in freezing injury in a warming climate that has been widely documented for spring and for the perennials vegetation such as forestin mid and high latitudes (Ball et al., 2012).
The mechanisms of the spring frost increase is attributed to the hastening of bud burst that considerably increase the vulnerability to lessacclimation/deacclimation processes. In this case the exposure to the gradual appearance

ofextreme minimum temperature. By contrast,the winter frost damage is linked to the warmerlow temperature results the in delayed acclimation through slower accumulation of resistance (Wedendrop et al., 2008) anddecreases the frost resistance.
Crop vulnerability seems also to be increased bymid-winter more frequent deacclimation to moderate elevation in temperature (≃5°C or less) in warmer climate and by the longer exposure of the crop to the fluctuating winter temperatures (Castel et al., 2017).
The aim of this work was to appreciate the yield performance and other traits and mainly the winter hardiness of several winter pea lines in the climatic conditions from NARDI-Fundulea.

MATERIALS AND METHODS

In 2017, 31 lines of peas F3 generations from four hybrid combination (Specter/F95-927; F98-492/Windham; F95-927/CHECO; Specter/CHECO) were tested in two comparative trials with 25 entries in three replications, on the each plot of 6 m^2 harvested area.
In parallel, 34 lines of peas F4 generations belonging to the same hybrid combinations were tested in two preliminary comparative trials with 25 entries in three replications, with the same size of the plot like in the F3 trials.
Of the same four hybrid combination but in F5 generation were tested 67 lines of peas in four preliminary micro-trials, each of them with 25 variants, one rep besides the parents of these lines: Specter, Windham, Checo, F95-927 and F98-492, on the each plot of 6m^2 harvested area.
The 2017 winter was mild enough, with a short period with negative temperatures of -23°C (the begining of January), but with a 20 cm snow loyer, which has protected the crop. There are no damages registered due to frost. The early spring was normal, fact that led to restart the vegetation under optimum conditions.
The level of resistance to winter hardiness was estimated in the field, early in the spring, in a scale 1 to 9, where score 1 is very resistance and 9 very susceptible. Plant height was measure in cm, total length of plant from the ground till the top to the end of flowering time. The earliness was appreciated like number of days from 1st January till the end of flowering time and yield as kg/ha.
The statistic analyses of data have been evaluated by correlations and linear regressions between study traits.

RESULTS AND DISCUSSIONS

Yield performances of the lines F3, F4 and F5 generation, tested in advance trials and respectively in preliminary trials in 2017, (Figure 1 and Figure 2) shows that the coefficients of correlation between generation are significant high (r=0.86 ***, F4/F3 and r=0.72*** F5/F3) that means a high enough heredity of this trait and possibility, in the breeding program, is not difficult, to select the new lines with improve yield performances.

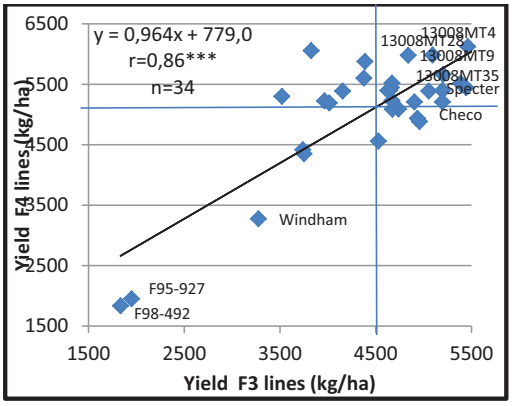

Figure 1. Correlation between yield of F3 and F4 lines selected from the four hybrid combinations of winter peas

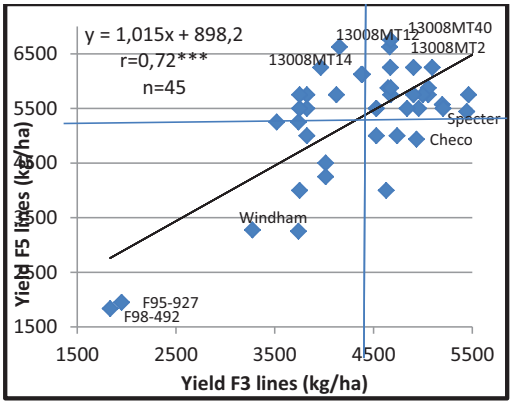

Figure 2. Correlation between yield of F3 and F5 lines selected from the four hybrid combinations of winter peas

Having in view that winter hardiness in winter peas is a very important trait, there was necessary to know in what way this trait could be recombined with other important agronomical characteristics, like earliness to flowering, plant height, grain yield as well as the relationship among other traits as plant height/earliness or yield/earliness.

In the Table1 are presented such correlations using the data collected from 31 F3 lines and 34 F4 lines in 2017.

The correlation between winter hardiness and yield either in F3 and F4 lines (Table1) was very distinct significantly negative (r=-0.61 and r=-0.77), what means that in winter peas is absolutely necessary to cultivate genotypes with good level of winter hardiness, to realize high and stable yields.

Also relationship between plant height and earliness should be positively strong enough in some case, what mains that it quite easily to be recombine such characteristics.

The correlation, between plant height and winter hardiness, was negative not significantly (r=-0.33 and r=-0.11), that suggests possibility to select the genotypes which recombine both traits.

The relationship between yield and earliness, in F3 and F4 peas lines was not significantly, (0.13 and 0.04), what mains, in some cases, that later types can realize high yield than earlier types.

Also, the relationship between winter hardiness and earliness, in the all cases, was not significant, that mains it is possible to select the winter pea form which recombine the both traits.

Table 1. Correlation coefficients among different traits in F3 and F4 lines selected from the four hybrid combinations of winter peas

The generation these genotypes	Correlation between different characters	The correlation coefficient
31 F3 lines tested in comparative trails in 2017	Winter hardiness/yield	**-0,61*****
	Winter hardiness/earliness	-0,32ns
	Winter hardiness/plant height	-0,33ns
	Plant height/earliness	0.18ns
	Yield/earliness	0.13ns
34 F4 lines tested in preliminary comparative trails in 2017	Winter hardiness/yield	**-0.77*****
	Winter hardiness/earliness	-0.32ns
	Winter hardiness/plant height	-0.11ns
	Plant height/earliness	**0.40***
	Yield/earliness	0.04ns

The correlation between yield and winter hardiness of F5 lines (Figure 3) shown a very strong enough negatively relationship (r=-0.48***). However, the distribution of the lines along regression line, demonstrated the possibility to select the new lines with the same level of winter hardiness like winter parents but with high level of yield than those.

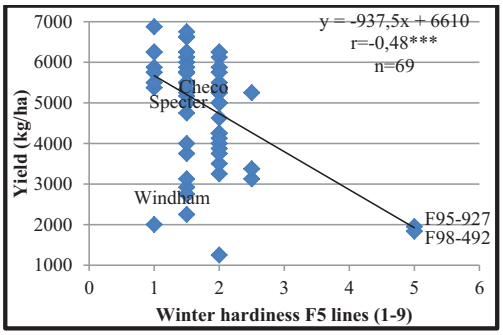

Figure 3. Correlation between winter hardiness and yield of F5 lines winter peas

Also, data from the Figure 4 indicated that is possible to select the perspective lines, with good yield potential but in the same time to recombine an acceptable earliness for Romanian climate conditions.

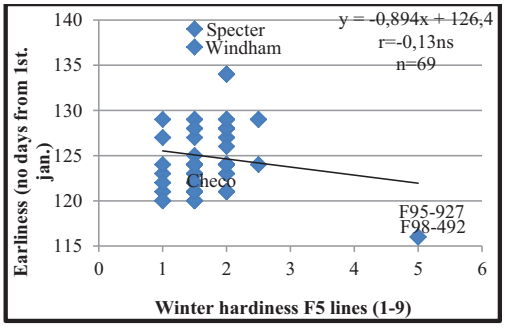

Figure 4. Correlation between winter hardiness and earliness of F5 lines winter peas

The data obtained till now form the study of relationship between winter hardiness and plant height, indicated the possibility of recombination of both traits -plant height and winter hardiness (Figure 5), suggesting that, in function of the end use the production, for forage need to be a tall variety with high biomass production or mid tall variety for grain type.

The date obtained between correlation between plant height and earliness to the F5 lines, shown a separation of the material în two

category, earlier lines with 50-100 cm plant height and later lines with 150-200 cm plant height (Figure 6).

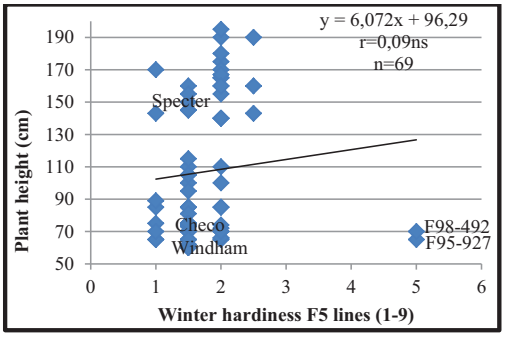

Figure 5. Correlation between winter hardiness and plant height of F5 winter peas lines

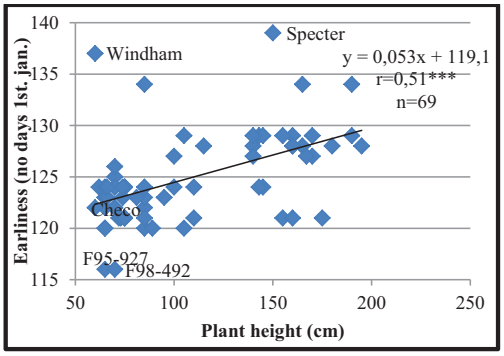

Figure 6. Correlation between plant height and earliness of F5 winter peas lines

The relationship between yield and earliness is negative (r= -0,23*), however ther are several which recombine the earliness with with high level of yield, that is important in the breeding winter peas program to select ssuch type of varieties (Figure 7).

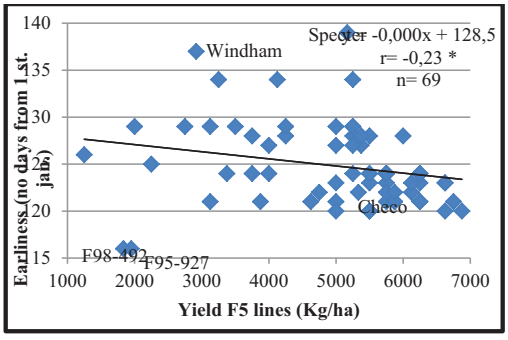

Figure 7. Correlation between yield and earliness of F5 lines winter peas

CONCLUSIONS

The data obtained in these studies, on the F3, F4 and F5 random lines from four hybrid combinations *(Specter/F95-927; F98492/Windham; F95-927/CHECO; Specter/CHECO)* shown existance the important lines of winter peas which posedhigh yield, good level of winter hardiness, plant height and earliness. In this study were remarked winter peas lines F5 with good level of winter hardiness, with high yield (6000-7000 kg/ha), earliness and with the plant height between 125-135 cm, this trait is very important for varieties of winter peas because can utilized both as pure crops and for cereal grain mixtures (high biomass).

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ALLELOPATHIC POTENTIAL OF VOLATILE/ESSENTIAL OILS AND HYDROSOLS OBTAINED FROM CULTURED MEDICINAL PLANTS

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Abstract

Using the chemical substances in weed control, diseases and pests, without discernment, has been responsible for environmental damage and human health. For these reasons, in the last years research has intensified its efforts to find alternative agriculture strategies. One of these is represented by the Integrated Weed Management System (IWMS), so that the capacity to combat by natural ways could have a wider and more valuable application. From the multidisciplinary and interdisciplinary point of view approach, some species of agricultural interest are already known for their allelopathic effects and can be used as instruments for weed management, disease and pests. A feasible alternative is represented by the identification of natural substances with allelopathic effects for the production of natural bio pesticides. Research done so far has highlighted the possibility of using volatile / essential oils and hydrosols extracted from medicinal and aromatic plants disproof in controlled environment, such as horticulture (in greenhouses and solariums). The advantage of using such natural compounds is the fast decomposing process in the environment and thus is less harmful and can be applied in organic farming.

Key words*: allelopathy, medicinal plants, essential oil.*

INTRODUCTION

In the production of medicinal and aromatic plants, the quality of the products is given by the content in active compounds.
When selecting a specie for a particular crop area, consideration is given to the complexity of the interaction of the different vegetation factors, so as to ensure an optimal ratio between the pedoclimatic conditions and the biological requirements of the plants.
There are avoided natural conditions which can increase vegetable biomass production to the detriment of the active principles.
Today, about 3,000 plants are used to obtain volatile oils, out of which 300 are world marketed (CBI, 2009a, Lelieveld, 2017).
The largest consumer of volatile oils is the United States, followed by Western European countries (France, Germany, Great Britain) and Japan (Holmes, 2005).
It is difficult to make a more accurate estimation of global production of volatile/essential oils, but in 2009 an estimate of the first 20 volatile oils was reported, which is much higher, over

104,000 tonnes (CBI, 2009b). Volatile oils were classified into 3 large groups based on volumes globally produced.
Production of the first group exceeds 100 tons / year (e.g.: *Mentha piperita, Lavandula hybrida, Citrus sinensis, C. aurantium*). The second group is between 50-100 tons / year (e.g.: *Salvia sclarea, Ocimum basilicum, Thymus vulgaris*). The third group is between 1-50 tons / year (e.g.: *Hyssopus officialis, Artemisia dracunculus, Carum carvi*) (Shrinivas and Kudli, 2008).
Data on the evolution of the surfaces and the production of medicinal and aromatic plants cultivated in Romania are shown in Figure 1 (Source: 2016 RSY-Statistical Yearbook of Romania, 2016).

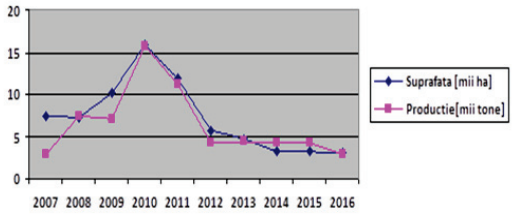

Figure 1. Correlation between aromatic plants surfaces and productions obtained during 2007-2016

Processing of medicinal plants can lead to: essential oils and hydrosols (floral waters), which are very concentrated mixtures of volatile chemicals; pharmaceutical products for pharmaceutical companies that produce drugs based on medicinal herbs extracted, or use plant derived compounds as raw material; herbal products such as extracts, teas, tinctures, capsules, etc.; nutraceuticals/functional foods that represent a group of products called health products or dietary supplements that are classified as fortified foods with health benefits other than basic diets; natural dyes. They are also increasingly used in other industries as: paints and varnishes, food, ecological industry and painting restoration; cosmetic and personal care products for cosmetic companies that produce a wide range of beauty and personal care products, hair care, perfumes and floral waters; recent group of cosmetics that contain products containing one or more bioactive compounds and which are used to enhance health and beauty. Some important ingredients used in the cosmetics industry are: oils, fats and waxes, essential oils and oleoresins, plant extracts and dyes; plant protection products in the form of extracts or compounds which can be directly used and others serve as precursors for the production of protective agents used against weeds, insects, pathogens (Vinod et al., 2014; Muthe et al., 2016).

EXTRACTION TECHNIQUES OF VOLATILE / ESSENTIAL OILS FROM MEDICINAL PLANTS

Volatile/essential oils are aromatic oily liquids obtained from various parts of medicinal plants, soluble in organic solvents and lipids, having a generally lower density than water. Among extraction techniques for obtaining volatile/essential oil, the most commonly used are:

Steam distillation - volatile substances are entrained by water vapours, even if they have high boiling points. A diffusion process of the volatile oil from the plant cells takes place before, depending on its location in the plant or on the chemical composition. When oil diffusion becomes more difficult, or when components have high viscosity and remain on the vessel walls, organic solvents (2-3%) such as benzene, hexane, etc. are also used. The laboratory uses Clevenger installations type, where the water recirculates, the plant being in constant contact with water. In industrial distillation plants there are fixed boilers installed on special platforms or mobile boilers so that planting can be done directly from the harvesting site. After mixing the volatile oil, it is collected in the Florentine vessels where the oil is decanting and separating (Tamas et al., 1996; Handa et. al., 2008).

The hydro diffusion - water vapours is forced to make a reverse circuit, which leads to saving time and energy, thus avoiding the degradation of the obtained oil quality (Tamas et al., 1996; Handa et. al., 2008).

Rotary Cones Column - distillation is done at lower temperatures, the plant material is loaded through the top, flows along the cones and it is spread over the surface of the rotating cones in a thin layer. The bottom-feed water vapours flows counter currently to the plant product and drives the volatile components into the condenser and then into the Florentine vessel (Tamas et. al., 1996; Huie C., 2002).

Vacuum microwave hydro distillation (VHMD) - azeotropic entrainment of volatile oil with vapours obtained only from the existing water in the vegetal material, is carried out in the extraction vessel and the distillation temperature is lower than 100°C. This technique is highly efficient, leading to high quality oil obtained in shorter extraction time (up to 10 time faster than classic hydro distillation) and saving energy, water, time and labour too (Tamas et al., 1996; Filly et. al., 2014).

Extraction with supercritical fluids - the most used supercritical fluid is considered to be CO_2, free of toxicity and with high dissolving power of volatile components in oils. Extraction

occurs spontaneously, without other solvents, at low temperature (Majdalani et al., 1993; Tamas et al., 1996). This extraction method protects very well the volatile oil constituents against thermal distortion.

Extraction with organic solvents involves the use of hexane, petroleum, petroleum ether or ethanol at about 50°C. Since some volatiles (pigments, waxes) are extracted too with volatile components, further steps are required to remove them. This technique is used on delicate plants with lower content of aromatic compounds and has the disadvantage of longer extraction period and high solvent consumption.

The quality of the oil and the yield of extraction are determined by a number of factors: the quality of the plant material, the available installations and the rigorous control of the extraction process parameters. The quality of the plant product is also influenced by a number of factors (Paun, 1988; Fleurentin et al., 1990; Tamas et al., 1996). Among these can be mentioned: pedoclimatic factors, applied crop technologies, photoperiodicity, the part of the plant where volatile oil accumulates, the used chemistries, the traumas produced by some pathogens and pests, the harvesting period, the process of processing the vegetal material etc.

CHEMICAL COMPOSITION OF VOLATILE/ESSENTIAL OILS

These very complex natural mixtures can contain about 20-60 compounds in different concentrations, mainly monoterpenes, sesquiterpenes and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides). Some volatile compounds include phenylpropanes and sulfur-specific or nitrogen-specific substances. Saturated or sulphuris compounds (e.g.: glucosinolates or isothiocyanate derivatives found in garlic and mustard oils) are also secondary metabolites of various plants. Generally, the volatile/essential oil composition is a balance between the different contained compounds, although in many species a constituent can prevail over all others. They are characterized by two or three main components in fairly high concentrations (20-70%), compared to other components present in small quantities. These important components determine the biological properties of the essential oils. The compounds include two groups of distinct biosynthetic origin (Croteau et al., 2000; Betts, 2001; Bowles, 2003). The main group is terpenes: monoterpenes such as monocyclic carbides (e.g.: cimenels, sabinens, etc.), bicyclic carbides (e.g.: alpha and beta pinen), acyclic alcohols (e.g.: citronellol, geraniol), phenols (e.g.: carvacrol, thymol) and terpenoids (e.g., ascaridol, menthol, etc.) and the other of the aromatic and aliphatic constituents (e.g., cinemaldehyde, chavicol, eugenol, estragole, anethol, etc.), characterised by low molecular weights. The main botanical families containing these compounds are: *Apiaceae* (e.g., *Parsley* with 7.71-10.56% apiol, *Anise* with 80-90% anethol, *Fennel* with 50-70% trans-anethol), *Lauraceae* (e.g. *Cinnamon* with 65-80% cinnamaldehyde), *Lamiaceae* (e.g. *Lavender* with more than 25% linalool). According to Chen et al. (2012), secondary metabolites of medicinal plants are predisposed to qualitative and quantitative variations, depending on several factors such as: genetic deviation, physiological conditions, season and harvest period and phenological development stage of the plant. Farming practices (spacing and harvesting period) critically influence quantitative characteristics of many medicinal and aromatic plants, which ultimately lead to general plant growth and increased yields. Long time, the optimum period for harvesting plant raw material was considered to be one of the most important factors influencing the accumulation of volatile oil in plants (Lee and Ding, 2016). Specialty literature shows that there is a direct correlation between the ontogeny of medicinal and aromatic plants and the temperature, daylight (Sangwan et al., 2001). In the study by Halva et al. (1992) it was reported that the dill growth and accumulation of essential oils is increased with light levels and the highest accumulation level was recorded under full sunlight. It is believed that the production of secondary metabolites is stimulated by the stressful environment. Meteorological parameters (temperature and precipitation) influence the quantity and composition of volatile oils in several medicinal and aromatic plants. Temperature and humidity are the most important factors

influencing the oil content of the *Lamiaceae* family (Verzea M., 2002). Cooler nights and hotter days have a negative effect on the oil content of several medicinal and aromatic plants. The hyssop (*Hyssopus officinalis* L.*)* achieves the maximum increase in leaf oil content under hot, sunny days. Poor clouds or low temperatures adversely affect both parameters. The variability of morphogenetic, ontogenetic, diurnal and ecological factors affects secondary metabolites of plants, especially essential oils and their compounds. In addition, the highest essential oil content is obtained during the warm period, between plant growth and complete blossoming (Süleyman et al., 2016). The question is whether the biological effects of volatile / essential oils are the result of the synergism of all competing molecules, or reflect only the action of the main molecules present at the highest levels according to chromatographic analyses. Most of the research papers report, only the concentration of the main constituents of essential oils such as: *terpineol, eugenol, timol, carvacrol, carvone, geraniol, linalool, citronelol, nerol, safrole, eucalyptol, limonene, cinnamaldehyde*. It is generally observed that those major compounds sufficiently reflect the biophysical and biological characteristics of the volatile oils from which they were isolated (Ipek et al., 2005), the magnitude of the effects depending only on their concentration when tested alone or contained in the oils they come from. The synergistic action of the various chemical compounds contained in a volatile oil, compared to the action of one or two main components of the volatile oil, seems to be questionable. However, it is possible that the activity of the main components to be modulated by other minor molecules (Santana-Rios et al., 2001; Hoet et al., 2006). Probably many components of essential oils play a role in defining perfume, density, texture, colour, cell penetration (Cal, 2006), lipophilic or hydrophilic attraction, and cell wall, membrane and cellular distribution. This latter feature is very important because the oil distribution in the cell determines the different types of reactions produced, depending on the place it occupies in the cell. For biological purposes, it is more appropriate to study the whole oil than a few components, because the concept of synergy seems to be significant.

ALLELOPATHIC POTENTIAL OF MEDICINAL PLANTS AND VOLATILE/ESSENTIAL OILS

The ability of plants to inhibit or stimulate the growth of other plants by releasing chemicals into the environment has been called allelopathy. So the beneficial or harmful effect of a plant on another plant by producing chemical compounds that it releases directly or indirectly to the environment is called allelopathy (Siddiqui et al., 2009). The allelopathic phenomenon has long been noticed, but its intensive, scientific research to identify the chemical compounds involved (allelopathic substances-secondary metabolites of low molecular weight plants present in different plant parts) is done since a few years ago. The allelopathic interactions between plants are of practical importance, especially in the case of plants of economic interest (such as medicinal plants), when other species are successively cultured on the same field. In this way, the allelopathic compounds derived from the remaining vegetable residues from the previous culture, have inhibitory/stimulating effects on the growth and development of other plant species (Netzly and Butler, 1986). Understanding and controlling such phenomena in the future creates the possibility of using these allelopathic compounds either as growth regulators or as natural bio-pesticides / pesticides. Recent studies have shown the allelopathic capacity of volatile oils and hydrosols (floral waters) obtained by various methods from medicinal plants. Volatile oils are spread in the plant, some families being very rich in such substances, both in number and quantity (e.g.: *Pinaceae, Lamiaceae, Umbeliferae, Myrtaceae, Lauraceae, Rutaceae, Caryophylaceae*, *Asteraceae* etc.). These allelochimic substances indirectly influence plant growth, inhibit the activity of microorganisms such as nitrifying bacteria and ectomycorrhizers that help to fix nitrogen (Hunter and Menges, 2002). Allelopathy is one of the factors that help a plant to settle in an ecosystem (Moktar Hossain et al., 2012). The use of synthetic herbicides everywhere in the world has led to the emergence of weed species

that are resistant to herbicides. Environmental concerns about herbicide safety have led to the finding of weed management systems that are not so dependent on herbicides. Considering these, allelopathy was recognized as a new approach, a new ecological weed management system (Pukclai and Kato-Noguchi, 2011). Allelopathy has a considerable role in agricultural ecosystems, so that the growth and development of crops, weeds and trees maybe influenced. Research papers has shown that the allelopathic effects are positive or negative, depending on the applied dose and the body. Several authors (Ruszkowski D., 2004; Arouiee H., 2010; Gahukar R.T., 2012) argued that allochimic substances released into the environment affect the plants closed to them, by reducing cell membranes, their permeability, interrupting the absorption of substances minerals and cause damage to the genetic material. In Asian and African countries, due to growing demands for wild native plants as herbal remedies, medicinal plants have become a component of agricultural ecosystems (Badmus and Afolayan, 2012). Many plant species have been investigated for their allelopathic potential, especially aromatic plants capable of producing a large amount of chemical substances (Campiglia et al., 2007). Different secondary metabolites known as allelochimic susceptors such as monoterpenes, sesquiterpenes and alpha-pines from essential oils of specific plants prevent germination of seeds and cause morphological and physiological changes in plant growth (Badmus and Afolayan, 2012). Many plant species have been investigated for their allelopathic potential, especially aromatic plants capable of producing a large amount of chemical substances (Campiglia et al., 2007). Different secondary metabolites known as allochimic susceptors such as monoterpenes, sesquiterpenes and alpha-pines from essential oils of specific plants prevent germination of seeds and cause morphological and physiological changes in plant growth (Badmus and Afolayan, 2012). Mahboobi and Heidarian (2016) have studied the allelopathic effects of medicinal plants on the germination and growth of seedlings of various weed species. Three different weed species (*Peganum harmala, Alyssum* spp*., Amaranthus retroflexus*), which were applied in five dry matter concentrations from four medicinal plants (*Menta, Rosmarin, Lavandula* and *Achilea*) were used. The effect of different concentrations of dry substances on germination, plumula length, root length, vigor and weight of plantule was studied. The results obtained indicated that the weeds show different sensitivities to the allelochimic substances present in the medicinal plants. Also, the inhibitory effects of the medicinal plants were different, which could be due to the type, quantity and characteristics of the allelophathic substances produced by these plants. The conclusions of this study have shown that at higher concentrations of medicinal herbs, more inhibitory effects on germination and growth of weed seedlings were observed and can be successfully used in organic crops and for the production of natural herbicides. Many studies (Piyo et al., 2009, Saad et al., 2012) investigated antifungal, antibacterial and antioxidant activity of volatile/essential oil obtained from basil-*Ocimum* spp. Seven fungi species have been isolated from plants: *Alternaria* sp*., Aspergillus flavus, Botrytis cinerea, Cladosporium herbarum, Eurotium amstelodami* and *Eurotium chevalieri. E. Chevalieri* was most sensitive to basil oil, while *A. flavus* was the most resistant (Jakowienko et al., 2011). Basil oil proved a strong antifungal activity against fungi, such as: *Penicillium aurantiogriseum, Penicillium glabrum, Penicillium chrys ogenum* and *Penicillium brevicompactum* (Kocić-Tanackov et al., 2012). The greatest sensitivity was recorded by the *P. chrysogenum* with complete inhibition of growth, at a concentration of basil oil extract of 1.5%. Basil oil at a concentration of 0.6% v / v had a 100% inhibition of mycelial growth on pathogenic fungi: *Fusarium moniliforme and Pyricularia arisea* and of 50% inhibitionat *Fusarium proliferatum*, but was not efficient against *Rhizoctonia solani* fungi. Volatile basil oil at a concentration of 0.8% v / v may inhibit germination of *F. monoliformespores* (91%) and *Alternaria brassicicola* (100%) (Piyo et al., 2009). Eugenol (a compound present in volatile essential oil) has been shown to have a very strong antifungal activity against *Absida glauca, Aspergillus nidulans, Aspergillus niger, Colletotrium chamus, F. monoliforme, Pestoltia psidi* and *Rhizopus nadssus* (Soković

et al., 2013). Linalool and anethol showed good antifungal activity at doses of 0.03-0.3 mg / mL and 1.3-2.8 μL / mL respectively in microdilution tests (Soković et al., 2013). Pest management is confronted with safety and efficacy challenges due to human and environmental hazards caused by the widespread use of synthetic insecticides. Insecticides obtained from plants are of great interest and developing (Patruica et al., 2017). Basil oil has shown increased toxicity to locusts (*Acrida exaltata*) and fruit flies (Chang et al., 2009). The LC50 average lethal concentration in basil oil against *Planococcus ficus* ranged from 44 to 47 mg/ml (Karamaouna et al., 2013). Basil oil is a potential control agent against mites (*Tetranychus urticae*) and white mussel (*Bemisla tabaci*) in greenhouse conditions. Basil oil has shown antifungal and insecticidal activity against *Aphis craccivora* Koch. Successful adoption of basil oil in the protection of food promises an ecological option compatible with international regulations on biosecurity. Extract and essential oils obtained from Hyssop-*Hyssopus* spp., show insecticidal properties against flies, beetles, green caterpillars, mites, larvae of worms, cabbage moths, mushrooms, etc. The plant attracts hummingbirds, butterflies, bees, etc. which are good pollinators for plants growing in the vicinity of hyssop. Hyssop oil has toxic, antibacterial and antifungal properties and is used against many plant diseases (e.g. *Phytophtora infestans*). The allelophatic activity of hyssop oil at different concentrations was evaluated against seed germination and root growth in crop species: radish (*Raphanus sativus* L. c. Saxa), garden mustard (*Lepidium sativum* L.) and salad (*Lactuca sativa* L.) (Almeida et al., 2010). In general, volatile oils obtained from medicinal plants containing low molecular weight compounds and lipophilic properties can easily penetrate through cell membranes to induce biological reactions. The hyssop plant - *Hyssopus officinalis*, possess antimicrobial activity *in vitro* against *Klebsiella* sp. and *Erwinia amylovora*, two important pathogens and it can be used in the treatment of plant diseases (Dehghanzadeh et al., 2012). In addition, *in vitro* and *in vivo* antifungal activity of hyssop oil, was evaluated against two phytopathogenic fungi, *Pyrenophora avenae and Pyricularia oryzae* - mycelial growth of fungi was completely inhibited by hyssop oil at a concentration of 0.4%. Also hyssop oil determined reduced germination of *Botrytis fabae* and *Uromyces viciae-fabae* (Letessier et al., 2001). Two volatile/essential hyssop oils demonstrated a significant antifungal activity against 13 strains of phytopathogens. Experimental reports have shown that 100% inhibition for all pathogens has been achieved when using 1600 ppm hyssop oil (Fraternale et al., 2004). The hyssop extracts have a higher antioxidant activity than the volatile/essential oils because the latter are mainly rich in oxygennated monoterpenes, which are known to have low activity against free radicals (Baj et al., 2010).

CONCLUSIONS

Due to the increased interest for natural products such as volatile/essential oils, it is important to know biological activity for developing new applications in human health, agriculture and environmental protection. The biological effects of volatile/essential oils could be the result of a synergy of all molecules, or could only reflect the activity of the main ones. Almost all literature cases analyze only the main constituents of volatile / essential oils obtained from medicinal herbs. In this sense, it may be better that oils to be studied as a whole rather than for some components, because the allelopathic interactions are of practical importance, especially in the case of plants of economic interest, such as medicinal plants. The use of volatile/essential oils to control weeds in organic farming seems promising, but these natural herbs react quickly, and their effectiveness is limited by their higher volatility. Alternative formulation such as microencapsulation can be developed in order to reduce the applied amounts and to increase the effectiveness in time by reducing volatility, reducing material handling, and slowing down the decomposing rate within the environment.

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SUSCEPTIBILITY OF SOME MELOLONTHINE SCARAB SPECIES TO ENTOMOPATHOGENIC FUNGUS *Beauveria brongniartii* (Sacc.) Petch AND *Metarhizium anisopliae* (Metsch.)

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Abstract

The melolonthine scarabs, Melolontha melolontha L., Amphimallon solstitiale L. and Anoxia villosa F. are well known as serious pest in orchards, vineyards, forests and fruit tree nurseries. The susceptibility of third instars larvae of M. melolontha, A. solstitiale and A. villosa to entomopathogenic fungus Beauveria brongniartii (three isolates) and Metarhizium anisopliae (three isolates) was evaluated in laboratory conditions by dipping the insects in aqueous suspensions of $1x10^7$ conidia/ml, respectively. The greatest mortality rates were observed to be caused by B. brongniartii isolates: 100% when M. melolontha larvae were treated and 60% mortality for A. villosa after 60 days of incubation. The most susceptible larvae species to M. anisopliae was A. villosa (35.5% mortality). The most resistant larvae species to all the fungal treatments was A. solstitiale at which mortality rate never exceeded 23.6% after 76 days of incubation.

Key words*: Melolontha melolontha, Amphimallon solstitiale, Anoxia villosa, Beauveria brongniartii, Metarhizium anisopliae, entomopathogenic fungus.*

INTRODUCTION

The melolonthine scarabs, *Melolontha melolontha* L, *Amphimallon solstitiale* L. and *Anoxia villosa* F. are well known as serious pest in Romanian forest nurseries (Simionescu et al., 2012; Arinton and Ciornei, 2015; Varga et al., 2015). Also called „white grubs" in larval stage, these polyphagous species feed on the roots of the plants and as adults they eat the leaves of young hardwoods and softwoods. Scarabs are a family of pest insects very difficult to control, on the one hand because of the resistance to chemical insecticides (Buss, 2006) and on the other hand because of the way of feeding, which makes difficult the penetration of insecticides in soil, to the root area. Considering these issues, the forest management added new regulations which prohibit or restrict the use of a large number of chemical insecticides.

Biological control using natural enemies of pest population has gained considerable attention over the past twenty years. Although entomopathogenic viruses, bacteria and nematodes have been investigated as potential insecticidal agents, entomopathogenic fungi were most often used in classical biological control programs (Hajek et al., 2007).

The entompathogenic fungi *B. brongniartii* and *M. anisopliae* are widespread soil borne pathogens which have been intensively studied for the biological control of a wide range of insect pests including scarabs (Rath, 1988; Raid and Cherry, 1992; Samuels et al., 1990; Yip et al., 1992; Ansari et al., 2006; Keller et al., 2003; Rodríguez-del-Bosque et al., 2005; Srikanth et al., 2010; Goble et al., 2015). Fungal infection begins with the attachment of conidia on insect's cuticle, which germinates by producing a germ tube and penetrate the integument by mechanical or enzymatical means, invading the host tissues. Once the fungus penetrates the host, it colonizes the haemocoele and multiplies, generating toxic metabolites and killing the host. Subsequently, the hyphae penetrate through the insect cuticle to the outside and, in proper conditions, form white (*B. brongniartii*) or green (*M. anisopliae*) mycelial masses on insect's body. In Europe,

biological products that are used in the biological control of whiteworms are Nema-green® (E-nema), B-Green® (Biobest), Terranem® (Koppert), to name a few. In Romania, the current strategy against whiteworm infestation includes forecasting measures (conducting soil surveys - during autumn), physico-mechanical measures (flooding uncultivated land, the use of trap plants) and agrotechnical measures (plowing and rotovating with rotary hoe) (Simionescu et al., 2000). Mechanical control is not possible in fields with young crops. In this study, laboratory bioassays were performed in order to select pathogenic isolates for subsequently field trials.

MATERIALS AND METHODS

Fungal isolates

The used fungal isolates belong to the entomopathogenic fungi collection of RDIPP and the place of isolation are described in Table 1.

Table 1. The origin of fungal isolates

Fungal name	Strain name	Host insect	Place of isolation
Metarhizium anisopliae	MaF	larva of *Anoxia villosa* Fabricius	Feteşti nursery (Ialomiţa county)
Metarhizium anisopliae	MaTVd	larva of *Anoxia* sp. Fabricius	Tudor Vladimirescu nursery (Galaţi county)
Metarhizium anisopliae	MaGp	adult of *Epicometis hirta* Poda	Timişoara county
Beauveria brongniartii	ICDPP# 2	larva of *Melolontha melolontha*	Roman nursery (Neamţ county)
Beauveria brongniartii	ICDPP# 3	larva of *Melolontha melolontha*	Dumbrava nursery (Neamţ county)
Beauveria brongniartii	ICDPP# 4	larva of *Melolontha melolontha*	Obicioara nursery (Bacău county)

To maintain the virulence, fungal isolates originated from pure cultures and maintained on the PDA medium were passed periodically through host insects.

For the preparation of conidian suspensions, 15 days age fungal slant cultures were used, resulting from stock cultures, subcultivated no more than 3 times, in order to avoid loss of virulence. Conidian suspensions were prepared by flooding the sporulated cultures with 5 ml aqueous solution of Tween 80 (0.01%) and vortexed for one minute at 1600 rpm with one gram of glass beads (2 mm in diameter), using a vortex-mixer (Velp Scientifica, Europa). The conidian suspensions were diluted for counting, using the Burker hemocytometer, under microscope (magnification 400 x).

Test insects

M. melolontha and *Amphimallon* sp. larvae were collected from nurseries in the counties Vâlcea and Vrancea, respectively, where the infestation was very high. *A. villosa* larvae were collected from a nursery in Feteşti (Ialomiţa county). Before being used in the experiment, larvae were quarantined for three weeks, with the exception of *A. villosa* larvae that were used immediately in the test. Only visible healthy individuals were used.

Biotesting

M. melolontha larvae were treated with *B.brongniartii*, respectively *Metarhizium* sp. by simultaneous dipping in 100 ml spores suspension of $1x10^7$ for 10 seconds, and then transferred into groups of 5, in disposable plastic food containers (8.5x10.5x5 cm). The boxes were filled with 300 g uncontaminated commercial soil-peat substrate and perforated for air exchange. Each replicate (x 4 boxes) consisted of five larvae from the same batch of insects. In each box were added small pieces of carrots for larval feeding. For the control variant, the larvae were treated with sterile distilled water and Tween 80 (0.05%). The boxes were incubated at 20 ± 2^0 C, in complete darkness. Mortality was recorded weekly for 60-76 days by overturning the entire contents of each box on sterile, single use substrates. The dead larvae were removed and placed in wet rooms to encourage the development of mycosis. The living larvae were returned in boxes and evaluated in the next week. Each larval species was treated in three different days. The entire experiment was performed on three different dates, using each time the conidian suspensions prepared in the test day, from the same batch of fungal cultures.

Statistical analysis

The mortality rates of fungal treated larvae were analyzed using the Kaplan Meier survival curves, and the log-rank test was applied to the significance threshold p <0.05 in the GrapfPadPrism version 5.00 for Windows (GraphPad Software, San Diego California USA). The individuals who survived until the end of the observation period were considered censored.

RESULTS AND DISCUSSIONS

Susceptibility of *M. melolontha* larva to *B. brongniartii* and *M. anisopliae* isolates

B. brongniartii (Sacc.) Petch fungus is well known to be the most important natural enemy of *M. melolontha* (Keller et al., 1997; Keller et al., 2003; Enkerly et al., 2004; Zimmermann, 2007) and a well-established active ingredient in bioinsecticide against insect pests (Faria and Wraight, 2007).

As expected, the highest *M. melolontha* larval mortality has been caused by the three strains of *B. brongniartii*.

Comparison of survival curves indicated an insignificant difference in susceptibility of *M. melolontha* larvae to treatment with different isolates of *B. brongniartii* (log-rank =3.54; p=0.17). A 100% mortality was recorded for larvae treated with ICDPP# 4 strain after 59 days of incubation. Those treated with the ICDPP# 3 strain recorded 89% mortality and the ICDPP # 2 strain determined a mortality of 68%. A single individual from the control variants was recorded with mycosis, 59 days post-treatment. The average survival periods for *M. melolontha* larvae treated with ICDPP *B. brongniartii* strains #2, #3 and #4 were 39, 37 and 38 days, respectively (Figure1).

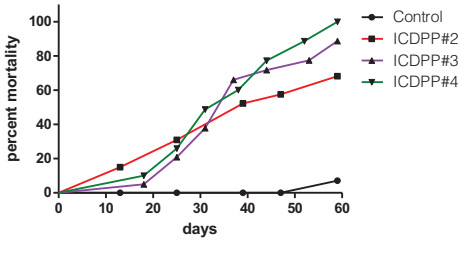

Figure 1. The mortality rate of *Melolontha melolontha* larvae (L3) treated with conidial suspension ($1x10^7$ conidia/ml) from different *Beauveria brongniartii* isolates

The mortality rate due to *M. anisopliae* infection was very low, ranging between 8% and 23% in the treated variants, after 53 days (Figure 2). For these values, average survival period could not be calculated because mortality rates were below 50%.

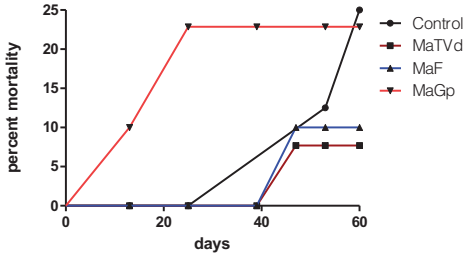

Figure 2. The mortality rate of *Melolontha melolontha* larvae (L3) treated with conidial suspension ($1x10^7$ conidia/ml) from different *Metarhizium anisopliae* isolates

Susceptibility of *A. villosa* larvae to *B. brongniartii* and *M. anisopliae* isolates

A. villosa larvae showed susceptibility to *B brongniartii* isolates and the highest mortality was of 60%, recorded among larvae treated with ICDPP # 4 isolate for which a median survival time of 59 days was obtained (Figure 3).

Comparison of survival curves indicated insignificant differences in the susceptibility of *A. villosa* larvae to treatment with different *B.brongniartia* isolates (log-rank = 0.10; p = 0.43).

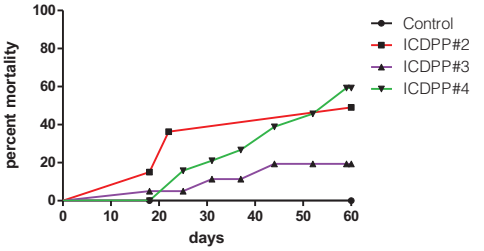

Figure 3. The mortality rate of *Anoxia villosa* larvae (L3) treated with conidial suspension ($1x10^7$ conidia/ml) from different *Beauveria brongniartii* isolates

M. anisopliae treatments led to infections of *A. villosa* larvae in very low percentages (<35.4%) and the mortality rate in control variant was 11.7% (Figure 4).

Comparison of survival curves showed insignificant differences in the susceptibility of

A. villosa larvae to treatments with various isolates of *M. anisopliae* (log-rank = 3.08, p = 0.21).

Although the MaF isolate originating from an *Anoxia* sp. larvae, its pathogenicity was very low under conditions of this study. This could happen because of an adaptation in time of this pathogen with his host. Also, no major larval mortality due to *M.anisopliae* was observed in field highly infested with *A. vilosa* from witch Maf isolate was obtained. Contrary to these results, when Raid and Cherry (1992) tested three isolate of *M. anisopliae* originated from different white grub species on *Ligyrus subtropicus* Blatchley larvae, only the isolate originating from *L. subtropicus* was pathogenic.

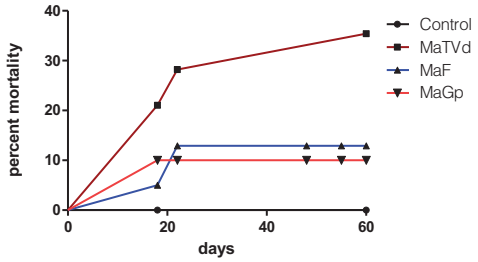

Figure 4. The mortality rate of *Anoxia villosa* larvae (L3) treated with conidial suspension ($1x10^7$ conidia/ml) from different *Metarhizium anisopliae* isolates

Susceptibility of *A. solstitiale* larvae to *B. brongniartii* and *M. anisopliae* isolates

Larvae of *A. solstitiale* showed a very low susceptibility to the *B. brongniartii* isolates, the highest mortality being 18%, after 60 days, and the mortality rate in the control variant was 5% (Figure 5).

Comparison of survival curves revealed insignificant differences in the susceptibility of *A. solstiliale* larvae to treatment with different isolates of *B. brongniartii* (log-rank = 1.07, p = 0.58). Pigmented points were observed on the cuticle of a live larva treated with *B.brongniartii.* These points could be melanin deposits occurring as a result of defense reactions at the penetration point of the fungus hyphae in the insect's cuticle (Gillespie et al., 1997). These defense reactions can explain the resistance of larvae to fungal attack.

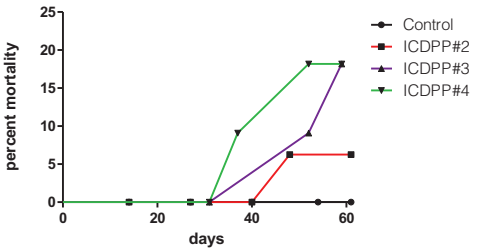

Figure 5. The mortality rate of *Amphimallon solstitiale* larvae (L3) treated with conidial suspension ($1x10^7$ conidia/ml) from different *Beauveria brongniartii* isolates

Treatments with *M. anisopliae* caused infection of *A. solstitiale* larvae in treated variants, but the mortality rate was very low. Mortality was also recorded in the control variants, which made the comparison of survival curves unjustifiable. After 60 days, the highest mortality (18%) was recorded in the variant treated with the MaTVd isolate, increasing to 23% after 76 days (Figure 6).

Results of laboratory studies on the screening and selection of virulent isolates of entomopathogenic fungi for the control of scarabs showed that the fungal isolates differed in pathogenicity and virulence against scarab-beetle species. In our tests both *B. brongniartii* and *M. anisoplie* endemic isolates proofed to be pathogenic for tested scarab species with different degrees of pathogenicity between fungal species of the same test insect species. No variation was found in pathogenicity of each isolate of the same fungal species.

Hadapad et al. (2005) reported differences in virulence of *B. brongniartii* isolates obtained from wide geographical and host origins, against the larvae of scarabs *M. melolontha* and *Holotrichia serrata* L. in screening and selection tests.

In our laboratory tests, *M. melolontha* and *A. villosa* larvae have shown greater susceptibility on *B. brongniartii* isolates compared with *M. anisopliae* isolates. Also, the results showed that *A. villosa* and *A. solstitiale* larvae (L3) are part of both *B. brongniartii* and *M. anisopliae* insect host spectrum. *B. brongniartii* is known to have a very narrow host specificity being a selective pathogen for *M. melolontha* (Hajek et al., 1994). In Romania, natural infected larvae of *M. melolontha* was reported by Ciornei et al. (2006). Larval stage diseases in field

experiments with endemic isolates of *B. brongniartii* and other scarabs such as *Anomala dubia* Scopoli and *Rhizotrogus aestivus* Olivier (Fătu et al., 2016) have also been reported.

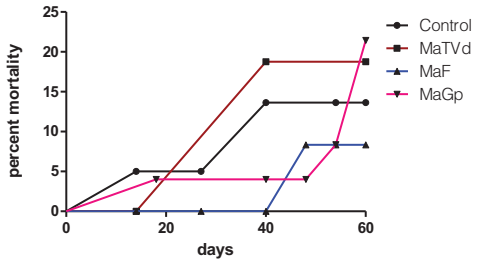

Figure 6. The mortality rate of *Amphimallon solstitiale* larvae (L3) treated with conidial suspension ($1x10^7$ conidia/ml) from different *Metarhizium anisopliae* isolates

In our experiments, *A. solstitiale* have proven to be resistant both to *Beauveria* and *Metarhizium* isolates. This could be explained by field insect populations that are continuously exposed to pathogens and may produce offspring with increased resistance to infection (Moret, 2006). So it is possible that *A. solstitiale* populations from Vrancea had been continuously confronted with *B. brongniartii* and *M. anisopliae* (data not shown). Another explanation might be the low susceptibility of the larval stage in which larvae of *A. solstitiale* were tested. Not all stages in an insect's life cycle are equally susceptible to infection by entomopathogenic Hyphomycetes (Inglis et al., 2001). Thus, in time mortality studies performed by Yubak Dhoj (2006) on fungus isolates indicated different pathogenicity of conidiospores and blastospores against three instars of white grubs *Maladera affinis* (Coleoptera, Scarabaeoidea), second instar larvae have shown greater susceptibility than first and third instars of infected grubs. The mortality percentage varied between 50%-80%. The most virulent isolates registered a LT_{50} of 2-4 weeks. Also Xiangcun Nong et al. (2011) founded no differences in susceptibility of all three instars of *Holotrichia oblita* Faldermann (Coleoptera: Scarabeidae) to some isolates of *M. anisopliae* and *B. brongniartii* but the younger instars of *A. corpulenta* (Coleoptera: Scarabeidae) were more sensitive than older instars to entomopathogenic fungi. In our experiment, the 3-rd instar larvae of *Melolontha* were very sensible to *B. brongniartii* isolates which is consistent with frequently literature reports, both in filed or laboratory (Hurpin and Vago, 1958; Ferron, 1967; Keller, 1997).

Although isolates of entomopathogenic fungi from *Amphimallon* larvae are reported (Keller, 2007; Kocacevik et al., 2015), reports on pathogenity of entomopathogenic fungus against this pest in field or laboratory are rare. Benker and Leuprecht (2005) obtained promising results in field trials with *B. brongniartii* (in the form of Melocont-Pilzgerste) against second larval stage of *M. melolontha* and *A. solstitiale*. The reduction of pest population was 80% of the starting number of the grubs in the variant treated with the fungus. Peters and Vlug (2005) have been successful in controlling L2 larvae of *A. solstitiale* using the nematode *Heterorhabditis bacteriophora*. Also Sezen et al. (2005) obtained high mortality for second and third instar larvae of *A. solstitiale* of 90% using *B. cereus* isolated from *A. solstitiale*, and of 100% using both mixture of this isolate of *B.cereus* with *B. sphaericus* and *B. thuringiensis,* within ten days.

Comparative studies on the susceptibility of soil-dwelling insect pests to *B. bassiana, B. brongniartii* and *M. anisopliae* led to different results. Beron and Diaz (2005) reported that different isolates of *B. bassiana* were generally more virulent to most soil-dwelling insect pests than *M. anisopliae*. Larvicidal bioassays on *Polyphylla fullo* (Coleoptera: Scarabaeidae) show also that *B. bassiana* is more effective than *M. anisopliae* both in young and older larvae (Erler and Ates, 2015). On the contrary, tests on *Anomala cincta* (Coleoptera: Melolonthidae) proved higher virulence of *M. anisopliae* compared with *B. bassiana* isolates (Guzman-Franco et al., 2012), according to the studies of Klein et al. (2000) which consider that *Metarhizium* species are better adapted to infect soil-dwelling insects than *Beauveria* species.

Besides pathogenic microorganisms, parasitoids like *Dexia rustica* F., *Dexiosoma caninum* F. (Walker 1944; Vanhara, 2009) and *Pexopsis aprica* Meig (Lutovinovas et al., 2014) were found to attack larvae and adults of *M. melolontha*, respectively. *Dexia rustica* also

infect *Amphimallon* species (Huiting et al., 2006). Also the protozoa *Nosema melolonthae* Krieg and *Adelina melolonthae* Tuzet (Hurpin, 1968) and neogregarian protozoa (Yaman et al., 2016) have been recorded from these hosts. Larvae of wasps like *Scolia hirta* Schrank develop within soil larvae of scarab beetle including *M. melolontha* (Banazsak and Twerd, 2009).

CONCLUSIONS

The third instar larvae of melolonthine scarabs, *M. melolontha*, *A. villosa* and *A. solstitiale* that were tested under laboratory conditions by immersion in conidial suspensions showed a different susceptibility to different entomopathogenic fungi: the larvae of *M. melolontha* and A. *villosa* showed a higher degree of susceptibility to *B.brongniartii* compared to *M. anisopliae*; *A. solstitiale* showed a low susceptibility to both fungal species. No significant differences in susceptibility of scarab larvae to different isolates of the same entomopathogenic species were registered.

The high mortalities of *Melolontha* and *Anoxia* third instar larvae indicate that isolates of *B. brongniartii* can be considered as future candidates for biological control of these white grub species. Further tests on young instar larvae of *Amphimallon* must be performed.

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VARIATION IN PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF DIFFERENT PLANT PARTS OF *Primula veris*

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Abstract
Primula veris L. (Cowslip primrose) is a species of flowering plant in the family Primulaceae. Usually, flowers and leaves from cowslip primrose are used for making sedative tea. The cowslip roots are also used in the treatment of respiratory tract problems, as an expectorant. In the present study we compared flowers, leaves and roots from Primula veris harvested in two different locations in the Western part of Romania (Julita and Nadas). In order to evaluate the phenolic content, antioxidant activity and the chemical composition, the ethanolic extracts have been obtained by maceration for 7 days. The results obtained indicated that ethanolic extracts of Primula veris presented high phenolic content that ranged between 131 to 168 mg GAE/L for roots, 136 to 159 mg GAE/L for leaves and 133 to 219 mg GAE/L for flowers. Remarkable percent of inhibition of DPPH has been obtained for all plants parts (as roots 16.4-23.2%, leaves 16.0-17% and flowers 14.9-24.7 %.) The following compounds have been quantified in all different plant parts: gallic acid, quercetin and kaempferol using Ultra-High Performance Liquid Chromatography (UHPLC).

Key words*: Primula veris, antioxidant activity, polyphenols.*

INTRODUCTION

Primula veris L. (cowslip) belongs to the *Primulaceae* family and is distributed throughout Europe and Asia with more than 400 species. Many plant parts have a long history of medicinal usage.
Primula veris has been mainly used for the production of herbal teas and preparations that are also considered dietary supplements. Due to its flavonoid and phenolic content, *Primula veris* is used in anti-inflammatory and expectorant treatments for cold and related sinusitis affections (Varela and Ibañez, 2009).
It is a well-known medicinal plant, with an expectorant activity, sedative, decongestant, diuretic, antioxidant activity (Bogenschutz-Godwin et al., 2002).
Cowslip is being used as tea (flowers and leaves), as liquid flower extract, flower and root tincture. The main compounds identified in *P. veris* leaves and roots are triterpene saponins as well as phenolic compounds, including flavonoids, phenolic acids and phenolic glycosides (Bączek et al., 2017).
Compounds identified in *P. veris* were: primverin, primulaverin, catechin, astragalin, chlorogenic acid (Bączek et al., 2017), rutin, kaempferol, hydroxydimethoxyflavone, quercetin-3-*O*-dihexoside (Bączek et al., 2017; El Morchid et al., 2014) 3′,4′,5′-trimethoxyflavone (Varela and Ibañez, 2009).
In this study, extracts obtained from aerial parts, flowers, leaves and roots from cowslip collected in western part of Romania, were extracted with ethanol and analyzed using UHPLC.
The composition in phenolic acids, total phenol content, as well as their antioxidant activity was determined.

MATERIALS AND METHODS

Flowers, leaves and roots of *Primula veris* were collected in 2014 and 2015 from two villages of Romania: Julita (46° 2' 0" North, 22° 8' 0" East) and Nadas (46° 13' 0" North, 21° 57' 0" East). Dried plant parts was stored in paper bags until further analysis and voucher specimens were taken and preserved at the

Institute of Technical and Natural Sciences Research-Development-Innovation of „Aurel Vlaicu" University of Arad.

The plant parts were dried at room temperature (20-25°C). The extracts were obtained through maceration. Briefly, 2 g of plant material was grinded and extracted with 40 mL ethanol for 7 days. Before further analysis all extracts were filtered through PTFE (0.45 µm) (Pag et al., 2014).

Total phenolic content

Total phenolic content of the extracts obtained was determined by using Folin-Ciocalteu method slightly modified (Baranauskiene et al., 2014; Pag et al., 2014; Tepe and Sokmen, 2007). Briefly, the aqueous extracts obtained were diluted with distilled water (1:25). To 1 ml sample were added 0.5 ml Folin-Ciocalteu reagent, 2 ml Na_2CO_3(20%) and 5 ml distilled water. The mixture was kept in the dark for 90 minutes, thereafter the absorbance was recorded against a blank prepared in the same conditions, at 765 nm, by using a UV-VIS double beam spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany). Gallic acid was used as reference. A calibration curve for gallic acid was obtained (20, 40, 100, 160, 200 mg/L), then the regression equation and the correlation coefficient were calculated and the results were expressed in mg GAE/L.Total phenolic content of the extracts was thereafter expressed as equivalents of gallic acid (mg GAE/L). All experiments were performed in triplicates.

DPPH radical scavenging activity

In order to determine the antioxidant activity of the extracts the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging capacity was assessed using a spectrophotometric method (Pag et al., 2014). The DPPH was dissolved in ethanol (0.2mM) and 3 ml of the resulting solution was mixed with 0.1 ml sample (20 mg/ml). Absorbance was recorded at 517 nm after 60 minutes incubation in the dark. Gallic acid was used as reference. A calibration curve for gallic acid was obtained (2.5 to 50 mg/L), then the regression equation and the correlation coefficient were calculated and the results were expressed in mg GAE/L. Inhibition of the DPPH stable free radical was calculated with Eq. (1):

$$\%Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} x100 \qquad (1)$$

where:

$Abs_{control}$ is absorbance of 0.2 mM DPPH in ethanol,

Abs_{sample} is absorbance of 0.2 mM DPPH +extract.

Chromatographic analysis

Gallic acid, quercitin and kaempferol were determined by using an UHPLC method. Briefly, a high performance liquid chromatograph (Nexera X2, Shimadzu, Tokyo, Japan) equipped with a diode array detector (M30A, Shimadzu, Tokyo, Japan) and a Nucleosil 100-3-C18 reversed-phase column (4.0 mm i.d. × 125 mm column length, 3 µm particle size, Macherey-Nagel GmbH, Duren, Germany) were used. The column temperature was maintained at 30ºC and the flow rate at 1 ml min^{-1}. The solvents used for the chromatographic elution consisted of ultra-pure water with 0.1% TFA (A) and acetonitrile (B). The chromatographic elution program used was as follows: 95% A and 5% B that was changed linear gradient to 42% B for 5 min., followed by a linear gradient to 35% B in 30 min. Thereafter, the eluent was changed to the initial composition of 95% A and 5% B linear gradient for 5 min. The measurements have been done between 200-600 nm wavelengths.

RESULTS AND DISCUSSIONS

Primula veris L. plants were harvested in 2014 and 2015 from two locations in the western part of Romania, Julita and Nadas. Ethanolic extracts from flowers, leaves and roots were obtained by maceration. The total phenolic content and antioxidant activity of the extracts were determined. In Table 1 are presented total phenolic content (mg GAE/L) and antioxidant activity (Inhibition %) of cowslip extracts obtained from flowers, leaves and roots. Total phenolic content of the extracts was determined by using Folin-Ciocalteu method. This method estimates the total content of all phenolics present in the analyzed extracts, including flavonoids, anthocyanins and non-flavonoid phenolic compounds.

As it is shown in Table 1, total phenolic content varied between different plant parts from 131 to 168 mg GAE/L for roots, 136 to 159 mg

GAE/L for leaves and 133 to 219 mg GAE/L for flowers. The highest content of phenols (218.5 mg GAE/L) was achieved in extracts obtained from flowers from Julita collected in 2015.

Table 1. Total phenolic content (mg GAE/L) of cowslip extracts obtained from flowers, leaves and roots collected in Julita and Nadas in 2014 and 2015

Plant parts	mg GAE/L	mg GAE/ 100 g plant material
roots Nadas 2014	131.73±1.23	263.46±1.16
roots Nadas 2015	167.69±1.75	335.38±1.56
roots Julita 2014	153.65±1.13	307.31±2.03
roots Julita 2015	149.23±1.04	298.46±1.96
leaves Nadas 2014	147.12±0.98	294.23±1.93
leaves Nadas 2015	136.15±0.87	272.31±1.82
leaves Julita 2014	149.42±0.62	298.85±1.87
leaves Julita 2015	159.04±0.99	318.08±2.08
flowers Nadas 2014	162.88±0.96	325.77±1.88
flowers Nadas 2015	170.00±1.43	340.00±1.76
flowers Julita 2014	133.27±1.29	266.54±1.46
flowers Julita 2015	218.46±1.14	436.92±2.88

The total phenolic content values obtained in the present study for the ethanolic extracts of cowslip are different than that reported for aqueous-ethanolic extracts of cowslip, namely 535.4 mg GAE/ 100 g for flowers (Tünde et al., 2015) and 155.8 mg GAE/g for ethanolic extracts ultrasonicated obtained from flowers (Chilku et al., 2017). The results obtained demonstrate the importance of solvent, solvent ratio and extraction conditions used to obtain extracts from plants.

In order to determine the antioxidant properties of the extracts we used the DPPH (1,1-diphenyl-2-picrylhydrazyl) colorimetric method. As it is depicted in Table 2 the inhibition varied between16.4-23.2% for roots, 16.0-17% for leaves and 14.9-24.7 %.for flowers. The antioxidant activity obtained in the present study is different than that reported for aqueous-ethanolic extracts of cowslip, Tünde et al., 2015 obtained 86.65 % (Tünde et al., 2015).

In Figure 1 are presented the concentration of kaempferol, quercetin and gallic acid (mg/L) of cowslip extracts obtained from flowers, leaves and roots determined through UHPLC method.

The highest quantity of gallic acid was found in cowslip: 21.22 mg/L for extracts obtained from flowers Nadas in 2015 and 18.78 mg/L for leaves plants harvested in Julita in 2015. Smaller quantities of gallic acids, 9.53 mg/L, were determined in the flowers extracts of cowslip harvested in Julita in 2015.

Table 2. Antioxidant activity (Inhibition % and mg GAE/L) of cowslip extracts obtained from flowers, leaves and roots and collected in Julita and Nadas in 2014 and 2015

Plant parts	Inhibition (%)	mg GAE / L
roots Nadas 2014	16.39±0.55	8.43±0.12
roots Nadas 2015	17.81±0.37	9.14±0.16
roots Julita 2014	18.87±0.54	9.67±0.18
roots Julita 2015	23.18±0.43	11.84±0.19
leaves Nadas 2014	16.84±0.41	8.65±0.12
leaves Nadas 2015	15.99±0.51	8.22±0.14
leaves Julita 2014	16.23±0.43	8.35±0.13
leaves Julita 2015	17.00±0.37	8.74±0.15
flowers Nadas 2014	18.04±0.23	9.25±0.18
flowers Nadas 2015	18.89±0.49	9.68±0.19
flowers Julita 2014	14.93±0.47	7.70±0.21
flowers Julita 2015	24.65±0.32	12.58±0.23

From the flavonoid family we determined the content of kaempferol and quercetin in cowslip extracts. The highest amount of quercetin and kaempferol was found in extract obtained from flowers harvested in Julita in 2015, 12.08 mg/L, respectively 3.60 mg/L. Kaempferol was not identified in extracts obtained from roots. Extracts obtained from roots harvested in both locations have had the lowest amount of quercetin. Bączek K. et al., 2017 identified by HPLC in *Primula veris* and *Primula elatior* the following phenolic compounds: catechin, chlorogenic acid, orientin, rutoside (quercetin 3- O-rutinoside), astragalin, isorhamnetin-3-O-glucoside and compounds specific for *Primula* species: primverin, primulaverin. Bączek K. et al., 2017 found also, that the contents of isorhamnetin-3-O-glucoside, astragalin, and catechin were distinctly higher in the flowers of *P. veris.* (Bączek et al., 2017). Ozkan et al., 2016 assess the content of catechin, rutin, gallic acid, protocatechuic, para hydroxy benzoic acid, vanillic acid, and p-coumaric acid by HPLC in *P. vulgaris* flowers. According to this analysis, rutin and p-coumaric acid seemed to be the main phenolic compound of this raw material.

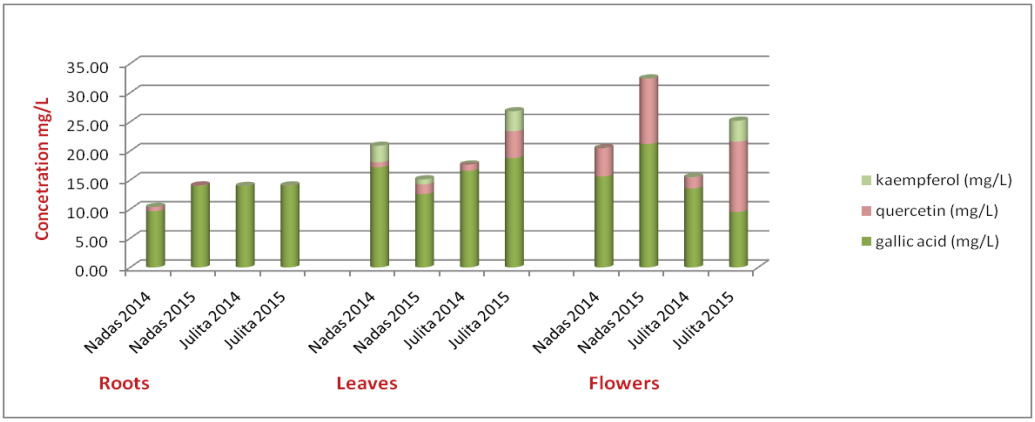

Figure 1. Concentration of gallic acid, quercetin and kaempferol determinated by UHPLC

CONCLUSIONS

The ethanolic extracts obtained from different parts of cowslip (flowers, leaves and roots) have had a remarkable quantity of phenolic content (mg GAE/L), determinated by Folin-Ciocalteu method, with a notable antioxidant activity. Chromatographic analysis revealed that extracts contain different amounts of kaempferol, quercetin and gallic acid.

In order to fully benefit from the plant bioactive compounds, we recommend the usage of flowers and leaves due to their high content.

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CHEMICAL COMPOSITION OF CAMEL MILK AND ITS BENEFICIAL EFFCTS ON HUMAN HEALTH

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Abstract

C*amel milk is considered one of the main components of the human diet in many parts of the World. The main components of whey proteins in camel milk and colostrum were similar to that in bovine, except for the lack in β-lactoglobulin. Due to its property camel milk has been also recommended to be consumed by children who are allergic to bovine milk. Other whey proteins which have been identified in camel milk include serum albumin, α-lactalbumin, immunoglobulins, lactophorin and peptidoglycan recognition protein. It has a high concentration of insulin used for diabetes. In addition, camel milk is used to stabilize the condition of patients with biliary atresia due to vitamin concentration at high concentration. Another application area of the camel milk due to its antibacterial and anti-viral properties is to use in human medicine. It has high concentration of insulin which is used for diabetes. Aditionally, camel milk is used for stabilize the conditions of biliary atresia patients due to its higher concentration of vitamin.*

Key words*: camel milk, chemical composition, beneficial effects, human health.*

INTRODUCTION

Camels were domesticated and developed approximately 5,000 years ago and throughout these years have played an integral role in the daily life of camel owners. They are distributed in Africa and Asia, where other livestock farming cannot be easily implemented (Gupta et al., 2015).

According to the recent statistics by the Food and Agriculture Organization (FAO), the total population of camels in the world is estimated to be about 20 million, with Somalia having the largest herd worldwide (FAO, 2008). Also, according to FAO data the production of camel milk is 5.3 million/liter in the world. At the present time, depending on the camel cultivation camel milk production is also becoming increasingly common. For this reason, the number of scientific research on camel milk have increased in recent years. Today, camels and their products have been using by humans for transport, traction power, milk, meat, fiber (wool and hair). At the same time, it is used as a raw material for textile industry.

Milk is a complete food for newborn mammals during the early stages of rapid development (Shah, 2000). Camel milk composition has been studied in different parts of the world including Saudi Arabia (Elamin & Wilcox, 1992; Haddadin et al., 2008; Mehaia et al., 1995; Omer & Eltinay, 2009; Sawaya et al., 1984; Shuiep et al., 2008). Literature data have shown wide ranges of variation in camel milk composition, these variations will be discussed later. Konuspayeva, Faye, and Loiseau (2009) conducted a meta analysis study and given the means of camel milk composition (Bactrian and Dromedary) for the period between 1905 and 2006.

According to the present studies, camel milk can precisely play a far more important role in the protection of malnutrition than it does. Expanding and elevation foodstuffs for the rapidly increasing human population is particularly uncertain in the hot and arid zones of the world- the very areas where the camel is one of the few animals not only to keep alive, but also to profit man. Before presenting data on milk production, both quantity and quality, one must consider in detail all the suitable knowledge about the camel in order to sense the full value that this animal can play in human diet. According to studies, the production of camel milk has significantly increased during the last few years with now pasteurized fresh camel milk in the

supermarket. These studies include that done to date of camel milk jaundice, asthma, in the treatment of various diseases such as tuberculosis it has been found to be helpful. In addition to this column cancer, diabetes, hypertension was identified that help to treat their patients (Hossam Ebaid et al., 2015). Nowadays, there is a general need to start a number of camel milk based functional products to the commercial markets due to increasing demand in recent years (Al haj and Al Kanhal, 2010). These products have to be clinically proven and scientifically evident supported (Ghosh, 2009). Camel milk has lots of functional properties. These are antioxidant activity, bioactivity, anti-cancer activity, hypoallergenicity activity.

Chemical composition of camel milk

The camel has the ability to produce more milk for a longer period of time in arid zones and dry lands (an environment of extreme temperature, drought, and lack of pasture) than in other domestic livestock species (Yagil and Etzion, 1980).

Most camel milk is drunk fresh. Also, it is consumed slightly sour or strongly soured. In generally, camels' milk is opaque white (Dihanyan, 1959; Kheraskov, 1961; Yagil and Etzion, 1980). Normally it has a sweet and keen taste, but sometimes it is salty (Rao, 1970). Sometimes the milk tastes watery. In particular countries there are concerns between the urban population concerning camel milk. It is considered as having an flavorless taste (Yasin and Wahid, 1957). It is foamy when afflicted slightly (Shalash, 1979). The varies in taste are caused by the type of forage and the presence of drinking water.

The pH of milk is around 6.5–6.7 (Shalash, 1979). This is parallel to the pH of sheeps' milk. It is also possible that the camel milk is kept at room temperature for a longer time without deterioration, since the acidity of camel milk is much slower than that of cow milk.

The first milk, called the colostrum, is white and slightly diluted as compared with the colostrum of cow (Yagil and Etzion, 1980). Other studies on the composition of the milk, contingent the stage of lactation, approve these data. During the first 2 days of lactation the total solid content decreased to 18.4%. This decreases in total solid was not draw on by a changing in fat content, as firstly the fat percentage was low, at 0.2%, and after 2 days of lactation fat percentage greatly increased to 5.8%; quite the decreases in total proteins and minerals was responsible.

Milk examined at monthly aperture until the 6th month of lactation, and at the end of the 14–17 months total lactation period, indicated that the average composition observed during the first month of lactation stayed stationary for the first 6 months. As a result of this study at the end of 14-17 months, average values of fat 1.079 %, protein 0.15 %, lactose 17.78 %, ash 2.60 %, and acidity 0.38 % have been observed (Ohri and Joshi, 1961).

In camel milk, the most important factor is water content. Camel's milk water content ripples from 84% (Knoess, 1976) to 90 percent. When, the diet remained unchanged throughout the year, studied only the effects of the lack of drinking water on camel milk, largely alters in water content of milk were based upon (Yagil and Etzion, 1980).

Generally, camel milk contains 2.9 to 5.5% fat, 2.5 to 4.5% protein, 2.9 to 5.8% lactose, 0.35 to 0.90% ash, 86.3 to 88.5% water, and 8.9 to 14.3% total solid non-fat (Khan & İkbal, 2001). Camel milk has similar protein content, lower lactose content (Elamin & Wilcox, 1992), and lower fat containing less saturated fatty acids (Gorban & Izzeldin, 1999) compared with cow's milk. Camel milk has greater contents of vitamin C (Mehaia, 1994), ash, and sodium, potassium, phosphorus, zinc, iron and manganese (Gorban & Izzeldin, 1997) than cow's milk.

Geographical root and seasonal variations are factors which influence most changes in composition of camel milk. Konuspayeva et al., (2009) studied the effect of geographical root on the composition of camel milk and the research indicated that camel milk from camels located in east Africa has more fat than milk from camels in Africa and western Asia. Seasonal variations also play a significant role in the composition of camel milk, also with camels of the same type and from the same district (Bakheit et al., 2008).

According to other research related to compositional, technological and nutritional aspects of dromedary camel milk; the average

values of camel milk composition reported from 1980 to 2009 are as follows: protein 3.1%; fat 3.5%; lactose 4.4%; ash 0.79% and total solids 11.9% (Adel et al., 2009)

Dry matter in milk

The dry matter in milk, in mean of 10.4 %, consists of fat, lactose, proteins and ash (Kouniba et al., 2005). Stage of lactation and season have a substantial impact on the daily production of milk, composition of fat, protein and dry matter (Zeleke, 2007).

In dry matter (average 15.06 %) of camel milk, the main ingredients in camel milk are protein (4.9 %), milk fat (5.60 %), lactose (5.85 %), mineral substances (0.99 %).

Milk proteins and lactose

The total amount of proteins varies from 2.15 to 4.90 % (Konuspayeva et al., 2009). The content of proteins in camel milk is influenced by the type and season. The content of protein is lowest in August (2.48 %), and highest in December and January (2.9 %) (Haddadin et al., 2008).

According to Gorban and Izzeldin (1999) the protein and lactose in camel milk compared with cow milk, although similar in both types of milk protein content, camel milk has a low level of lactose and less saturated fatty acid composition. Milk from the thirsty camel has a seriously decreased percentage of protein (Yagil and Etzion, 1980). This situation shows the direct effect of drinking water on the composition of milk.

Also, it must be emphasized that protein content of the feed will directly affect that of milk. The substances of methionine, valine, phenylalanine, arginine and leucine are higher than in cow milk.

Whey proteins

In general, the composition of camel milk whey protein is different to that of bovine milk whey, where camel milk is deficient in β-lactoglobulin, as also observed for human milk (El-Agamy et al., 2006).

In bovine milk whey proteins, β-lactoglobulin is the main component (50%) and α-lactalbumin is the second (25%), whereas in camel milk whey, β-lactoglobulin is deficient (El-Agamy, 2000; Farah ve Atkins, 1992; Kappeler et al., 2004; Merin et al., 2001) and α-lactalbumin is the main component.

Camel milk is different from cow milk in point of chemical composition but it contains all essential nutrients as cow milk (El-Agamy, 1996), besides its high whey proteins such as lactoferrin and immunoglobulin that are give to it the high antimicrobial properties. In equated, camel milk includes more proteins and whey protein than cow milk (Farah et al., 1993, Walstra et al., 1999). The quantity of whey proteins of camel milks and cow milks are respectively, at 0.9 to 1.0 % and 0.7 to 0.8 % (Mohamed, 1990). This is primarily due to the higher content of albumin and lactoferrin (Farah, 1993). The functional properties of bovine milk proteins with camel milk proteins, the proteins were separated and characterized and found that an important thermodynamic property related to the heat stability (Beg et al. 1986). They showed that the whey proteins of camel milk were found to be much more heat stable than proteins of cow's milk (Farah and Atkins, 1992).

Casein

Casein forms approximately 80% of cow milk proteins (Hipp et al., 1952), while camel milk casein content is 52–87 % (Al haj and Al Kanhal, 2010). The cow milk main casein fractions are αs1-, αs2-, β- and κ-casein in ratio 4:1:4:1 (Walstra et al., 1984) and the amino acid numbers of residues in these four casein fractions were 199, 207, 209, 169, in order of as compared to 207, 178, 217 and 162, in order of for camel casein (Kappeler et al.,1998).

The content of casein fractions is very different between each fractions it is showed that the amount of κ-casein fraction of camel milk is of about 5% of the total casein, compared with about 13.6% in bovine casein (Ramet, 2001). Camel milk casein is different from cow's milk casein in terms of micellar size distribution. This large difference in the κ-casein content impairs the cheese making properties (Mohamed, 1990), There is little information available on the ability of camel milk to undergo enzymatic coagulation. When compared with cow milk camel milk casein and their fractions were found to be weak in crude protein (Pant and Chandra, 1980).

Concentration of nonessential amino acids of κ-casein in cow milk is higher than in camel milk, outside arginin the concentration is higher in camel milk of κ-casein. Cows milk κ-casein includes a higher concentration of essential amino acids in comparison of camel milk, outside for lysine whose concentration is higher in the camel milk of κ-casein (Salmen et al., 2012).

However, it would be untimely to dispute the effect of this difference in relation to the preparation of camel milk products. Therefore various biochemical aspects of camel milk should be considered and additional studies should be done.

Vitamin

In the studies performed about the vitamin concentration of camel milk showed that the camel milk contains less vitamin A than cows milk while the content of vitamin E is about the same level with comparison of camel milk (Farah et al., 1992), and the level of vitamin C is in average three times higher than that of cow milk (Stahl et al., 2006).

The availability of a relatively abundant amount of vitamin C (average 37.4 mg/l) in camel milk is of significant relevance from the nutritional viewpoint in the arid areas where fruits and vegetables containing vitamin C are scarce.

Also according to bovine milk, the niacin (B_3) content was noticed that to be higher in camel milk (Haddadin et al., 2008; Sawaya et al., 1984).

Milk fat

Milk fat is emulgated in milk, which means that it is found in the form of fat globules dispersed in milk serum. The diameter of fat globules varies between 1.2-4.2 μ. The amount of fat in camel milk ranges between 1.8% and 5.0 % (Khaskheli et al., 2005), with a mean of 2.63 %., compared camel milk with cow's milk and found that fat globules of cow's milk similar to the distribution of camel milk fat globules.

In compared with cow milk, camel milk fat contains less short-chain fatty acids (Abu-Lehia et al., 1989) and a lower concentration of carotene (Stahl et al., 2006). Due to the lower concentration of carotene, camel milk is significantly white. Camel milk also includes a higher concentration of long-chain fatty acids compared to cow milk (Konuspayeva et al., 2008). Similarly, mean values of unsaturated fatty acids (43 %) are higher in camel milk than cow milk, especially essential fatty acids (Haddadin et al., 2008). In cow milk, the amount of saturated fatty acids is higher (69.9 %) than in camel milk (67.7 %) (Konuspayeva et al., 2008).

Gast et al. (1969) asserted that the value of camel milk is to be found in the high concentrations of volatile acids and, especially, linoleic acid and the polyunsaturated acids, which are essential for human nutrition and health.

Additionally, the dispersion state of milk fat has a considerable impact on the technological processes in dairy products as it determines the texture, flavor, and physicochemical properties of cheese and butter.

The beneficial effect of camel milk on the human health

Camel milk used for medical purposes for cure material of diseases. Camel milk can also be considered as a promising new protein source for children who are allergic to cow milk protein, and camel milk infant formula can be taken into account. Kappeler (1998) reported that camel milk is free of β-lg, which is considered as one of the major antigens of cow milk proteins responsible for the incidence of hypersensitivity reactions (allergy) in babies. Camel milk fat is mainly consists of polyunsaturated fatty acids that are completely homogenized and gives the milk a smooth white appearance.

The proteins of camel milk are the decisive components for preventing and curing food allergies because camel milk contains no β-lactoglobulin and a different β-casein, these two components in cow milk that are responsible for allergies.

Camel milk contains a number of immunoglobulins that are compatible with human ones which is reduce children's allergic reactions and strengthen their future response to foods. The importance of treating food allergies by using camel milk in children is therefore found in its non-allergenic properties and the child's immunologic rehabilitation. Two main categories of hypersensitivity are

that allergy and autoimmune disease. Strongly and rapidly the immune system develops and it is challenged at a young age would also be contributing factors.

Milk allergy is caused by the immune system reacting to the protein in the milk as a threat to the body. Most allergic people produce immunoglobulin E as an antibodies. In vitro tests have shown that camel milk reduces anti-immunoglobulins in the blood.

Hamers-Casterman et al. (1993) described the magnificent immune system of the camel, which is different from of all other mammals. IgG2 and IgG3 consist of only two heavy chains and there are no light chains. There is a single V domain (VHH) (Riechmann and Muyldermans, 1999).

Camel VHH has a long suppmentary determining region (CDR3) loop, compensating for absence of the VL. Conventional antibodies seldom exert a complete neutralizing activity against enzyme antigens. Camel IgG has full neutralizing activity. Camel hypervariable regions have increased the repertoire of antigen binding sites (Muyldermans et al., 2001). VHH domains of the camel are better suited to enzyme inhibitors than human antibody fragments (Riechmann and Muyldermans, 1999).

Viral enzymes play an important role in triggering diseases, their neutralization can prevent their replication. Variable domain antibody fraction of camel is a potent and selective inhibitor of the hepatitis C enzyme system (Martin et al., 1997). The size of the antibodies is a major flaw in the development of immunotherapy. Larger antibodies cannot reach their target. The camel's antibodies have the antigen similarity as human antibodies but they are ten times smaller (Jassim and Naji, 2001).

In camel milk there are many protective proteins that exert immunologic, bactericidal and viricidal properties (Kappeler et al., 1998). The most important of these are lactoferrin, lactoperoxidase, PGRP and NAGase.

Camel milk is also rich in vitamin C, calcium and iron. Diabetic patients start insulin therapy, they have to take it permanently and usually insulin dose continue to increase as time progresses. Clinical research on the use of camel milk by patients with type 1 diabetes has indicated that drinking camel milk daily decreases the blood glucose level and reduces insulin requirement by 30%. Clearly, that camel milk provides an insulin-like protein in a different form than in other mammals and gives some other therapeutic compounds that raise the health of diabetic patients.

As a unique property of camel milk, the insulin-like protein could be protected in the stomach and absorbed efficiently into blood stream to reach the target. This is why camel milk does not coagulate in an acidic environment and it has a higher buffering capacity than the other ruminant milks. Camel milk also contains approximately 52 micro unit/ml of insulin-like protein compared to cow milk (16.32 micro unit/ml) which imitates insulin interaction with its receptor, and it has a higher content of zinc which has a key role in insulin secretory activity in pancreatic beta cells.

Beg et al. (1986) found that the amino acid series of some camel milk protein is rich in half cystine, which has a cursory similarity with the insulin family of peptides. Camel milk has high concentration of vitamin C which is used for stabilize the conditions of biliary atresia patients. It also has a positive effect of chronic fatigue.

According to studies of Agrawal et al. (2003) in order to detect effective of camel milk on glycemic control and treatment of type-1 diabetes disease, camel milk demonstrated effective extention in the management of type 1 diabetes as there was important reduction in doses of insulin, diabetes quality of life however, there was no change in lipid profile and insulin levels. It is based on that one of the camel milk protein has many characteristics similar to insulin (Beg et al., 1986) and it does not compose coagulum in acidic medium (Wangoh, 1993). This lack of coagulum formation consents the camel milk to pass quickly through stomach together with the certain like protein/insulin and remains present for absorption in intestine.

According to studies of Agrawal et al. (2003), observed a significant improvement of camel milk treatment and the positive effects in weight gain.

These positive results are due to the nourishing qualities of camel milk. Camel milk was found

to include about 52 units/litre insulin (Singh, 2008) and it may be the cause for smaller necessity of insulin in camel milk group.

Beg et al. (1986) has based upon that amino acid sequence of some of the camel milk protein. According to this study, camel milk is rich in half cystine, which has superficial similarity with insulin family of peptides. Lots of experiments shows that its therapeutic impact may be due to lack of coagulum creation of camel milk in acidic medium. Especially considering the level of insulin for diabetic patients is important to note the value of camel milk could be a success.

CONCLUSIONS

The production of camel milk has gradually increased due to an increased interest by consumers in recent years.

Camel milk was found to be different in some aspects from milk of other animal species, such as bovine milk.

Use of camel milk is widely spread not only during production of different kinds of milk products but also as cure material of different kinds of diseases such as cancer, diabetes, hypertension, autism dropsy, jaundice, tuberculosis, asthma.

One of the more important property of camel milk is suitable for people who have problem with milk protein allergy.

Therefore it is consumed especially for infants and babies.

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MICROENCAPSULATION OF ESSENTIAL OILS OBTAINED FROM NATURAL HERBAL FOR USE IN THE FOOD INDUSTRY

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Abstract

Microencapsulation is a tehnological process in which solid, liquid or gaseous substances of small size are completely surrounded by individual polymeric coatings to avoid physical and chemical and for maintain the biological and physicochemical properties of core materials. Microencapsulation can be applied with success to entrap natural compounds, like vegetal extracts. Essential oils could have antibacterial and antifungal properties and have screened as potential sources of novel antimicrobial compounds. In food science and biotechnology, microencapsulation involves incorporating of natural ingredients, volatile additives, polyphenols, enzymes, bacteria into small capsules, giving them stability, protection and preservation, against nutritional losses, acting as antimicrobial agents. The aim of this literature review is to describe and functional properties and the benefits of various oils with antimicrobial activity obtained from natural herbals, application of encapsulated oils in food industry and microencapsulation techniques.

Key words: *biotechnology, microencapsulation, essential oils, antimicrobial activity.*

INTRODUCTION

Microencapsulation is used as a potential solution to solving punctual technological problems, most often leading the development of innovative processes or to the development of new products (Li SP et al., 1988; Finch CA, 1985; Arshady R. , 1993). Microencapsulation is more and more applicable in the field of biotechnology, especially in food and agriculture. In recent decades, encapsulation of active compounds has become a process of great interest and importance, being suitable for food, chemical, pharmaceutical and cosmetic ingredients. In the food field, microencapsulation is used to extend the shelf life of flavored, spices in dry mixtures; isolates additives used for baked goods, which are released only under the influence of heat; protecting vitamins; masking the taste, smell or color.

Microencapsulation can be successfully applied to encompass natural compounds such as essential oils or plant extracts containing polyphenols with antimicrobial properties well known for use in food packaging.

In food science and biotechnology, microencapsulation involves incorporating natural ingredients, volatile additives, polyphenols, enzymes, bacteria into small capsules, giving them stability, protection and preservation, against nutritional losses, acting as antimicrobial agents.

Essential oils from natural herbs and theirs benefits

Essential oils from natural herbal, the odorous, volatile products of an aromatic plant's secondary metabolism are well-known antimicrobial agents that could be used to control food spoilage and foodborne pathogenic bacteria. They have long been served as flavouring agents in food and beverages, and due to their content of antimicrobial compounds, they possess potential as natural agents for food preservation. The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds, which also in pure form exhibit antibacterial or antifungal activity. Given the fact that consumers demand less use of chemicals in food products, more attention has been given to the search for naturally occurring substances able to act as alternative antimicrobials and antioxidants. Plant essential oils are becoming more popular as naturally derived antimicrobial agents**.**

Oregano essential oil

According to (Olmedo et al., 2014; Muriel-Galet et al., 2015), oregano essential oil contain ingredients, including carvacrol, thymol, α-

terpinene, γ -terpinene, terpinen-4-ol, p-cymene, α-terpineol, and sabinene. Carvacrol and thymol constituting about 78% to 82% of the total oil, are the principal phenolic compounds responsible for antioxidant and antimicrobial activities. According to the researchers, adding a higher amount of oregano oil increased the retention of α-tocopherol in the meat (Botsoglou et al., 2003). According to the studies, oregano oil is one of the oils with high antimicrobial activity, its phenolic components permeate and depolarize the bacterial cytoplasmic membrane, leading to cell death. According to Rodriguez-Garcia et al. (2015) the antimicrobial efficacy of essential oil is in the order: oregano/ clove / coriander/ cinnamon > thyme > mint > rosemary > mustard > cilantro/ sage.

Asensio et al. (2015), found that completation of the oil in organic cottage cheese decreased, during storage, chemical deterioration. Also oregano essential oils hold antifungal and insecticidal properties and can be used for the prevention of neurodegenerative excitement (Almeida et al., 2013). Oregano oil due to its preservative effects and pleasant flavor is used as a food ingredient. *Basil oil* according to researchers has a powerful medicinal value (Baliga et al., 2013). Its main components of phenolic and terpenoid derivatives include methyl eugenol (42.58%) followed by caryophyllene (26.88%) and eugenol (10.66%). Basil oil has antibacterial, antioxidant (Chanwitheesuk et al., 2005) and antifungal (Kumar et al., 2010) properties. Khanna et al. (2010) and Baliga et al. (2013) found that basil oils can also inhibit cholesterol synthesis and improve digestive performance. According to Sutaphanit and Chitprasert (2014) microencapsulation of basil oil with gelatin insured protection against chemical and physical loss under fast storage conditions at 60°C for 49 days. *Rosemary oil (Rosmarinus officinalis* L.*)* Rosemary is the most used medicinal and aromatic plant in the world, because of its phenolic compounds and essential oil (Rozman and Jersek, 2009). The research related to rosemary essential oil has mainly focused on its , antifungal (Soylu et al., 2010), antibacterial (Jiang et al., 2011), anticancer (Degner et al., 2009), insecticidal (Zoubiri and Baaliouamer, 2011), gastric, antiseptic, anti-inflammatory, antioxidant and antiviral properties (Barni et al., 2012). Also, rosemary oil helps in perception improving (Moss et al., 2003). Microencapsulation of rosemary oil accomplish functional activity with high retention volatiles. *Cinnamon leaf oil* is appreciated for its flavor in addition to it antimicrobial properties (Singh et al., 2007; Ayala-Zavala et al., 2008). Antifungal and antioxidant properties of cinnamon leaf oil are due to volatile components such as eugenol and cinnamaldehyde (Combrinck et al., 2011). Cinnamon leaf oil it has been found to have antimicrobial (Matan et al., 2006), anti-inflammatory (Gunawardena et al., 2014) and antidiabetic properties (Ping et al., 2010). The U.S. Food and Drug Administration consider cinnamon oil as a Generally Recognized as Safe compound (Tzortzakis, 2009). Microencapsulation of cinnamon leaf and garlic oil with β-cyclodextrin reveal good antifungal activity against *Alternaria alternata*. Due to improved stability, solubility, and bioavailability, cinnamon leaf and garlic oil microcapsules could have important applications in the food industry (Ayala-Zavala et al., 2008).

Thyme oil. Thyme is a known source of essential oil and also a phytonogenic food additive. Essential oil of thyme is widely used in food and the flavor industry, as well as in the manufacture of cosmetics and perfumes. Its antimicrobial and antioxidant activities are mainly attributed to the presence of carvacrol, cinnamaldehyde, thymol, geraniol and eugenol (Sipailiene et al., 2006; Navarrete et al., 2010). Jouki et al. (2014) have found that the incorporation of essential thyme oil into edible films has increased safety and the shelf-life of ready to eat foods.

Essential oils have the potential to be used in the food industry as a preservative to ncrease the shelf life and prevent damage of products because they are a source of natural antimicrobial substances. The essential oils could also reduce side effects by their replacement of chemical preservatives (Abhishek K.D. et al., 2016). A variety of molecules derived from essential oils have bioactive properties with antibacterial activity that can be used directly in food products. In Table 1 few examples of latest studied essential oils and thir composition and properties are given.

Table1. Essential oils from natural herbal, components and activity

Oil type	Species	Components	Properties of essential oils	References
Oregano oil	*Origanum vulgare*	Carvacrol, thymol, α-terpinene, γ -terpinene, terpinen-4-ol, p-cymene, α-terpineol, and sabinene.	antioxidant, antifungal	Olmedo et al., 2014; Muriel-Galet et al., 2015
Basil oil	*Ocimum basilicum*	Methyl eugenol, caryophyllene, eugenol	antioxidant, antibacterial, antifungal	Baliga et al., 2013; Chanwitheesuk et al., 2005; Kumar et al., 2010
Rosemary oil	*Rosmarinus officinalis L.*	Camphene, (*α* & *β*-Pinene), limonene, & camphor	antifungal, antibacterial	Rozman and Jersek, 2009; de Barros Fernandes et al., 2014; Fernandes et al., 2013; Soylu et al., b2010; Jiang et al., 2011
Cinnamon leaf oil	*Cinnamomum zeylanicum*	Eugenol, cinnamaldehyde	antifungal, antioxidant	Ayala-Zavala et al., 2008; Singh et al., 2007; Combrinck et al., 2011
Thyme oil	*Thymus vulgaris*	Carvacrol, cinnamaldehyde, thymol, geraniol and eugenol	antioxidant, antimicrobial	Sipailiene et al., 2006, Navarrete et al.,2010

Materials used as encapsulation matrices

Carbohydrates. For microencapsulation the most commonly used shell materials are carbohydrates such as starches and maltodextrins. Carbohydrate based materials because they have poor interfacial properties have to be chemically modified to improve surface activity.

Hydrolysed *starches* are depolymerised ingredients produced by hydrolysing starch with acid and/or enzymes. These wall materials offer the advantage of being inexpensive; low viscosity at high solids; and excellent protection to encapsulated core materials.The degree of protection is directly related to the dextrose equivalent of the hydrolysed starch, higher-dextrose systems are less permeable to oxygen and result in powders with higher encapsulation efficiencies (Dalgleish D.G., 2006).

Cyclodextrins have also been used in encapsulation of food oils and flavours. They are cyclic molecules containing six (alpha-), seven (beta-) or eight (gamma-) glucose monomers that are produced from starch. These monomers are connected to each other, giving a ring structure that is relatively rigid and has a hollow cavity with the ability to encapsulate other molecules. Many reports have demonstrated that inclusion complexes are virtually completely stable to oxidation compared to other wall materials. Reineccius et al. found that c-cyclodextrin generally functioned better than (alpha-) and b-cyclodextrins in terms of initial flavor retention.

Encapsulation in *β-cyclodextrin* is a method for controlling the odor and reactivity of active compounds during the release of natural antimicrobial compounds. β-cyclodextrin is a cyclic molecule made up of 7 D-glucose monomers linked via a cone (1,4) bond. A hydrophobe is a cavity, while the outer face is hydrophilic. These properties have made β-cyclodextrin an option for encapsulation from several organic and inorganic compounds. Encapsulation in β -cyclodextrin is considered as a molecular complex, in which the hydrophobic active constituents of the essential oil can interact in the hydrophobic cavity of β-cyclodextrin, indicating that when forming the capsule, the outer molecule is hydrophilic.

Proteins. Functional properties of the proteins, including the ability to interact with water solubility, film forming and emulsifying and stabilizing emulsion droplets, have many of the desirable characteristics for a wall material. One of the commonly used proteins is gelatine According to research, in recent years for the potential of new wall materials, for the encapsulation of flavors and oils, were studied other proteins, especially soy and milk proteins, such as whey protein concentrate, skimmed milk powder and caseinates. These proteins change their structure during emulsification through unfolding and adsorption at the oil water interface and by forming resistant multilayer around oil droplets and also with the help of repulsive forces, make significantly stable emulsions which are critical for encapsulation purposes. Investigations have proven proteins to function well for oils.

Gums. Acacia gum, usually known as gum arabic, due to its excellent emulsifying properties is mostly used gum. Due their emulsion stabilization and film forming properties of gums make them a suitable microencapsulation agent. The constituent of gum Arabic are L-rhamnose, D-glucuronic acid, L-arabinose and D-galactose with approximately 2% protein, which is responsible for emulsifying properties of gum arabic (Dickinson E., 2003). According to Krishnan et al. (2005) gum arabic compared maltodextrins and modified starch, have been found to be a better wall material for encapsulation of cardamom oleoresin, the resulting microcapsules exhibit a free-flowing character. Gum arabic have shown good properties as wall material for encapsulation of cumin oleoresin by spray-drying (Amr M. et al., 2015). Usually, gum arabic is preferred because it produces stable emulsions with most oils over a wide pH range and forms a visible film at the oil interface. Gum arabic because of this emulsifying efficiency, has been usually used to encapsulate lipids. Gum Arabic is ideally for the microencapsulation of lipids because of both its surface activity and its film forming properties.

Microencapsulation techniques for essential oils

There are several methods of producing the microcapsule using different types of coating materials as well as generating particles of different sizes, thickness and core permeability, thus adjusting the release. Generally, these techniques are divided into two categories: physical methods and chemical methods. Chemical methods can also be subdivided into physico-chemical and physico-mechanical techniques.

Emulsification is a method used in a extensive variety of pharmaceutical and food products. Emulsion technology is an important step in the microencapsulation of oils. In general, emulsification is applied to encapsulate bioacids in aqueous solutions, which can be directly used in liquid form. Emulsions are prepared by homogenizing the oil, water and emulsifier, using a homogenizer (Augustin et al., 2006).

Microencapsulation of essential oils by using *supercritical fluid technology* is of great relevance to the pharmaceutical, cosmetic and food industry. This method has several inherent advantages: nontoxicity, mild solvent removal, product degradation, and the process utilizes a wide variety of materials that produce controlled particle sizes and morphologies.

Spray drying is a physico-chemical method and is the most frequently used technique to encapsulate flavors. Spray drying can be described as a simple process, capable of producing a wide range of good yield microcapsules, including microcapsules loaded with aromatic oils or aromaTonon et al. (2011) found that spray-drying is a technique that involves the atomization of emulsions into a drying chamber at a relatively high temperature, which leads to water evaporation and, therefore, crust formed at fast rate and quasi-instantaneous entrapment of oil. The process involves four steps: preparing a dispersion or emulsion; homogeneity of dispersion; atomizing the feed emulsion; and dehydration of the atomized particles. Spray drying is the microencapsulation technique most commonly used in the food industry and is used to encapsulate a wide range of ingredients (Beristain et al., 2001, 2002).

The *coacervation* technique can be divided into two main groups: aqueous and organic. Aqueous phase coacervation can only be used to encapsulate water-soluble materials. Organic coacervation allows the encapsulation of a water-soluble material but requires the use of organic solvents.

Freeze-drying is a simple process and is used for the dehydration of almost all heat-sensitive materials and aromas like oils. Before drying, the oil is dissolved in water and frozen between -90°C and -40°C (Heinzelmann et al., 2000; Amr M. et al., 2015) and then the surrounding pressure is reduced and enough heat is added to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase (Oetjen and Haseley, 2004).

In situ polymerization according to the researchers has become the most used method for the preparation of microcapsules and functional fibers. In situ polymerization is a microencapsulation method which, by adding a reactant inside or outside the core material,

leads to the formation of a wall (Amr M. et al., 2015). *In situ* polymerization differs from other encapsulation polymerization processes because no reactant is included in the base material. According to the researchers, Amr M. et al. (2015) microcapsule formation is performed with an oil emulsion in a melamine-formaldehyde resin solution and sonication process to emulsify the oil in the aqueous phase, then the resin is added with stirring and then the pH of the emulsion to the acid finally forming the microcapsule shells. By using this method, the melamine reaction with formaldehyde is promoted at the oil droplet interface, producing a crosslinked melamine-formaldehyde polymer film as a microcapsule shell.

CONCLUSIONS

Microencapsulation is an important tool for the preparation of high quality oil products and health benefits in the food industry to improve their chemical, oxidative and thermal stability.
Essential oils are natural products that consist of complex mixtures of many volatile molecules. Essential oils due to their source of natural antimicrobial substances are used in the food industry as a preservative to prevent damage and to increase the shelf life of products. Despite their many applications, essential oils are very sensitive to environmental factors when used as such, and encapsulation is a relevant alternative that enhances essential oils stability. Various microencapsulation techniques have been successfully used to achieve this purpose.
According to studies, the most commonly used matrices for microencapsulation are carbohydrates such as maltodextrins and starches because it offers the advantage of being cheap; have low viscosity and excellent protection for encapsulated base materials.

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SOME RELEVANT QUALITY INDICATORS OF RED WINE FROM THREE GRAPES CULTIVARS – A MINIREVIEW

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Abstract

In the last years studies point out the fact that the red wine is one of the most consumed beverages over the world. Appreciation of quality in food products is a complex process and is often based on multi-dimensional facets, whose measurement requires clearly validated scales. Factors that influence the overall perception of wines in terms of quality are: geographical origin – ground type, climate, grape variety - and authenticity. One of the most important indicator of the red wine is polyphenolic compounds, such as flavonoids, anthocyanins and tannins present in large quantities in wine, especially in red wines; their composition in wine being influenced by the varieties, the vintage and the wineries. The aim of the present work is to make a short review about three types of wines: Cabernet Sauvignon, Merlot and Feteasca neagra quality attributes in order to establish the relationship of the intrinsic characteristics of each individual product and the extrinsic attributes and to adapt the scale to each considered product.

Key words*: red wine, South Region of Romania, Romania red wines, wine quality factors, geographical origin.*

INTRODUCTION

Nowadays, regardless of the geographical location of the vineyards in the world, it is not possible to produce high quality wines without taking into account the quality status of the grape at harvest (Pons et al., 2017). Geographical origin and authenticity are both factors influencing the overall perception of grapes and wines in terms of quality. Given the fact that the natural diffusional movement of elemental traces follows a pattern, moving from rocks to soil, and from the soil to the grape, allows for wines to be differentiated through the elemental analysis of their provenance soils. (Geana et. al., 2012). Overall wine is one of the most consumed beverages in the world (Hosu et al., 2013).

In recent years, the assessment of wine traceability and authenticity became a prerequisite in many countries. The consumers have been increasingly interested in information on the characteristics and the quality of foods, especially with regard to composition, nutritional properties and origin (Charlton et al., 2010; Versari et al., 2014). The wine industry is a particular example in which authenticity has been extensively investigated because wine is a product widely consumed over the world and which can be easily adulterated (Makris et al., 2006). The wine authenticity is guaranteed by strict guidelines elaborated by responsible national authorities, and includes sensory evaluation and several chemical analyses (Versari et al., 2014). Also, wine is a complex beverage recognized for its beneficial effects on human health, contributing to an improvement in the quality of life (Hosu et al., 2015).

Otherwise, wine is an alcoholic beverage that contains various polyphenols extracted from grapes during the processes of vinification (Rastija and Medić-Šarić, 2009a). The polyphenolic compounds, such as flavonoids, anthocyanins and tannins are considered to have antioxidant activity, protecting the body cells against oxidative stress. These compounds are present in large quantities in wine, especially in red wines, which may explain so-called French paradox. Moreover, polyphenolic compounds are responsible for the quality of red wines, influencing their astringency, bitterness and colour. The viticulture practices, different oenological techniques, the varieties and the harvesting year of grapes and the wineries influence the polyphenolic composition of press wines (Cliff et al., 2007).

In the case of wine, bitterness and astringency are amongst the least understood perceptions. This can be due to a number of different

reasons related to their complexity and multimodality, probably also because they induce fatigue generating great individual variability among consumer perception, but maybe also because most often previous research has neglected interactions with other stimuli such as aroma or taste. (de-la-Fuente-Blanco et al., 2017).

That is why the quality perception is influenced by the characteristics of the product which have been mainly classified into intrinsic and extrinsic factors (Charters and Pettigrew, 2007). Intrinsic cues are those related to the product itself (physical part of it) and its organoleptic properties such as aroma, in-mouth properties or colour. Extrinsic cues refer to properties which are not physically part of the product such as package design or region of origin. For the specific case of wine, intrinsic cues of previously experienced wines are determinant in repurchase situations (Mueller et al., 2010). The importance of extrinsic properties lies on the fact that at wine purchase the consumer is rarely able to taste wine and thus has to rely on extrinsic cues to infer wine quality (Sáenz-Navajas et al., 2016).

Quality perception through sensory properties it is very important, but fermentation is the most critical value adding activity in the winemaking process, with significant technical, equipment and human resource demanding to associate with the process (Muhlack et al., 2013). Although ethanol is the main product of wine fermentation, the concentration and composition of phenolic compounds such as tannins and anthocyanins, as well as aroma and flavour components have the greatest influence on the overall sensorial quality of the young wine (Bisson and Karpel, 2010; Cheynier et al., 2006; Garde-Cerdan and Ancín-Azpilicueta, 2006; Gonzalez-Barreiro et al., 2015). As such, understanding the impact of parameters that affect the concentration of these compounds during winemaking is vital for producing a final product of desired quality and composition. In conclusion, not only anthocyanins and proanthocyanidins, who are very important to analyse, can drastically change the characteristics of the resulting wine, but also the others parameters because each grape variety has a specific characterisctic compounds responsible for the quality of red wines, influencing their astringency, bitterness and colour. (Setford et al., 2017).

Also tannin, acid, and ethanol are fundamental components driving overall aroma, taste and mouthfeel in red wine. Specific wine or vinicultural production practices modify these components prior to, or during vinification. The extraction of grape derived tannin is dictated by the management and maceration (Sacchi et al., 2005). Ethanol, the result of sugar fermentation, is modified by altering juice sugar concentration during fermentation or harvesting at various fruit maturities. Acidity is also commonly adjusted prior to fermentation through the addition of tartaric acid (Frost et al., 2017).

The aim of this study is to make a critical review about quality indicators (physical-chemical due sensorial parameters) of these three types of wines: Cabernet Sauvignon, Merlot and Feteasca neagra red wines.

CABERNET SAUVIGNON, MERLOT AND FETEASCA NEAGRA GRAPES CHARACTERISTICS

Grapes characteristics

According to the latest research several grapevine varieties like Cabernet Sauvignon, Merlot, Feteasca Neagra, Pinot noir, Burgundy, Cadarca, were investigated during 2006-2007, 2008-2009, 2011,2012, 2013-2014 years, in order to obtain wines with denomination of origin controlled in different wine centers: Murflatar, Jidvei, Halewood wineries (Artem et al., 2014; Chira et al., 2010; Dobrei et al., 2016; Petropulos et al., 2013). The studies present the evolution of routine quality control parameters - sugars content, acids, titratable acidity, sugar-acidity index and phenolic compounds - anthocyanins and polyphenolic index. The reported results were useful to find the optimum moment for grape harvest ensuring the production of high quality wines and to show that the antioxidant content of samples depends on the analyzed material and on the grape variety. The latest research revealed that phenolic compounds from the three red grape varieties play an important role in the quality of red wine, particularly on colour and astringency and also are responsible for the sanogenic or multiple benefic effects on human health after a moderate consumption of wine. By their

physico-chemical attributes the phenolic compounds are rightly considered the most important group of chemical compounds in grapes, after sugars and acids. The type and concentration of phenolic compounds in wine depends on grape variety, ripening, atmospheric conditions, viticultural and vinification techniques. In the studied wines, phenolic acids represented by galic and syringic acids were reported in relative lower amounts, between 0.10 and 1.04 mg/L for galic acid and 0.10 and 1.33 mg/L with important amounts in Feteasca neagra wine variety. (Artem et al., 2014; Hosu et al., 2014, Rodríguez-Delgado et al., 2002).

Cabernet Sauvignon is one of the world's most widely recognized red wine grape variety, being grown in America, Australia, Asia and Europe. Grape bunches are tronconical or conical shaped, with rare grains rachides. The average weight of the bunches is 100-140 g. The grapes have spherical shape, franc taste and thick skins, colored in dark red-purple, with intense pruine and they have a long vegetation period (180-190 days) and the climate of the growing season affects how early the grapes will be harvested (in Romania, the grapes ripen usually in September). The sugar concentration and the total acidity can reach 240 g/L, and 5.0-5.5 g/L H_2SO_4, respectively. Cabernet Sauvignon can be grown in a variety of climates, being resistant to frost, drought and gray mold, but is affected by rot (Patic, 2006).

Figure 1. Cabernet Sauvignon grapes (Mike Roberts, 2016)

Merlot is an old variety of red wine grape from Gironde-Bordeaux wine-growing region. The name Merlot is thought to derive from the "Old French" word for young blackbird, merlot, a diminutive of merle, the „blackbird" (*Turdus merula*), probably from the colour of the grape. Beyond France it is also grown in Italy, Eastern Europe and New World, especially California. It grows in many regions where also grow Cabernet Sauvignon but tends to be cultivated in the cooler parts of those areas. In areas that are too warm, Merlot will ripen too early. Merlot grapes are identified by their loose bunches of large spherical berries. The colour has less of a blue/black hue than Cabernet Sauvignon grapes and with a thinner skin and fewer types of tannin. Grapes have a middle vegetation period (170-180 days), a large force of growth and develop rich foliage. A characteristic of the Merlot grape is the propensity to quickly overripe. It normally ripens up to two weeks earlier than Cabernet Sauvignon. Compared to Cabernet, Merlot grapes tend to have a higher sugar content 205-240 g/L, and total acidity of 4.5-5.5 g/L H2SO4. Merlot thrives in cold soil, particularly ferrous clay. The grapes tend to bud early which gives it some risk to cold frost and its thin skin increases its susceptibility to rot. If bad weather occurs during flowering, the Merlot wine is prone to develop colour (Patic, 2006).

Figure 2. Merlot grapes (http://sedimentality.com/variety-focus/red-wine-grapes/merlot/)

Feteasca Neagra is a dark-skinned grape variety native to the Republic of Moldova and Romania, although it is now more widely planted in the latter. It is considered to make some of the top red Romanian wines, exhibiting spicy, smoky fruit characters and good tannin structure. The grapes are medium to large, cylindrical-conical bunch with spherical, medium-sized berries and dark

purple skins. Although it is a vigorous vine, it is resistant to frost, drought and rot, Feteasca Neagra has quite a low productivity (sometimes around 30 % of regular). For this reason, when pruning the vines, a large number of buds are left. Feteasca Neagra reaches maturity shortly before Merlot, generally after September 15th, with a growing season of about 160-170 days. This variety easily accumulates significant amounts of sugar (230 -240 g/l) and has good acidity of over 7 g/l tartric acid. Favourable conditions for the maturation of this variety are provided by sunny slopes, where the - accumulation of anthocyanins reaches optimum levels. More often, bunches don't mature uniformly, so they should be granted special attention at harvest (Patic, 2006).

Figure 3. Feteasca neagra grapes (Răzvan Avram, 2017)

Grapes quality parameters

The ripening period of the grapes is different from one year to another and from one vineyard to another, depending mainly on the climate. For this reason, it is necessary to follow the evolution of maturation of each variety, every year. Harvesting of the grapes is very important and it must be done timely because, generally, the quantity and quality of the harvest depends on it. Grapes full maturation is reached when the weights of grape berries achieve a maximum value and the evolution curve begins to decrease. At this moment, the sugar content of the grapes is also at its maximum. The evolution of sugar remains stationary for a few days and total acidity is reduced substantially and the evolution curve indicates a slow decrease of acidity. Reaching full maturity varied from variety to variety depending on the genetic traits. First varieties that reach ripeness in the Murfatlar region are for example, Pinot noir and Feteasca Neagra, followed by Cabernet Sauvignon and Merlot. Although, from the vineyards Murfatlar, Ploiești (Halewood Wineries) and Jidvei red wines, harvest of 2011, 2012, 2013 showed significant amounts of polyphenols, they contribute to color formation, stability and sensory characteristics thereof. Red wines Cabernet Sauvignon from Murfatlar, Pinot Noir from Jidvei and Merlot from Murfatlar had the most significant amounts of glycerol, so from the sensory point of view they can be characterized as unctuous wine, full bodied and with a sweet taste effect (Artem et al., 2014, Hosu et al., 2011, Stegarus et Tita, 2015).

Climate in the years of Dobrei et al. (2016) research was very different with extremes influences on the vine, which made it possible to observe experimental variants responses to climatic stress conditions, and favorable conditions.

Following parameters have been investigated: the weight of 100 berries (g), sugars (g/L), total acidity expressed as tartaric acid (g/L) and phenolic maturity reflected in total anthocyanin (mg/L), polyphenols index.

Sugars content was determined using an electronic refractometer, total acidity was evaluated volumetrically, the weight of 100 berries was done gravimetrically. Total anthocyanin and polyphenols index were achieved according to ITV method [1] and is based on the extraction of phenolic compounds in acidic conditions (ethanol 95% and hydrochloric acid (HCl) 0.1% v/v), at room temperature, for two hours. Anthocyanin concentration was estimated by measuring the absorbance of the extract solution after dilution 1:20 with 1% HCl solution, at 520 nm, while polyphenol index was estimated by measuring the absorbance of the extract after a dilution 1:100 with distillate water, using a spectrophotometer with quartz cuvette of 1 cm (Artem et al., 2014).

2013 harvest year was noted as an year when the five varieties of grapes for red wines showed a high potential for accumulation of sugars with values above 214 g/L at harvest for Feteasca Neagra, Pinot Noir, Merlot and Cabernet Sauvignon varieties. For all investigated varieties, sugar accumulation rate

was more intense at the beginning of ripening and then decreased gradually as approaching full maturity. As regards the accumulation of sugars and anthocyanins in grapes there are certain correlations, the cultivars being differentiated quantitatively by their genetic traits, but decisively influenced by the specific conditions of the production year. Accumulation of phenolic compounds in grapes evaluated by the anthocyanin content and polyphenols index ranged between 312.1-589.3 mg/L for total antocyanins, with higher values for Cabernet Sauvignon variety. The study was carried out with samples from a total of 24 wine samples (Cabernet-Sauvignon cultivar) and a total of 7 wine samples (Merlot cultivar), although there were measured the Pinot Noir samples. Wines are all of known ageing periods and they are kept under similar conditions during and after the wine-making process. From the results obtained we can say that Pinot Noir has a lower astringency, same as Merlot. Cabernet Sauvignon wines from Sâmbureşti and Jidvei may be tougher characters, astringent, compared with those from Murfatlar, where values are lower in polyphenol (Artem et al., 2014, Chira et al., 2010, Hosu et al., 2011, Stegarus et Tita, 2015). In the next years was verified the influence of the seeds and skins extracts, wine and grape variety on the antioxidants content of samples and to estimate statistically the relationships between grape varieties based on their antioxidant activity. The results showed that the antioxidants content of seeds for all grape varieties was higher than the antioxidants content of wine. The antioxidants content of seeds and skins were reported to the antioxidant content of the wine. The Merlot variety was found to have the highest diversity of antioxidants in the grape, having in same time the highest content of antioxidants. Regarding the red wines, the most significant amounts of higher alcohols are in samples from Dobrogea, Oltenia followed by those from Muntenia and Transylvania. Volatile fatty acids present very similar values in wines from Oltenia, Dobrogea, Muntenia and significantly superior values in wines from Transylvania. Wines from Dobrogea and Muntenia present the most significant amount of esters, followed closely by those in Transylvania; the lowest content of esters have the ones from Oltenia.

The aldehydes were identified in high concentrations in red wines from Transylvania, followed by those in Oltenia, Dobrogea and Muntenia, and terpene compounds were found in wines from Muntenia and Transylvania. Lowest temper compounds quantity was identified in red wines from Dobrogea. It can be said that depending on the region which these wines are from, although it is the same variety, their aromatic structure sometimes differ greatly (Hosu et al., 2014; Stegarus et Tita, 2015).

The sugar and anthocyanins accumulation in grapes showed certain similarities, these being related to genetic nature of each variety, but also decisively influenced by the specific conditions of production year (Artem et al., 2014).

Year 2014 was less favourable for grapevine growing, with excess rainfall, while 2015 was a dry year. Feteasca neagra registered the highest sugar concentration (Dobrei et al., 2016).

The results of 2014 year also show that 'Cabernet Sauvignon' and 'Merlot' grapes varieties are very different in terms of antioxidants content (Hosu et al., 2014).

CABERNET SAUVIGNON, MERLOT AND FETEASCA NEAGRA WINES

Today, Romania is an important European wine producer and therefore the wine industry is facing increasing competition worldwide due to globalization of food markets. In this highly competitive market, the wines authenticity has become a key factor in establishing its effective cost. Thus, accurate methods for wine analysis that may certify the quality and authenticity of Romanian wines are mandatory (Geana et al., 2015). The chemical profile of a wine is derived from the grape, the fermentation microflora, secondary microbial fermentations that may occur, aging and storage conditions (Styger et al., 2011).

Wines quality parameters

Phenolic compounds are responsible for sensory characteristics in wine, such as colour, mouthfeel, and flavour (Li et al., 2009).

Wine flavor is composed by a wide variety of compounds with different aromatic properties which presence and concentration depends on a

number of factors including grape cultivar, composition of grape must, yeast strain, fermentation conditions, winemaking practices, wine aging and storage conditions, among others (Moreira et al., 2016). Flavor constitutes one part of the intrinsic quality of wines and drives consumer preference. One of the main characteristics of great red wines is their aromatic complexity, with nuances such as herbaceous, green pepper, blackcurrant, blackberry, or figs and prunes. It is generally recognized that grape intrinsic composition, in terms of flavor and flavor precursors gives wine specific volatile compounds composition (Pons et al., 2017).

Aroma is a key attribute for professionals and consumers and is therefore one of the major attribute driving the intrinsic quality of wine. In recent years, research in enology and in wine flavor chemistry has made it possible to identify and quantify hundreds of volatile compounds including terpenes, C13-norisoprenoids, thiols, carbonyls, pyrazines and benzene derivatives. Wine flavor, resulting in the combination of volatile compounds found in grapes, produced during fermentations and also aging (González-Barreiro et al., 2015), cannot be fully described without an understanding of the role played by its individual molecular components, their concentrations, odor thresholds and interactions with other compounds (Ferreira et al., 2002; Pineau et al., 2007).

The grape varieties selected for identification of compounds associated with the dried fruit character were *Vitis vinifera* L. cv. Merlot and Cabernet Sauvignon. Sensory analyses were performed by a panel of five judges recruited from the staff of the research unit. All panelists from Bordeaux area had extensive experience in wine tasting and had regularly participated in sensory panels with red Bordeaux wines. All the assessments were performed at room temperature in individual booths under daylight. Wine and must (50 mL) were presented in standard 'XL5-type' tasting glasses with glass covers identified by random three-digit codes and assessed within 15 min of pouring. Each must and wine were submitted to the panelists just after the bottle was opened. During the two sessions, organized in the same week, they were asked to evaluate the intensity of dried fruit aroma on a 0–5 scale (0: no odor, 1: discrete odor, 2: just perceived odor, 3: recognized odor, 4: clear odor, 5: strong odor) (Pons et al., 2017).

An independent and specific descriptive sensory analysis was conducted to confirm that the aroma vectors as well as taste (bitter and sour) and astringency stimuli generate specific aroma/flavour differences and did not change others. Results, however, revealed the existence of a quite limited number of sensory interactions affecting exclusively bitterness (bitterness sourness and bitterness-animal), while confirmed that in the red wine context, astringency is driven almost exclusively by polyphenols and that it is not influenced by taste or aroma interactions (de-la-Fuente-Blanco et al., 2017).

Phenolic compounds appear as the grape changes colour, substituting the chlorophyll. They are of great oenological importance and play a key role in determining the quality of the wine. Along with their nutritional and pharmacological properties they also account for characteristics like colour, aroma, taste and astringency (Bartolomé et al., 2004; Harborne and Baxter, 1999). Their antioxidant properties also have positive effects on a wine's stability (Cheynier, 2001; Waterhouse, 2002). The total content of polyphenols is also an indication as to whether the wine can be aged (Mulero et al., 2015). Generally, wine phenolic compounds are composed of two main groups, anthocyanins and non-anthocyanin phenolic compounds (namely, hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols and stilbenes) (Gao et al., 2014).

Anthocyanins, which are found in the grape skin of nonteinturier cultivars and transferred to the must during the first days of winemaking, are the principal responsible for the color of red wine (Briz-Cid et al., 2014).

The young red wines show the highest content of monomeric anthocyanins (responsible for the red color wines in the first stages of the life of wine (Alcalde-Eon et al., 2007; Torchio et al., 2011) but they are involved in different reactions (copigmentation, polymerization, winemaking and further into the wine aging) that can change its concentration (Briz-Cid et al., 2014).

Grapes of the *Vitis* type are relatively rich in phenolic compounds compared to other edible fruits. The grape essentially contains non flavonoid compounds in the pulp and flavonoid compounds in the skin, seeds and stems. It is estimated that seeds contain 65% of the polyphenols of the bunch, the stem 22%, the skin 12% and the pulp just 1% (Hidalgo Togores, 2003). Hence, the technological transformation the grape undergoes conditions the extraction of these compounds and, therefore, contributes to the polyphenolic content of the wines. Vinification involves musts and wines being in constant evolution. The phenolic content of the wine depends on the raw material and the type of vinification followed, which affects physical phenomena (diffusion from the solid parts, extraction of wood compounds, etc.), and chemical and biochemical phenomena (oxidation, degradation, condensation etc.) (Mulero et al., 2015).
The anthocyanic content of Cabernet Sauvignon wines were significantly higher than for other varieties. In all wine varieties from Murfatlar vineyard, the most abundant anthocyanin was malvidin-3-O-glucoside (Mv), being in agreement with other published results (Fanzone et al., 2012), possible due to the fact that this anthocyanin is thought to be a more stable compound than the other. The second most abundant anthocyanin was malvidin 3-O-acetylated glucoside, in all wines. Higher values of delphinidin-3-glucoside (De) were found in Cabernet Sauvignon variety followed by the Feteasca Neagra and Pinot Noir varieties. Contents of cyanidin-3-glucoside (Cy) were nearly the same in all varieties, while petunidin-3-glucoside (Pt) was higher in Feteasca Neagra and Cabernet Sauvignon varieties and peonidin-3-glucoside (Pe) was higher in Pinot Noir varieties. Feteasca Neagra and Mamaia varieties showed lower content of acylated anthocyanins, compared to the others, while coumarylated anthocyanins were higher in Feteasca Neagra and Merlot varieties (Geana et al., 2015). The addition of tannins was shown to increase total polyphenols levels and total tannins levels. No significant effect was observed on the monomeric flavanols because the added tannins are condensed tannins which cannot release monomeric flavanols (Ghanem et al., 2017)

Tannin, acid, and ethanol are fundamental components driving overall aroma, taste and mouthfeel in red wine. Specific wine or vinicultural production practices modify these components prior to, or during vinification. The extraction of grape derived tannin is dictated by cap management and maceration (Sacchi et al., 2005). Ethanol, the result of sugar fermentation, is modified by altering juice sugar concentration during fermentation or harvesting at various fruit maturities. Acidity is also commonly adjusted prior to fermentation through the addition of tartaric acid (Frost et al., 2017).
Tannin concentration is correlated with wine bitterness and astringency (Vidal et al., 2003, Kennedy et al., 2006;). Villamor et al. (2013) evaluated three tannin concentrations in a model red wine showing that increased tannin content increased the perceived intensity of drying and bitter.
Acidity has been shown to alter bitter and sour perception, but pH is also associated with altering astringency (Fischer and Noble, 1994; Fontoin et al., 2008; Gawel and Van Sluyter et al., 2013).
Ethanol content has been shown to decrease astringency, but increase bitterness (Fontoin et al., 2008; Vidal et al., 2003).
This review was centred on three varieties of wine grapes to obtain three red wines Cabernet Sauvignon, Merlot and Feteasca neagra. The Merlot variety was found to have the highest diversity of antioxidants in the grape, having in same time the highest content of antioxidants.
The most intense colour was shown by Cabernet Sauvignon and Feteasca neagra wines - these two being also the varieties with the highest content of anthocyanins; middle colour attributes presented Merlot wine variety. Quality of raw material has a decisive role for the production of quality wines. Red wines obtained in 2013 harvest are dry wines with a highalcohol level. The total acidity, expressed as tartaric acid, had the highest value for Feteasca neagra variety; low volatile acidity indicate a correct fermentation processes in terms of alcoholic fermentation and malolactic fermentation. Furthermore, the unreduced extract values certify the quality of obtained wines and their qualification as wines with denomination of protected origin. The type and

concentration of phenolic compounds in wine depends on grape variety, ripening, atmospheric conditions, viticultural and vinification techniques (Rodríguez-Delgado et al., 2002). In the studied wines, phenolic acids represented by galic and syringic acids were reported in relative lower amounts with important amounts in Feteasca neagra wine variety. Higher levels of total polyphenols were reported for Cabernet Sauvignon, followed by Feteasca neagra variety (Artem et al., 2014).

Wine quality is mainly defined by sensory attributes, which are determined by the physical and chemical characteristics of the wine. Since phenolic compounds are essential constituents of wine and are responsible for important organoleptic characteristics such as color, astringency and bitterness, they constitute an important quality parameter of red wine. In wine, they are mainly composed of anthocyanins, including monomeric anthocyanins and their derivatives, and non-anthocyanin phenolic compounds which include hydroxylbenzoic and hydroxycinnamic
acids (and their derivatives), flavanols and flavonols. The color of young red wine is mainly a result of the quantity and quality of
monomeric anthocyanins, while astringency and bitterness is related to flavanols and phenolic acids (He et al., 2012a, 2012b).

Intrinsic sensory cues driving global quality involved colour (red colour), aroma (defective and roasted aroma) and in-mouth (astringency) properties. It is interesting to note that visual and in-mouth sensory cues differed depending on the information that experts had access to when judging wine. Red colour of wines was a significant parameter taken into account (together with other sensory parameters) when evaluating the global quality of wines (Sáenz-Navajas et al., 2015).

CONCLUSIONS

Regarding the wine age, Cabernet Sauvignon and Merlot wines showed highest phenolics amount in 2006 and 2007, respectively, suggesting that the content of phenolics does not depend only on wine age, but also on the initial phenolic compounds levels, the conditions during storage, as well as the applied techniques for winemaking (Petropulos et al., 2013)

In the harvest 2011, 2012, 2013 years the results showed that the red wines contain significant amounts of higher alcohols in the samples of Dobrogea, Oltenia followed by those from Wallachia and Transylvania. Volatile fatty acids present very similar values in wines from Oltenia, Dobrogea, Muntenia and significantly superior wines from Transylvania. Dobrogea and Muntenia wines from presenting the most significant amount of esters, followed closely by those in Transylvania and the lowest content of esters that from Oltenia. Aldehydes were identified in high concentrations in red wines from Transylvania, followed by those in Oltenia, Dobrogea and Muntenia and terpene compounds in wines from Wallachia and Transylvania. It can be said that depending on the region from which these wines, although it is the same variety, their structure aromatic sometimes differ greatly. Sugars determined red wines studied them within the category of dry and semi-dry area resulting values were included in the current standards, glucose, fructose actually showing similar values resulting from biochemical processes that occur during alcoholic fermentation. Variation values in sugar for the same sort of wine in different years can be explained by climatic conditions specific ripening and maturation of grapes, precipitation and temperature variations thereof (Stegarus et Tita, 2015).

The year of 2013 was noted as an year in which the grape varieties for red wines showed a high potential for accumulation of sugars, with values above 214 g/L at harvest for Feteasca neagra, Pinot noir, Merlot and Cabernet Sauvignon, the highest value reached in Feteasca neagra variety. Red wines produced in 2013 were dry wines with a high alcohol level, of more than 13.0% vol. The most intense colour was shown by Cabernet Sauvignon and Feteasca neagra wines, these being also the varieties with the highest content of anthocyanins; middle colour presented Merlot wine variety and lower intensity was found for Pinot noir, which presented also the smallest values of anthocyanins (Artem et al., 2014)

Young wines of 2013 and 2014 harvests can be well characterized based on anthocyanin,

parameters that are in higher amounts in young wines compared with aged wines. Certain anthocyanins and anthocyanin ratios (De, Pe, Pec and Pt/Mv, Mvc/Mv) coupled with sugars like glucose (3.83 ppm 1H NMR signal) can be estimated as variables for differentiation of aged wines from 2010. Isotopic variables (d18O and d13C) and amino acids like alanine (1.47 ppm and 1.45 ppm 1H NMR signals) represent useful parameters for 2012 vintage differentiation, while isotopic variables ((D/H)II and R) and sugars (3.18 ppm 1H NMR signal and 62.58 ppm 13C NMR signal) were highlighted for 2011 vintage differentiation (Geana et al., 2015)

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STUDY ON INCIDENCE OF GENETICALLY MODIFIED PLANTS LIKE RAW MATERIALS, IN ROMANIA, DURING 2012 - 2017

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Abstract

The paper aimed to present the distribution of the incidence of genetically modified vegetable like raw materials in Romania, during the period 2012 - 2017, including Calarasi, Iasi, Salaj, Satu-Mare, Suceava, Tulcea, Constanta, Bucuresti counties. It is based on the statistical data provided by National Sanitary Veterinary and Food Safety Authority, submitted by specialized molecular biology laboratories. The data have been processed into the following indicators: species, type of material or product, type of detection and identification method, GMO's specific genetic structures and transformation events identified, degree of transformation compared to the European Union rate of 0,9% for necessary labeling. During the analyzed period, a number of samples ranging from 200 to 300 were analyzed annually. The raw material samples were grains, groats, flours from genetically modified plants, as well as finished products of food or feed, like biscuits, chocolate, bakery products. For the analyzed samples, genetic transformation processes were detected, but without exceeding the limit of 0.9% and not labeled as GMO. As a conclusion, on the Romanian market are safe genetically modified foods and feeds and the traceability and labeling of GMOs are respected.

Key words*: distribution, GMO, raw materials, method, Romania.*

INTRODUCTION

It is well known that the genetically modified food or feed contains as whole or in part genetically modified organisms (GMO) and for this reason special regulation need to be respected. GMO means an organism that possesses foreign genes obtained through the use of modern biotechnology (i.e. genetic engineering) (Cornea, 2010).

European Union legislation lists the conditions for the commercialization of such products through intra-Community trade. Well established traceability and labelling procedures for all genetically modified organisms must be complied in order to provide to consumers the right information and free choice.

The following regulations are the basic legislative framework adopted at European Union level in the field of Genetically Modified Organisms:

• Council Directive no. 2001/18 / EC on the deliberate introduction into the environment of genetically modified organisms;

• Regulation of the European Parliament and of the Council no. 1829/2003 on genetically modified food and feed;

• Regulation of the European Parliament and of the Council no. 1830/2003 on the traceability and labelling of genetically modified organisms and the traceability and labelling of food and feed products, produced from genetically modified organisms.

At European level, the leading role is given to the scientific opinions of the European Food Safety Authority (EFSA) on genetically modified organisms that are issued as a result of the risk assessment which genetically modified organisms may have for public health, animal health and environmental protection. The risk assessment show that for certain conditions of use initially established, the genetically modified product is safe. Procedures of evaluation and authorization of genetically modified organisms are transparent and not have the time limit. (https://www.ansvsa.ro, November 2017)

The risk assessment is based on scientific information at international level, based on well-defined criteria.

(https://www.efsa.europa.eu/en/science/scientific-committee-and-panels, November 2017)
The scientific opinions of the EFSA detail the scientific information analysed, which led to the approval of the marketing of genetically modified products on the European market. 58 genetically modified plants (containing GM events) are authorized for marketing in the member states of European Union. (https://www.efsa.europa.eu/en/science/gmo, November 2017)
Generally, the methods to obtain the transgenic plants are based on the use of *Agrobacterium* system (Cornea, 2010; Kado, 2015; Parmar et al., 2017).
The scientific publications with a major impact on the safety of GMOs authorized by EFSA, are permanently monitored. Until now, the conclusions of the approved opinions have not been changed.
Depending on the transformation event, validated methods for detection and quantification of GMO content of food and feed are available (http://gmo-crl.jrc.ec.europa.eu/gmomethods/, January 2018). Such reference methods are based mainly on molecular techniques (PCR, qPCR) (Levy et al., 2014) and could be applied to a various plant species (maize, soybean, rice, tomato, papaya, carnation, cotton) for specific transformation event or sequences (Bonfini et al., 2012).
At national level, control of the correct traceability and labelling of genetically modified foods and feeds is the responsibility of the National Sanitary Veterinary and Food Safety Authority(NSVFSA) (www.ansvsa.ro).
The aim of the present study is to analyse the distribution of the incidence of genetically modified plant raw materials in Romania and includes data provided by the molecular biology and genetically modified organisms laboratories for the period 2012-2017.

MATERIALS AND METHODS

The data were collected from laboratories that can perform analyses to identify or quantify foods and feeds that represent or may contain genetically modified organisms (Sanitary Veterinary and Food Safety Laboratory (SVFSL) Calarasi, SVFSL Constanta, SVFSL Iasi, SVFSL Salaj, SVFSL Satu-Mare, SVFSL Suceava, SVFSL Tulcea and the National Reference Laboratory for GMOs from the Institute of Diagnosis and Animal Health were processed and statistically interpreted in order to develop an distribution of the results of the analysed samples.
The period evaluated of study is 2012-2017.
The analyses performed for the GMO detection in the Romanian laboratories through standardized methods are:
• Detection by PCR techniques of genetic GMO-specific genetic structures in food and feed (Screening real time PCR for the identification of GMOs in food and feed of plant origin);
• The identification and quantification of GMOs in foods and feed containing corn;
• Identification of DNA specific for GTS 40-3-2 line (Roundup Ready) from food and feed containing soy;
• Quantification of DNA specific GTS 40-3-2 line (Roundup Ready) from food and feed containing soy;
• Detection of p35S and tNOS elements in food and feed.
The identification and quantification methods used are as follows:
• ISO 21569 - Methods of analysis for GMO detection - qualitative methods
• ISO 21570 - Methods of analysis for the quantification of GMOs - quantitative methods
• ISO 21571 - Methods for the extraction of nucleic acid.
However, currently, the most used methods for the detection and quantification of GMOs in food and feed are:
• Identification of genetically modified soybeans or corn (RENAR accredited method), involving the detection of p35S and tNOS genetic elements by PCR;
• Genetically modified DNA quantification in foods and feed containing soybeans specific to the GTS 40-3-2 (Roundup Ready) line by Real-Time PCR (RENAR accredited method).
The following indicators have been used in order to characterize the incidence of GMOs: plant species, type of material or product, type of detection and identification method, GMO's specific genetic structures and transformation events.
The research methodology used in this study has the following main aspects:

• *bibliographic study of the internal literature;*
• *collecting the concrete information within the researched area;*
• *ordering, processing and presentation of results in synthetic form;*
• *analysis and interpretation of results and formulation of conclusions.*

RESULTS AND DISCUSSIONS

In accordance with the Report on the Assessment of the Economic Performance of Genetically Modified Genetic Plants, published on the European Commission's website(https://ec.europa.eu/food/sites/food/files/plant/docs/gmo rep-stud 2011 report econ-perf.pdf, September 2015), the global area seeded with genetically modified plants has grown significantly annually since 1996, when it was first grown for commercial purposes on an area of 2.8 million hectares. In 2016 this area grew to 185 million hectares (ISAAA, 2016 https://www.isaaa.org/resources/publications/briefs/52/download/isaaa-brief-52-2016.pdf, December 2017). Soy, maize, rapeseed and cotton were mainly grown.

Regarding soybean, it is known that more than 36 million tonnes are needed for animal feed in Europe. Annually, the Member State cultures produce 1.4 million tonnes of non-genetically modified soybean. Genetically modified soybeans are not authorized for cultivation in the European Union but are imported very large quantities soybean (18.5 million tonnes of soybean flour and 13.5 million tonnes of soybeans in 2013). (https://www.ansvsa.ro, November 2017).

In Romania, the National Sanitary Veterinary and Food Safety Authority, as regulatory and controlling authority in the field of genetically modified food and feed, has the role of ensuring that only genetically modified food and feed authorized is introduced on the national market and the provisions traceability and labelling are respected by food and feed business operators. Within the eight laboratories specialized in molecular biology are analysed both the samples taken in the GMO official control program and the samples taken by the food and feed industry operators in the self-control process.

In the last 6 years (Table 1) were analysed a total of 5184 samples of food or feed that may contain parts of genetically modified organisms of plant origin (used as raw material in the production process).

According to the data provided by the County Laboratories and the Reference Laboratory of the Institute of Diagnosis and Animal Health (IDSA), the number of samples analysed varied from 1070 in 2013 to 756 in 2016.

Table 1. Number of samples analysed annually for GMO detection, during 2012-2017

	2012	2013	2014	2015	2016	2017	Total
Calarasi	46	53	22	30	32	40	**223**
Constanta	31	32	23	34	38	29	**187**
Iasi	41	141	150	124	105	109	**670**
Satu Mare	40	38	29	9	12	13	**141**
Salaj	216	249	208	251	268	313	**1505**
Suceava	59	64	50	37	25	11	**246**
Tulcea	29	57	21	26	23	26	**182**
IDSA	509	436	313	278	253	241	**2030**
Total	**971**	**1070**	**816**	**789**	**756**	**782**	**5184**

The detailed evidence of the number of samples transmitted from the 7 County Laboratories of Molecular Biology and GMO and the Reference Laboratory of IDSA showed a total of samples processed in each laboratory (Figure 1), as follows: for Calarasi County 223 analysed samples for identification of genetic modification events, for Constanta County 187 samples, for Iasi County 670 samples, for Satu Mare county 141 samples, for Salaj county 1505 samples, for Suceava county 246 samples, for Tulcea county 182 samples and for IDSA 2030 samples.

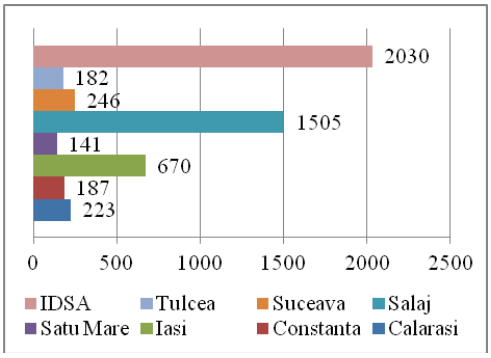

Figure 1. The total number of analysed samples (by counties) for the identification of GMOs (2012-2017)

The largest number of samples were analysed in the laboratories of IDSA, but also in Salaj and Iasi counties.
The total number of samples processed in all the 8 laboratories is close and has an average of 864 samples per year, except 2013 (Figure 2). The interlaboratory difference is approximately ± 100 samples per year.

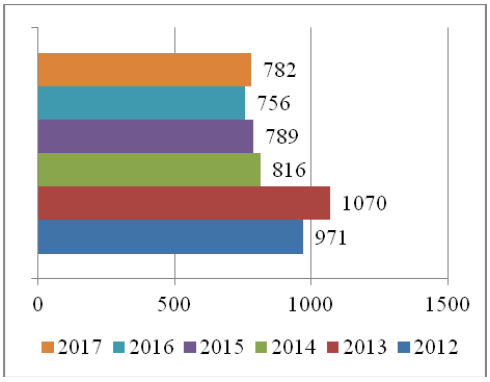

Figure 2. The total number of samples analyzed (annually) for the identification of GMOs

Regarding the identification of the sequences of the promoter P-35S and the T-NOS terminator specific to the genetically modified plants, the distribution of the number of samples processed annually in each of the 8 laboratories in Romania is shown in Figure 3.

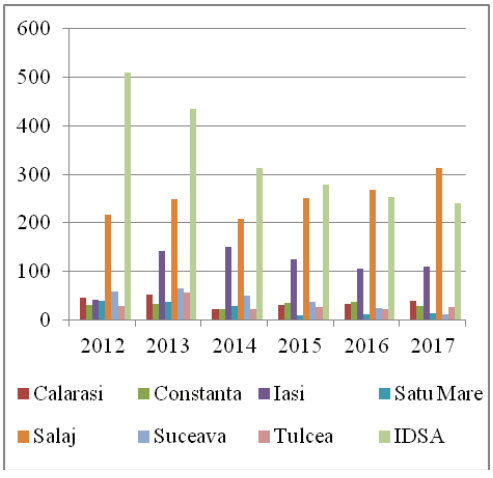

Figure 3. Annually distribution of the number of samples analysed for GMO identification

The largest number of samples (509) was processed in 2012 by the reference laboratory for molecular biology and GMO at the Institute of Diagnosis and Animal Health.
Regarding the plant species examined for transformation events analyses have been carried out to identify GMOs, raw material of plant origin, food or feed containing genetically modified soybeans (2678 samples) and genetically modified maize (2286 samples).
The types of vegetal material analysed were:
• for soy: beans, wheat flour, protein isolate, textured, milk, fibres, granules, cubes, snuff, slices, bread, chocolate, pate, sauce, salami, sausages, bakery brewers, cakes, waffles, biscuits, pudding, lecithin, canned meat, pastry premix and cream;
• for corn: grains, malt, popcorn, puff, chips, oil, flakes, sweet corn, flour, starch, fodder yeast, compound feed.
The total number of samples processed by each of the 8 laboratories (Table 2) shows that in Romania soy is more used in animal feed and food production, compared to corn.
This idea is sustained by the fact that in the laboratories from Calarasi, Satu Mare and Suceava were processed only samples to identify the DNA sequence specific to genetically modified soybean GTS 40-3-2 (Roundup Ready).

Table 2. Total number of samples of raw materials of soy and corn processed during 2012 - 2017

	Species	Total
Calarasi	soy	223
Constanta	soy	142
	corn	45
Iasi	soy	246
	corn	424
Satu Mare	soy	141
Salaj	soy	952
	corn	553
Suceava	soy	246
Tulcea	soy	176
	corn	6
IDSA	soy	772
	corn	1258

Moreover, the highest number of samples examined for genetically modified soybeans was in the Salaj laboratory (952 samples) and at IDSA laboratory (772 samples) (Figure 4.)

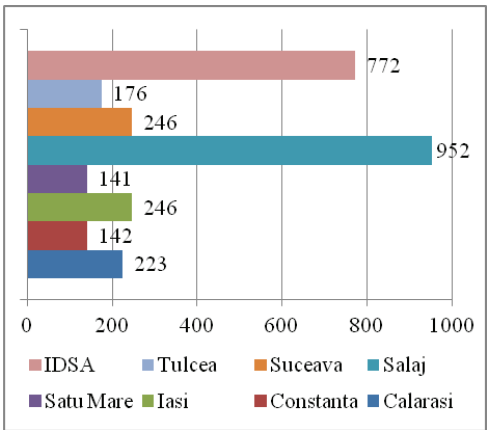

Figure 4. The total number of samples analysed for the identification the specific modified DNA of line GTS 40-3-2 (Roundup Ready) during 2012-2017

The samples processed to identify genetically modified corn (Figure 5) are shared between three laboratories that correspond to the historical geographic areas. Half of the samples were analysed by IDSA (1258 samples), and the other half of the samples are split between laboratories from Salaj (553 samples) and Iasi (424 samples).

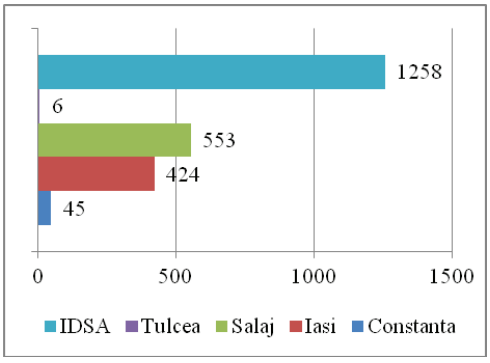

Figure 5. The total number of samples analyzed for P-35 S and T-NOS identification from corn-containing raw materials during 2012-2017

From the analysis of the statistical data regarding the number of samples of raw materials of vegetal origin (Table 3) it is found that each laboratory has processed annually a relatively constant number of samples, but their averages are quite different from one county to another, ranging from dozens of samples. The working capacity is very different between the 8 laboratories.

Table 3. Number of samples raw materials of vegetable origin (soy and corn) analysed (P-35 S, T-NOS)

	Species	2012	2013	2014	2015	2016	2017
Calarasi	soy	46	53	22	30	32	40
Constanta	soy	31	32	23	18	20	18
	corn	-	-	-	16	18	11
Iasi	soy	40	47	44	41	40	34
	corn	1	94	106	83	65	75
Satu Mare	soy	40	38	29	9	12	13
Salaj	soy	121	138	126	180	168	219
	corn	95	111	82	71	100	94
Suceava	soy	59	64	50	37	25	11
	corn						
Tulcea	soy	27	57	21	26	19	26
	corn	2	-	-	-	4	-
IDSA	soy	168	131	142	117	117	97
	corn	341	305	171	161	136	144

In 2012, for the identification of genetically modified corn a maximum of 341 samples were processed at the IDSA and a minimum of 1 sample were processed by the Iasi laboratory.
Figure 6 and 7 reveal the distribution of the number of samples processed to detect genetically modified soybeans is much higher than the number of samples processed to identify genetically modified corn.
The average is relatively uniform for genetically modified soy in all the laboratories (Figure 6).

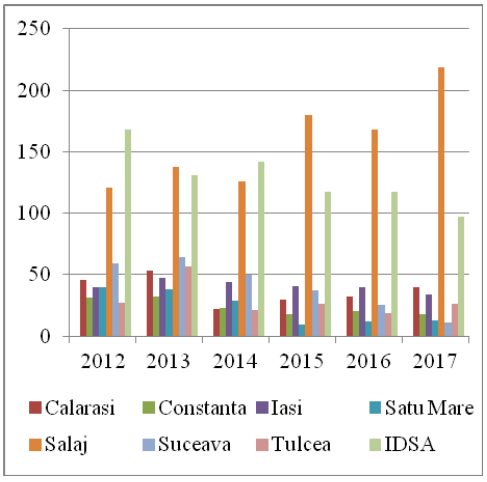

Figure 6. Counties distribution of the number of samples of raw materials containing soybean

Differences were recorded for the identification of genetically modified corn: in at least two laboratories no corn samples were examined (Figure 7).

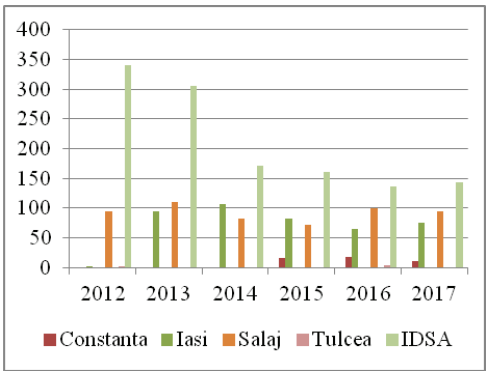

Figure 7. Counties distribution of the number of samples of raw materials containing corn

In Romania is approved for cultivation only maize MON 810, but the introduction on the national market and the use of GMO as raw materials in various forms, in feed and food industry is allowed, according to current legislation.

EU legislation obliges food and feed business operators to label all genetically modified products if their presence exceeds 0.9%. [3] For this reason the possible presence of GM events have to be identified throughout the food chain.

During the analysed period (2012-2017) it was shown that all the samples processed to identify genetically modified corn had a negative result (under the 0.9% limit). However, positive results were recorded in several samples examined for the identification of genetically modified soybean (Table 4). The highest positive number of samples was detected in Salaj laboratory (29 samples).

Table 4. Total number by counties of samples with positive result on identification for GMO

	Species	Total
Calarasi	soy	10
Constanta	soy	10
	corn	-
Iasi	soy	14
	corn	-
Satu Mare	soy	-
Salaj	soy	29
	corn	-
Suceava	soy	20
Tulcea	soy	25
	corn	-

The representation of the distribution on each laboratory that had positive results for food or feed containing genetically modified soybean (Figure 8) shows an approximately uniform distribution for the historical-geographic areas in Romania. Of the 2898 samples analysed, 108 of them had a positive result, which represents only 3%, which means that a small amount of genetically modified soybeans has been imported into our country through intra-community trade or import.

The proportion of positive results for GM soybean from the total of samples processed at County Laboratories of Molecular Biology and GMO is low, ranging from 3% for Salaj laboratory to 14.2% for Tulcea laboratory. At Calarasi laboratory this proportion is 4.5%, at Constanta laboratory is 7%, at Iasi laboratory is 5.7%, at Suceava laboratory is 8.1%.

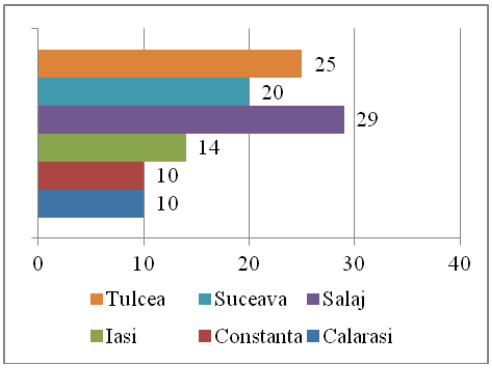

Figure 8. The total number of positive samples for GMO identification in 2012-2017

The annual distribution of positive results (Table 5) shows a maximum of 13 samples in 2017 at Tulcea laboratory and 13 samples in 2015 at Suceava laboratory.

Table 5. Number of samples by counties and annually with positive result on identification for GMO

	Species	2012	2013	2014	2015	2016	2017
Calarasi	soy	2	1	-	3	3	1
Constanta	soy	7	2	1	-	-	-
	corn	-	-	-	-	-	-
Iasi	soy	4	1	4	1	4	-
	corn	-	-	-	-	-	-
Satu Mare	soy	-	-	-	-	-	-
Salaj	soy	4	11	8	4	2	-
	corn	-	-	-	-	-	-
Suceava	soy	-	-	4	13	3	-
Tulcea	soy	-	4	2	2	4	13
	corn	-	-	-	-	-	-

From the analysis of these results, for the period 2012 - 2017, a similar amount of food and feed containing genetically modified soybeans was introduced annually in our country. We note, therefore, that the food industry and livestock activity had a relatively constant activity during this time period.
Distribution of the number of samples that had a positive result for the identification of genetically modified soybean (Figure 9) reported annually and for each laboratory reveals a non-homogeneous pattern.
In 2017, a number of samples with a positive result were detected only for Tulcea laboratory. The same situation was in 2015 at Suceava laboratory and in 2013 at Salaj laboratory. A homogeneous distribution is only in 2016.

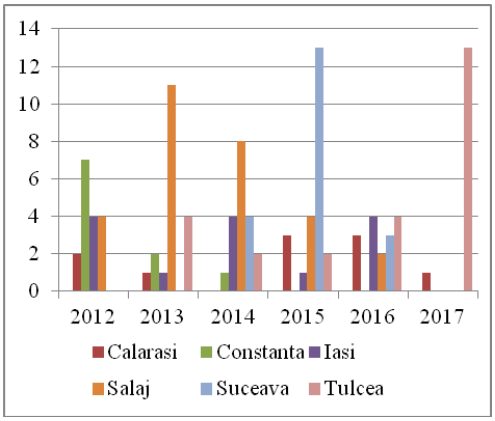

Figure 9. Counties and Annually distribution of the number of samples with positive result on GMO identification

CONCLUSIONS

In Romania is allowed for cultivation only one transformation event (maize MON810), but they are allowed to be placed on the national market and used like animal feed and in the food industry. They can be used to obtain food products and can therefore be found throughout the food chain.
During 2012-2017, eight molecular biology laboratories processed 5184 samples of raw materials, food or feed of plant origin to identify specific genetic modification sequences.
The methods used for the detection and quantification of GMOs (soybean or corn) in food and feed are RENAR accredited.
In Romania, there is a representative laboratory for the number of processed samples for the three historical-geographic areas: Muntenia, Transylvania and Moldova.
The number of samples processed for identifying genetically modified soybeans (2678 samples) is higher than the number of samples processed to identify genetically modified corn (2286 samples).
For the identification of genetically modified soybeans, the Salaj laboratory (952 samples) and IDSA (772 samples) were processed the largest number of samples in comparison with all the other laboratories.
The samples processed to identify genetically modified corn had been processed half of the samples were analysed by IDSA (1258 samples), and the other half of the samples are split between laboratories from Salaj (553 samples) and Iasi (424 samples).
All samples processed to identify genetically modified corn have had a negative result (under the limit of 0.9%), which reveals that this products respected the current legislation (during 2012-2017).
A small amount of genetically modified soybeans has been imported into our country through intra-community trade or import: by the 2898 samples analysed, 108 (3%) had a positive result.
The data presented in this work confirm the compliance of the legislation in GMO domain in Romania, regarding not only the cultivation but also the detection and quantification.

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CLASSIC VERSUS MODERN TOOLS TO STUDY MICROBIAL POPULATION DYNAMICS DURING FOOD FERMENTATION PROCESSES

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Abstract

For a better understanding of microbial processes and dynamics of microbial population in fermented food is essential the taxonomical definition of their content. It is difficult to estimate true microbial diversity due to inability to cultivate most of the viable bacteria or to evaluate stressed cells. The most appropriate approach it seems to be the integration of phenotypic and genotyping data, while the molecular methods alone are not enough to establish distinct boundaries among phyllogenetically related species. It is important for identification of microbial strains to connect physiological, morphological and biochemical features as well as the aspects of its genetic profile. The most common genotypic and phenotypic methods are reviewed in this paper, highlighting on the suitable techniques which can be used to differentiate among microbial strains.

Key words*: molecular techniques, food fermentation, genotyping, phenotyping.*

INTRODUCTION

Fermentation is a metabolic process in which an organism converts a carbohydrate, such as starch or sugar, into an alcohol or an acid. For example, the yeast performs fermentation to obtain energy by converting sugar into alcohol. Lactic bacteria perform fermentation, transforming carbohydrates into lactic acid. This process is used to produce wine, beer, yogurt and other products.

Fermentation is a natural process. People have been fermenting to produce products like wine, honey, cheese and beer long before the biochemical process is understood. In the 1850s Louis Pasteur became the first scientist to study fermentation when it was shown to be caused by living cells.

The most important fermentations involved in food production are alcoholic fermentation, lactic fermentation and acetic fermentation. In the alcoholic fermentation yeast and certain bacteria perform the fermentation of carbohydrates in which pyruvic acid is broken into ethanol and carbon dioxide; this process is specific to bread, wine or beer production.

In the lactic fermentation, the lactose is converted through pyruvic acid in lactic acid. This type of fermentation is used for the production of cheese and dairy products. Acetic fermentation is another type of fermentation and is produced by acetic bacteria, and as an intermediate product results acetic acid.

By the acetic fermentation of the wine we get the vinegar. Acetic fermentation is also used to conserve pickles. Although it is considered fermentation, it is carried out in the presence of oxygen.

During these fermentations, which are conducted on natural sources of carbohydrates (grapes, milk, cereals etc.), the microbial biodiversity and levels are in a continuous changing.

To get the best final product it is important to conduct an optimal fermentation process, in which the microorganisms involved are an important factor.

To characterize the fermentation microbial biodiversity the approach is complex and should be taken into account phenotypic and genotypic methods and to establish correlation between the results of these methods (Girafa et al., 2004; Cocolin et al., 2013; Donelli et al., 2013; Matei et al., 2018).

In the following will be presented both the phenotypic and genotypic tools useful in characterizing the microbial diversity and levels.

MATERIALS AND METHODS

Online information research was conducted by the use of different database collections and on-searching engines (Google Academic, Web of Knowledge, PubMed and ScienceDirect). The information has been structured according to the approach used in the characterization of the microbial diversity.

RESULTS AND DISCUSSIONS

Phenotypic studies have been broadly used during years, this is why the presented data will mainly focus on the molecular tools used in the characterization of microbial biodiversity during food fermentations.

(1) Phenotypic methods

Phenotypic characterization of microbial strains is based on data supplied by all the typing methods not based on DNA or RNA, including chemotaxonomic methods that are able to give information on chemical constituents of microbial cells. Thus, the classical phenotypic tests are important sources of taxa, from species up to genus and family. In many cases the set of all the morphological, physiological and biochemical features of a strains allows the recognition of taxa. These phenotypic characteristics in specific microbial groups, such as lactobacilli and bifidobacteria, are not enough to completely describe or differentiate taxa and must be performed in addition to genotypic analysis (Tannock, 1999; Mastromarino et al., 2002)

The morphological investigation of a microorganism both by light and electron microscopy provides information on cell shape, flagella and inclusion bodies while color, dimension and form of its colonies are detected macroscopically on a suitable agar plate. Physiological data useful for classification purposes include growth temperature, pH value, salt concentration and oxygen requirement whereas biochemical features of interest include enzymatic activity, gas production and compound metabolism (Yang et al., 2010; Nomura et al., 1999).

For rapid phenotypic characterisation in practice are used API stripes, which are test kits for identification of Gram positive and Gram negative bacteria and yeast produced by Biomerieux company. The system offers a large and robust database now accessible through the Internet-based APIWEB™ service. According to the most common protocols, carbohydrate fermentation analysis for lactobacilli is carried out using API 50 CH, a research strip that enables the study of the metabolism of 49 carbohydrates and is able to identify *Lactobacillus* species within 48 hours. However, some epidemiological studies have reported shortcomings in the use of this methodology due to the possibility of identifying lactobacilli belonging to different species as the same microorganism (Vasquez et al., 2002), thus fermentative profile seems to be inaccurate method for identification and classification of *Lactobacillus* species which therefore needs to be performed additional genotypic analysis (Pavlova et al., 2002).

FAME (fatty acid methyl esters) analysis (Miller, 1982) has been successful used since fatty acids are the major constituent of lipids and lipopolysaccharides in microbial cells and have been used for taxonomic purposes. In fact, more than 300 different chemical structures of fatty acids and their variability in chain length, double-bond position and substituent groups has been very useful for the characterization of bacterial taxa (Suzuki et al., 1993). This is a cheap and rapid method with high degree of automation that was recently used to investigate the diversity of 94 *L. reuteri* isolates (Hilmi Hanan et al., 2007).

(2) Genotypic methods

The application of molecular biology methods has greatly improved the bacterial identification and classification, by genotyping directed toward to DNA or RNA molecules.

The currently available molecular-based typing methods are mainly based on restriction analysis of the bacterial DNA, polymerase chain reaction (PCR) amplification of specific targets and identification of DNA sequence polymorphisms. Table 1 presents the use of different molecular tools in the characterization of microbial dynamic during fermentation.

Random Amplification of Polimorphic DNA (RAPD-PCR) is a PCR method based on segments of DNA that are amplified randomly, for the identification of the genetic variation;

by the use of a single arbitrary primer in a PCR reaction is resulting the amplification of many DNA products. This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocylic amplification. RAPD-PCR method was used to differentiate between probiotic *Lactobacillus* in wine (Plessas et al., 2017), identifying *Oenocococcus oeni*, *Leuconostoc mesenteroides* in wine (Ruiz et al., 2008; Lucena-Padros et al., 2014); genotypes have been found in olive fermentations of *L. pentosus, L. paracollinoides, L. rapi, Pediococcus* sp., *Staphylococcus* sp., *Candida thaimueangensis, S. cerevisiae, Hanseniaspora* sp. (Lucena-Padros et al., 2014), bio-typing of *Lactobacillus sakei, L.paracasei, L.curvatus, L. plantarum, L. fermentum* in traditional fermented sausage (Tremonte et al., 2017; Pisacone et al., 2015), characterization of *L. brevis, L. plantarum, L. pentosus, L. fermentum* in eggplant (Sesena et al., 2005), characterization of *Weissella* sp., *Pediococcus* sp, *Lactococcus* sp., *Lactobacillus* in Mexican fermented beverage (Väkeväinen et al., 2018), rapid identification of *L. casei, L paracasei, L. plantarum, L. rhamnosus, L. helveticus, L. fermentum, L. brevis, Streptococcus thermophilus, Enterococcus faecalis, Lactococcus lactis* in dairy (Rossetti et al., 2005) and identification of *L. plantarum, L. sanfranciscensis, Leuconostoc mesenteroides, L. fermentum, Weissella cibaria, L. pentosus, L. brevis, L. paraplantarum* in sourdough fermentation (Rizzello et al., 2014).Limitations of the method are: mismatches between the primer and the template may result in the total absence of PCR product, RAPD-PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is the most worldwide used molecular method, being introduced approximately 25 years ago. These techniques consist of amplification of the genes encoding the 16S rRNA from the matrix containing different bacterial populations, followed by separation of the DNA fragments on gel electrophoresis, molecules with different number of pair base will migrate on different position generating a patterns which can provide a preliminary view of predominant species. PCR-DGGE has been a useful method for identification of *Lactobacillus sp., Yarrowia lipolytica, Debaryomyces hansenii, Rhodotorula mucilaginosa*, *Candida stellata, S.cerevisiae, L. curvatus, L. plantarum, S. xylosus* in Ciauscolo, a traditional Italian salami (Silvestri et al., 2007; Aquilanti et al., 2007), identification and characterization of *Lactobacillus sanfranciscensis, Candida milleri, Sacharomyces cerevisiae,* in sourdough (Palla et al., 2017), for detection of *Aspergillus niger, Botrytis cinerea, Hanseniaspora uvarum, S. cerevisiae, C. stelatta, Leuconostoc mesenteroides, O. oeni, S. cerevisiae* (Andorra et al., 2010; Perez-Martin et al., 2014; Portillo et al., 2016), identification of *Leuconostoc mesenteroides, Tetragenococcus halophylus*, *Enterococcus farcium, Enterococcus faecium, B. subtilis, B. licheniformis, Mucor plumbeus*, *Aspergillus oryzae, Debaromyces hansenii,* in soybean paste (Kim et al., 2009; Do Ham et al., 2012) and characterization of *L. sakei, L. paracasei, L. curvatus, L. plantarum*, *L. fermentum, S. xylosus, S. saprophyticus, S. pasteuri, S. epidermis, S. simulans, S. equorum* in traditional sausage (Pisacane et al., 2015; Fonseca et al., 2013). This method requires long time to be performed, works well only with short fragments less than 600 bp, thus limiting phylogenetic characterization, results difficult to reproduce between gels and laboratories.

Real-Time qPCR method is using primers pair specific for a desired targeted sequence and internal probe, labelled with fluorescent dye, with each amplification cycle, the fluorescence intensity is increasing, which is collected by the instrument system. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the „Ct" or threshold cycle. This Ct value can be directly correlated to the starting target concentration of

the sample. This method has been used for the detection of *Streptococcus thermophilus* and *Lactococcus lactis* in dairy (Pega et al., 2017), detection of *A. niger, Botrytis cinerea,*

Table 1. Method used to describe the dynamic of microbial population in different food matrix

Method/Matrix	Microorganism	References
PCR-DGGE		
Italian salami	*Lactobacillus* sp., *Yarrowia lipolytica, Debaryomyces hansenii, Rhodotorula mucilaginosa*, *Candida stellata, S. cerevisiae, L. curvatus, L. plantarum, S. xylosus*	Plessas et al., 2017
Sourdough	*Lactobacillus sanfranciscensis, Candida milleri*, *Sacharomyces cerevisiae*	Aquilanti et al., 2007 Palla et al., 2017
Wine	*Aspergillus niger, Botrytis cinirea*, *Hanseniaspora uvarum, S. cerevisiae, C. stelatta*	Andorra et al., 2010
Cocoa bean	*L. plantarum, L. fermentum, Acetobacter pasteurianus*	Lefeber et al., 2011
Sorghum	*Lactococcus lactis, Weissella cibaria, L. curvatus*, *Enterobacter* sp.	Madoroba et al., 2011
Sausage	*L. sakei, L. plantarum, Weissella hellenica, Leuconostoc mesenteroides*	Tremonte et al., 2017
Cassava dough	*L. plantarum, L. fermentum, L. pentosus, L. case, L. acidophilus*	Oguntoyinbo et al., 2010
Shenqu	*Pediococcus acidilactis, Rhizopus* sp., *Aspergillus oryzae, Enterobacter* sp., *Klebsiella* sp., *Erwinia* sp., *Pantoea vagan*	Lin et al., 2017
Wine	*Gluconobacter* sp., *Acetobacter* sp., *Gluconoacetobacter* sp., *Bifidobacterium* sp., *Hanseniaspora* sp., *Sacharomyces* sp., *Candida* sp.	Portillo et al., 2016
Soybean paste	*Leuconostoc mesenteroides, Tetragenococcus halophylus, Enterococcus farcium, Enterococcus faecium, B. subtilis, B. licheniformis, Mucor plumbeus*	Kim et al., 2009 Do Ham et al., 2012
Traditional sausage	*Aspergillus oryzae, Debaromyces hansenii, L. sakei, L. paracasei, L. curvatus, L. plantarum*, *L. fermentum, S. xylosus, S. saprophyticus, S. pasteuri, S. epidermis, S. simulans, S. equorum*	Pisacane et al., 2015 Fonseca et al., 2013
Wine	*Leuconostoc mesenteroides, O. oeni, S. cerevisiae*	Perez-Martin et al., 2014
Chinese liquor	*Methanocorpusculum* sp., *Methanobrevibacter* sp., *L. acetotolerans, L. alimentarius, Clostridium kluyveri, Clostridium sartagoforme, Methanobacterium* sp., *Methanoculleus* sp.	Ding et al., 2015 Zheng et al., 2013
Alcohol fermentation	*Rhizopus orizae, R. microsporus*, *Absidia corymbifera, Amylomyces sp., S. cerevisiae, Pichia anomala, Candida tropicalis, Clavispora lusitanie, Pediococcus pentosaceus, L. plantarum, L. brevis, Weisella confuse, B. subtilis, Acetobacter orientalis, A. pasteurianus*	Thanh et al., 2008
Olive fermentation	*L. plantarum, Marinilactibacillus* sp., *Propionibacterium olivae, Alkalibacterium* sp., *Halolactobacillus* sp., *Pediococcus acidilactici*	Lucena-Padros et al., 2015
Leek fermentation	*Leuconostoc mesenteroides, L. sakei, L. plantarum*, *L. brevis, L. parabrevis*	Wouters et al., 2013
Palm wine	*S. cerevisiae, S. ludwigii, Zygosacharomyces bailii, Hanseniospora uvarum, Candida parasilopsis, C. fermentati, Pichia fermentans*	Stringini et al., 2009
RAPD-PCR		
Feta cheese	*Lactobacillus* sp.	Plessas et al., 2017
Wine fermentation	*Oenococcus oeni, Leuconostoc mesenteroides*	Ruiz et al., 2008
Olive fermentation	*L. pentosus, L. paracollinoides*, *L. rapi, Pediococcus* sp., *Staphylococcus* sp., *Candida thaimueangensis, S. cerevisiae, Hanseniospora* sp.	Lucena-Padros et al., 2014
Fermented sausage	*L. sakei, L. plantarum, Weisella hellenica*, *Leuconostoc mesenteroides, S. xylosus, S.saprophyticus, S. pasteuri, S. epidermis, S. simulans, S. equorum*	Tremonte et al., 2017 Pisacone et al., 2015
Eggplant	*L. brevis, L. plantarum, L. pentosus, L. fermentum*	Sesena et al., 2005
Fermented beverage	*Weissella* sp., *Pediococcus* sp., *Lactococcus* sp., *Lactobacillus* sp.	Väkeväinen et al., 2018
Italian sausage	*Staphylococcus xylosus*	Iacumin et al., 2006
Dairy	*L. casei, L. paracasei, L. plantarum, L. rhamnosus, L. helveticus, L. fermentatum, L. brevis, S. thermophilus, Lactococcus lactis*	Rossetti et al., 2005
Sourdough fermentation	*L. plantarum, L. sanfranciscensis, L. fermentum*, *Leuconostoc mesenteroides, Weissella cibaria, L. pentosus, L. brevis, L. paraplantarum*	Rizzelo et al., 2014
qPCR		
Dairy	*Streptococcus thermophilus, Lactococcus lactis*	Pega et al., 2017
Wine	*A. niger, Botrytis cinerea, Hanseniospora uvarum, S. cerevisiae, Candida stellata*	Andorra et al., 2010
Cocoa bean	*L. sakei, L. plantarum, Weissella hellenica*, *Leuconstoc mesenteroides*	Schewendiman et al., 2017
Sourdough	*L. curvatus, L. brevis, L. pontis, Weissella* sp., *Pediococcus pentosaceus, L. plantarum, S. cerevisiae*	Michel et al., 2016 Lin et al., 2014 Sienwerts et al., 2018
Wine	*Gluconobacter* sp., *Acetobacter* sp., *Gluconoacetobacter* sp., *Bifidobacterium* sp., *Hanseniaspora* sp., *Sacharomyces* sp., *Candida* sp.	Portillo et al., 2016 Andorra et al., 2011
Spanish sausage	*Staphylococcus equorum, L. sakei*	Fonseca et al., 2013
Cheese milk	*Propionibacterium freudenreichii, P. thoenii, P. jensenii*, *P. acidipropionici*	Turgay et al., 2018

Tabel 1 (Continued)

White cheese	*Saccharomyces cerevisiae, Enterococcus* sp., *L. brevis, L. curvatus*	Kadiroglu et al., 2014 Ladero et al., 2010
Fish sauce	*Virgibacillus halodentrificans, Tetragenococcus halophilus*	Udomsil et al., 2016
Sau-PCR		
Wine fermentation	*Saccharomyces cerevisiae*	Perrone et al., 2013
Sausages	*Staphylococcus xylosus*	Iacumin et al., 2006
T-RFLP		
Wine fermentation	*S. cerevisiae, H. uvarum, Pichia minuta, Sacharomycodes ludwigii, Candida zemplinina*	Sun and Liu, 2014
PFGE		
Wine fermentation	*S. cerevisiae, H. uvarum, Pichia minuta, Sacharomycodes ludwigii, Candida zemplinina*	Sun and Liu, 2014
Youghurt	*L. delbrueckii, S. thermophilus*	Rademaker et al.,2006
Cells-qPCR		
Wine	*B. bruxellensis, S. cerevisiae, Z. bailii, L. plantarum, Oenococcus oeni, A. aceti, Gluconobacter oxydans*	Soares-Santos et al.,2017, 2018
ARDRA–ITS RFLP		
Sourdough	*L. sanfranciscensis, Candida milleri, S. cerevisiae*	Palla et al., 2017
Cocoa fermentation	*B. subtilis, B. pumilus, B. sphaericus, B. cereus*, *B. thuringiensis, B. fusiformis*	Ouattara et al., 2011
FISH		
Olive	*L. plantarum, L. paraplantarum, L. pentosus*	Ercolini et al., 2006
Wine	*S. cerevisiae, Hanseniospora guilliermondii*	Andorra et al., 2011
MS – PCR		
Food spoilage	*L. plantarum, L. paraplantarum, L. pentosus*	Dakal et al., 2018
PCR-RFLP		
Food spoilage	*L. plantarum, L. paraplantarum, L. pentosus*	Dakal et al., 2018
PMA – qPCR		
Wine	*S. cerevisiae, B. bruxellensis, O. oeni, L. plantarum, Acetobacter paseurianus*	Rizzotti et al., 2015
Box		
Sourdough	*L. curvatus, L. brevis, L. pontis, Weissella* sp., *Pediococcus pentosaceus*	Michel et al., 2016
Box – PCR		
Enzyme food	*Bacillus coagulans, L. plantarum, L. oris, S. epidermis*	Zhu et al., 2014
Flow cytometry		
Wine	*S. cerevisiae, B. bruxellensis, Candida vini, L. plantarum, L. casei, L. brevis, O. oeni, Acetobacter* sp., *Gluconobacter* sp., *Gluconoacetobacter* sp., *S. cerevisiae, Hanseniospora guilliermondii*	Longin et al., 2017 Andorra et al., 2011
Nested – PCR		
Shenqu	*Pediococcus acidilactici, Rhizopus* sp., *Aspergillus oryzae, Enterobacter* sp., *Klebsiella* sp., *Erwinia* sp., *Pantoea vagan*	Lin et al., 2017
Soybean paste	*Leuconostoc mesenteriodes, Tetragenococcus halophylus, E. faecium, B. subtilis, B. licheniformis, Mucor plumbeus, A. oryzae*	Kim et al., 2009
Rep – PCR		
Italian sausage	*Staphylococcus xylosus*	Iacumin et al., 2006
Cocoa bean	*S. cerevisiae, Candida ethanolica, L. fermentum, L. plantarum, Acetobacter pasteurianus, Acetobacter syzygii, Hanseniospora uvarum, Pichia manshurica*	Visintin et al., 2016

Hanseniaspora uvarum, S. cerevisiae, Candida stellata in wine fermentation (Andorra et al., 2010; Andorra et al., 2011; Portillo et al., 2016), *L. plantarum* and *L. fermentum* in cocoa bean fermentation (Schwendimanet al., 2015), *L curvatus, L. brevis, L. pontis, Weissella* sp., *Pediococcus pentosaceus* in sourdough (Michel et al., 2016; Sienwerts et al., 2018), *Staphylococcus equorum, L. sakei* in Spanish sausage Chorizo (Fonseca et al., 2013), *Propioni bacterium freudenreichii, P. thoenii, P. jensenii*, *P. acidipropionici* in cheese milk (Turgay et al., 2018; Kadiroglu et al., 2014), *Virgibacillus halodenitrificans, Tetragenococcus halophilus* in fish sauce (Udomsil et al., 2016).

Sau-PCR technique is based on the digestion of genomic DNA with the restriction endonuclease Sau3AI and subsequent amplification with primers whose core sequence is based on the Sau3AI recognition site. This method has been used for investigation of the dominance behaviour of *Saccharomyces cerevisiae* strains during wine fermentation (Perrone et al., 2013) and

characterization of *Staphylococcus xylosus* isolated from naturally fermented Italian sausages (Iacumin et al., 2006)

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) is a method that analyzes variation among 16s rRNA genes from different bacteria, being based on the restriction endonuclease digestion of fluorescent end-labeled PCR products. Restriction fragments are separated by gel electrophoresis and the fluorescence signal is quantified. Distinct patterns are obtained as each fragment represents each species present. This method has been used in investigation of yeasts species: *Saccharomyces* sp., *Hanseniospora uvarum, Pichia minuta, Saccharomycodes ludwigii, Candida zemplinina* in wine fermentation (Sun and Liu, 2014) and for assesement of *L. delbrueckii, S. thermophiles* in youghurt (Rademaker et al., 2006).

Pulsed-Field Gel Electrophoresis (PFGE) is a highly discriminative molecular typing technique that is used in worldwide. PFGE is based upon the variable migration of large DNA restriction fragments in an electrical field of alternating polarity. By comparing the DNA fingerprints of two isolates, it can be investigated if they belong to the same strain or if they are genetically unrelated. According to Ruiz et al., 2008, PFGE method has been used to study intraspecific genetic diversity of *Oenococcus oeni* and *Leuconostoc mesenteroides* from malolactic fermentation of Cencibel wines. Oguntoyinbo et al., 2010, studied dynamics of *L. plantarum, l. fermentum, L. pentosus, L. casei, L. acidophilus* during the spontaneous fermentation of cassava dough.

Cells-qPCR is a quantitative PCR assay and has been developed for rapid detection and quantification of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) from grape must and wine that does not require DNA extraction. This method is robust, reliable, fast and specific method to detect and quantify different bacteria and yeasts, like *B. bruxellensis, S. cerevisiae, L. plantarum, Oenococcus oeni, Acetobacter aceti, Gluconobacter oxydans, Zygosaccharomyces bailii*, overcoming the presence of inhibitors like polyphenols and ethanol (Soares-Santos et al., 2017; Soares-Santos et al., 2018).

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is a tool to study microbial diversity that relies on DNA polymorphism. Fragments of 16S rDNA gene, obtained by applying either universal or genus-specific primer sets, are amplified and digested by restriction endonucleases, followed by separation of the resulting fragments on high-density agarose or acrylamide gels. The emerging profiles are then used either to cluster the community into genotypic groups or for strain typing. ARDRA method has been used to describe *Lactobacillus sanfranciscensis, Candida milleri, S. cerevisiae*, in sourdough (Palla et al., 2017), *B. subtilis, B. pumilus, B. sphaericus, B. cereus, B. thuringiensis, B. fusiformis* has been isolated and identified from cocoa fermentation (Ouattara et al., 2011).

Flow Cytometry (FCM) is a rapid and sensitive technique that measures each cell size. FCM technique is based on sorting of the stained cells through a process called hydrodynamic focusing in a narrow steam, the cells are then hit with a laser beam and florescence emitted is detected by several photomultipliers. This method has been used to quantify pathogen, spoilage microorganisms and microorganisms of interest such as *S. cerevisiae, B. bruxellensis, Candida vini, L. plantarum, L. casei, L. brevis, O. oeni*, *Acetobacter* sp., *Gluconobacter* sp., *Gluconoacetobacter* sp. from wine (Longin et al., 2017; Andorra et al., 2011).

The Fluorescence in Situ Hybridization (FISH) with rRNA targeted oligonucleotide probes has been developed over the last years, a number of variants of this basic technique have been described until now. Microbial cells are treated with appropriate chemical fixative and then immobilized onto microscopic slides. Probes used are 15-20 nucleotides in length and are labeled covalently at the 5'-end with a florescent dye. After hybridization and washing, specifically stained cells are observed by epiflourescence microscopy. This method

has been used for detection of *L. plantarum, L. paraplantarum, L. pentosus, L. acidophilus, L. brevis, L. casei, L. curvatus, L. fermentum, L. paracasei, L. reuteri, L. rhamnosus* in natural fermentation of olives (Ercolini et al., 2006), Analysis of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* during wine fermentation (Andorra et al., 2011).

CONCLUSIONS

Phenotypic and genotypic analysis can contribute to characterize any microbe at species and strain level; this can be obtained by the combination of different identification and classification procedures and then to discrimination by molecular techniques.

The most used method is PCR-DGGE, followed by q-PCR and RAPD-PCR among genotyping methods, being a very useful tool for detection of probiotic bacteria, spoilage bacteria and pathogens bacteria during fermentation processes, the other molecular methods being less used.

It is necessary to expand possibilities to investigate microbial diversity within natural populations by analysing less conserved genes. Culture-independent methods can not completely avoid biases from estimating microbial diversity introduced by maceration and blending of the food sample, dilution of the homogenate, plating of dilution onto agar media and isolation and identification of colonies.

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SCREENING OF KILLER ACTIVITY IN YEAST STRAINS ISOLATED FROM FERMENTED AND NON-FERMENTED FOODS AGAINST SPOILING FOOD YEASTS

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Abstract

Killer activity is one of the mechanisms of antagonism among yeasts during fermentations. A variety of yeast species secrete a compound which is protein produced outside the cell and is called the killer toxin. Killer toxins are protein in nature and active at low pH. These toxins have a lethal effect against yeasts, bacteria and the moulds, except for their own species. Killer yeasts can be used to prevent the contamination of many spoilage yeasts which are sensitive to killer toxins in food processing via this mechanism during the food fermentations. In this way, it is possible to control of the food spoilage microorganisms and prolongation of the spoilage processes in foods by using of killer yeasts or direct using of killer toxins. The shelf life of foods can be extended by using killer features of yeast, so food resources can be better assessed and economic losses can be prevented. Therefore, it is important to determine the killer yeasts which are effective against spoilage food yeast. For these reasons, it is aimed to determine the effective killer yeast strains against spoiling food yeasts in this study.

In the study, yeast isolation was performed to obtain spoilage yeasts from various spoiled fermented foods. At the same time, in this study, the yeasts, which were isolated from different products and obtained from Süleyman Demirel University Food Engineering Department laboratory, were evaluated to determine the killer activities against these spoilage yeasts isolated from various spoilage fermented products.

It has been determined that those yeasts, which are effective against sensitive spoilage food yeasts, were investigated by using the agar diffusion method in the solid medium and have a low, medium and high spectrum activities as a result of quantitative experiments.

The study was carried out under the 30°C incubation temperature and pH 4 acidic conditions to the determine the killer activity. The results that were obtained in the study were calculated and evaluated over the zone radius. It was determined that the yeasts obtained from laboratory, showed 50% killer activity against spoilage yeasts as a results of quantitative analyses in solid medium. These yeast isolates were classified according to their effect degrees. It was determined that the yeasts obtained from Süleyman Demirel University Department of Food Engineering, showed killer activity against spoilage yeasts isolated from spoilaged food products as a results of in vitro experiments.

Key words*: spoilage yeast, antagonism, killer yeasts, food fermentation, biocontrol.*

INTRODUCTION

The yeasts are commonly found in nature as one of the groups of microorganisms. Yeast cells are widely obtained from the natural environments such as soil, water, fruit, fermented foods, animal and human biologic systems. However, it is important that the yeasts strains are used in different food products (Van Vuuren and Jacobs, 1991). In recently, the killer properties of the yeast strains have attracted more attention. Many strains of yeasts secrete toxins called as killer toxins (Bevan and Makower, 1963).

Many yeasts strains are evaluated as killer yeast because of their abilities to produce killer toxin. It was well-known that killer toxins produced by some yeast strains are low molecular mass proteins or glycoproteins. These proteins cause the death of sensitive cells of the same or related yeast genera and of the fungi and bacteria cells. The killer yeast strains are resistant to their own toxin and can be sensitive to the toxins produced by other killer yeasts (Bevan and Makower, 1963; Marquina et al., 2002).

Many studies indicate that the killer phenomenon is especially widespread among yeasts. Also, it can be found the killer character in natural yeast isolates and laboratory yeast strain collections (Florentina and Gageanu, 2011).

In many studies have been determinated that killer yeasts have been identified in many genera, such as: *Williopsis, Kluyveromyces,*

Debaryomyces, Saccharomyces, Torulopsis, Hanseniaspora, Metschnikowia, Pichia, Hansenula, Cryptococcus, Zygosaccharomyces and Ustilago. Several research articles have shown that killer yeasts can be applied to control growth of undesirable yeasts in food productions (Bevan and Makover, 1963; Woods et al., 1968; Woods et al., 1974; İzgü et al., 1996; İzgü et al., 2004; Santos et al., 2004; Hatoum et al., 2012).

The determination of killer yeast strains can also provide important information for combating different food deteriorate processes caused by certain spoiling strains of the yeasts (Van Vuuren and Jacobs, 1991; Garcia-Garibay et al., 2009; Ullivarri et al., 2011; Hatoum et al., 2012).

The main aims of the present study are to determine killer activity of laboratory yeasts isolated from different foods in previous studies against spoiling yeast strains. Yeast strains obtained from Microbiology Laboratory at Süleyman Demirel University in Turkey were screened for their killer activity against yeast strains which could cause spoiling in vinegar, olive and pickle food products.

In this study, killer characteristic was screened by *in vitro* tests in twenty-five strains which were previously isolated from different food products.

Killer activity of 25 isolates previously isolated from different food sources against nineteen yeast isolates which have potential spoilage character was analyzed.

For this reason, these nineteen yeast strains were isolated from spoiled fermented foods in this study.

The aim of our trial was to develop an effective biological and natural tool by using killer strains as biocontrol material against undesirable yeast strains.

MATERIALS AND METHODS

Materials

Yeast strains

Twenty-six yeast strains used in present study as a killer toxin producers were isolated from different fermented or non- fermented food products in previous studies in our laboratory. These strains were conserved in the Department of Food Engineering, Suleyman Demirel University in Turkey. Laboratory strain codes and isolation sources of yeasts were demonstrated in Table 1.

Table 1. Laboratory strain codes and isolation sources of yeasts

Laboratory strain codes	Isolation sources of yeasts
LM-1	bread dough
LM-3	Milk
LM-4	Milk
LM-5	Milk
LM-6	Milk
LM-7	Yoghurt
LM-8	Yoghurt
LM-9	Cheese
LM-10	Cheese
LM-11	home-made vinegar
LM-12	Pickle
LM-13	Kefir
LM-14	Kefir
LM-15	tarhana (traditional product)
LM-16	tarhana (traditional product)
LM-17	Pickle
LM-18	turnip (traditional product)
LM-19	goruk (traditional product)
LM-20	goruk (traditional product)
LM-21	goruk (traditional product)
LM-22	Kefir
LM-23	fermented olive
LM-24	fermented olive
LM-25	Bousa
LM-26	Bousa

LM: Laboratory yeast strain

Nineteen yeast strains were also used in present study as a spoilage yeast strains. Spoilage strain codes and isolation sources of yeasts were demonstrated in Table 2.

Table 2. Spoilage strain codes and isolation sources of yeasts

Spoilage strain codes	Isolation sources of yeasts
BM-41	fermented olive
BM-42	fermented olive
BM-43	fermented olive
BM-44	fermented olive
BM-45	fermented olive
BM-46	fermented olive
BM-47a	fermented olive
BM-47b	fermented olive
BM-48	pickle
BM-49	pickle
BM-50	pickle
BM-51	pickle
BM-52	pickle
BM-53	pickle
BM-54	pickle
BM-55	pickle
BM-56	pickle
BM-57	home-made vinegar
BM-58	home-made vinegar

BM: Potential spoilage yeast strain

Culture media

Growth medium was YEPD broth medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose. The medium was buffered to pH 4.0 with 0.1 M citrate-phosphate buffer, and YEPD-MB agar (medium containing 0.003% (w/v) methylene blue and 2% (w/v) agar) was used in assays for the killer activity (Ullivarri et al., 2014).

Malt extract broth (MEB), yeast peptone dextrose broth (YPDB), potatoes dextrose agar (PDA), dichloran rose bengal chloramphenicol agar (DRBC), malt extract agar (MEA), yeast peptone dextrose agar (YPDA) mediums were also used to activate and cultivate all yeast isolates.

Methods

Isolation of yeast strains

Sampling. Spoilage yeast strains were isolated from spoiled food samples. Different spoiled samples were collected during experimental studies. These foods (cucumber, cabbage, pepper pickles, home- made vinegar, fermented olive samples) were purchased from local market place in various regions in Isparta-Turkey.

Isolation of spoilage yeast strains

The spoilage yeast strains were isolated by routine methods. Ten grams or milliliters of the spoiled food products were homogenized with ninety milliliters of sterile 0.85% NaCl solution and then were diluted. After suitable dilution of the cell cultures, the dilute was plated on appropriate solid medium and the plates were incubated at 28-30^{0}C for 5 days. Different colonies were selected according to colony properties of yeast strains from the plates and were transferred to the solid medium slants, respectively. Yeasts cells were also counted in appropriate solid medium.

Screening of yeasts strains for killer activity

The killer activity was investigated by method described below. Spoilage yeast strains were grown in YEPD broth medium at 28-30 ^{0}C for 24-48 h and the cells from the culture were suspended in sterile 0.85% NaCl solution and cell density was adjusted to 10^5-10^6 cells/ml. Adjusted and standardized cell densities of yeast strains were used. Each yeast culture was mixed with YEPD-MB containing 2 % (w/v) agar, and the mixture was poured in a sterile Petri dish containing the assay medium. The plates were incubated for 1-2 h until the agar hardened. For determination of the killing activity, the potential killer yeast strain cells (obtained from Department of Food Engineering laboratory) were inoculated onto the YEPD-MB containing 2 % (w/v) agar assay medium which cells of the spoilage yeast strain were mixed. The Petri dishes were incubated at 28-30^{0}C for 2-5 days, and checked daily (Gulbiniene et al., 2004).

Measurement of killer toxin activity

We assayed killer toxin activity with an agar diffusion test that was mentioned above. Finally, in the end of the incubation time, the diameter of the inhibition zone was used as a measure of the yeast killer activity. A killer effect was considered when the clear killing zone of inhibition around the tested isolates appeared on the Petri cups. Also, yeast strains which were surrounded by bluish colored cells which to be potential sensitive strains were considered as killer strains. Killer activity was measured by subtracting diameter of the yeast colony from diameter of the inhibition zone.

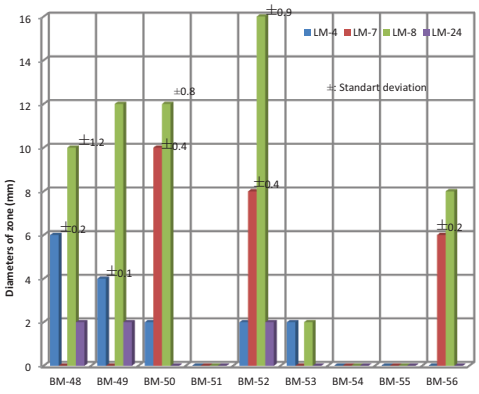
RESULTS AND DISCUSSIONS

The results of the obtained killer activity tests were evaluated according to three different product groups (pickle, table olive and vinegar). In each product group, it was tried to detected the killer yeast strains separately. In addition, potential best killer strains to be used in fermentation processes against spoilage yeasts were detected.

Killer activity experiments between potential killer strains and spoilage yeasts isolated from spoiled pickles

There was great variation in killer effect percentages against yeasts that have spoilage potential. There were killer effect percentage values between 11-67 %, except LM-11, LM-12, LM-19 and LM-23 strains, towards spoilage yeast strains isolated from different pickle products. Most killer effect percentage value was 66.7 %. LM-11, LM-12, LM-19 and

LM-23 yeast strains were not able to kill any of potential spoilage strains used in the study.
The highest killer activity was observed in LM-8 and LM-17 strains. The highest killer activity was also observed in LM-4 and LM-13 yeast strains. Therefore, these strains were considered to be superior killer yeast strains as suitable yeasts to be used as starter cultures in pickle fermentation.
According to results of diameter rate of clear zone as shown in Figure 1, the highest killer activity was determined in LM-8, LM-13, LM-3 and LM-7, respectively. Concurrently, it was revealed that LM-8 yeast ensured similar diameter rate of clear zone when compared with the results were obtain from tests by reference yeast strain.
Also it was observed that BM-49 spoilage yeast strain isolated from spoiled pickle products was the most sensitive strain from the tested laboratory strains.



BM: Spoilage Yeast ; LM: laboratory Yeast, Diameters of zone (mm), ±: Standart deviation

Figure 1. The diameter of clear zones of killer toxin on potential spoilage yeast strains isolated from spoiled fermented pickle products

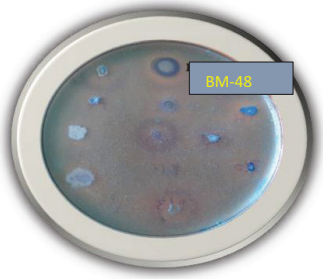


Figure 2. The image of inhibition zones of killer toxins secreted by laboratory and reference strains on a potential spoilage yeast strain BM-48 at pH 4.0

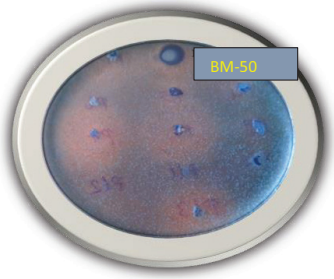


Figure 3. The image of inhibition zones of killer toxins secreted by laboratory and reference strains on a potential spoilage yeast strain BM-50 at pH 4.0

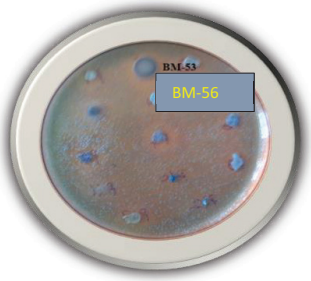


Figure 4. The image of inhibition zones of killer toxins secreted by laboratory and reference strains on a potential spoilage yeast strain BM-56 at pH 4

Killer activity experiments between potential killer strains and spoilage yeasts isolated from spoiled table yeasts

Similarly, according to the results of analyses carried on potential spoilage yeast strain isolated from spoiled fermented olives, it was determined that there were great variations in killer effect percentages against spoilage yeasts.
There were killer effect percentage values between 14-72 %, except LM-23, LM-25 and LM-26 strains, against spoilage yeast strains isolated from spoiled table olives. At the end of the study, it was detected that LM-5, LM-6 and LM-8 strains were the most active against the spoilage strains isolated form spoiled table olives.
Most killer effect percentage value was 71.5 % and LM-8 yeast strain shown the highest killer effect. LM-23, LM-25 and LM-26 strains were not able to kill any of potential spoilage strains that were used in this stage of the study.
The highest killer activity was also observed in LM-5, LM-15 and LM-21 yeast strains. For this reason, these strains and LM-8 yeast strain were considered to be superior killer yeast

strains as suitable yeasts to be used as starter cultures in pickle fermentation.
It was observed in this part of the study that LM-23, LM-25 and LM-26 yeast strains were not able to kill any of potential spoilage strains that were used in the study.
It was also appreciated that the highest killer activity was in LM-9 and LM-10, according to the diameter ratio of the clear zone.
Also, killer activity was observed in reference killer strain. Reference killer strain shown inhibition effect against only 6 strains of all to be spoilage yeasts isolated from table olives. It was measured killer activity values of reference strain according to the diameter ratio of the clear zone and it was found between 6-12 mm.
According to mentioned inhibition zone diameter range, yeast strains were appreciated as suitable potential killer strains in this study and were considered to have similar activity to reference strain also. In addition, it was observed that BM-43, BM-47, BM-45 and BM-41 spoilage yeast strains isolated from table olive products were most sensitive strains to the tested laboratory strains.

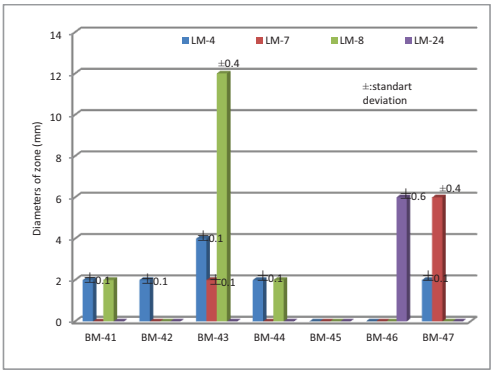


Figure 5. The diameter of clear zones of killer toxin on potential spoilage yeast strains isolated from spoiled table olive products

Killer activity experiments between potential killer strains and spoilage yeasts isolated from spoiled vinegar

Killer activity assay was also performed against two different spoilage yeast strains (BM-57 and BM-58) isolated from the home- made vinegar samples. Tested 25 laboratory yeast strains and reference strain were evaluated to killer effects towards two spoilage yeast strains.

Among yeasts isolates that were tested to determine killer strains, lowest killer activity was detected in this product group.
It was determined that there was killer effect percentage value at 50 % in some test yeast strains.
These strains, LM-4, LM-5, LM-7, LM-8, LM-14, and LM-24, were able to kill one of the potential spoilage strains used in the study.
It was also observed that other remaining tested strains were not killer against any spoilage strains.
It was detected that BM-58 spoilage yeast isolate was most resistance strain from the tested laboratory strains.
As a result, there was no observed killer activity of 25 yeast against BM-58 strain. However, it was affected by reference strain.

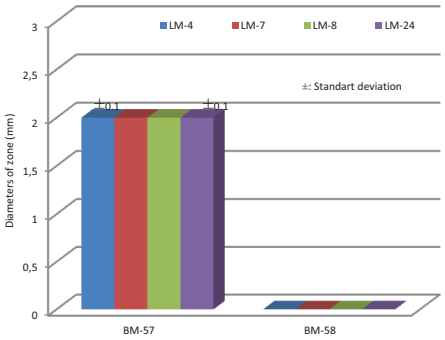


Figure 6. The diameter of clear zones of killer toxin on potential spoilage yeast strains isolated from spoiled vinegar products

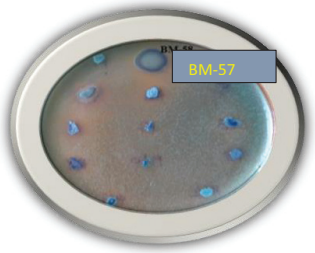


Figure 7. The image of inhibition zones of killer toxins secreted by laboratory and reference strains on a potential spoilage yeast strain BM-57 at pH 4.0

CONCLUSIONS

Killer yeast strains or their toxins have been recommended to control spoilage yeasts and

other undesirable microorganisms in food industry. In our study, it was determined that potential killer strains can be used in food industry by pre -screening killer activity of laboratory yeast isolates. Some of tested yeast strains were considered as new industrially killer strains according to results of our study. Among the killer yeasts isolated from different food products a wide range of killer activity was observed. These laboratory yeast strains could be recommended to biological control of undesirable yeasts in especially pickles, table olives and vinegar fermentations.

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FOOD SAFETY

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DESIGNING AN INFORMATIC SYSTEM FOR THE FUNCTIONING OF A VETERINARY PHARMACY IN THE RURAL ENVIRONMENT IN ROMANIA. CASE STUDY PORK TRANSABILITY

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Abstract

The design approach of an information system for monitoring pig meat traceability (SIMTCP) can not ignore the feeding of animals.
Starting from this premise, in this paper are prezented the modeling assumptions, considerations and UML diagrams for processes of feeding pigs at farm level. Scientific research results were obtained in the detail analisys phase of SIMTCP, during of the deployment of the research project „Designing an information system to monitoring the traceability in pork production", financed by the Competitive Grants Scheme (SCG), developed with the support of Modernization of Information and Knowledge Systems in Agriculture Project (MAKIS).
At the end of communication are worded conclusions and guidelines for future research in the areas of product traceability, food safety and new information and communication technologies, taking into account trends and requirements of a sustainable global economy.

Key words: *information systems design, UML, pork traceability, feeding traceability, new IC&T.*

INTRODUCTION

The european regulation defines traceability as "the ability to trace and follow a food, feed, food-producing animal or substance intended to be or expected to be incorporated into a food or feed, through all stages of production, processing, and distribution" and applies to all food and feed except primary production for private domestic use or private domestic consumption. All food and feed companies are legally bound to have traceability systems (European Union, 2002, cited by Meisinger et al., 2008).
Traceability for swine livestock is recognized like an effective component of any food safety control system, serving consumers' increasingly requires concerning a better quality of pork. The practical value of a traceability informational system is affirmed by improving consumers' trust in meat quality and by the encouragement of pork industry (Zhao, Teng and Wang, 2009).
The analysis of causes of disease or of quality non-compliance of meat has highlighted the need to obtain information on the modality and origin of animal feeding and water ([TRA1]).
Development of society and the improvement of living standards, consumers' demand for high quality meat products is constantly increasing ([TRA1])..
Over 90% of specialists participating in a seminar on traceability theme in Dublin (2008) concluded that full traceability will become increasingly important for feed additive and feed suppliers within the European marketplace. Also, over 50% considered that trace mineral and energy regulations will pose the biggest challenge to pig production in the future ([TRA2]).
Our research methodology included revealing state of knowledge by literature and materials of mass - media review. Also, ideas, information were extracted in workshops framework, organized with farmers and representatives of the authorities (Ministry of Agriculture and Rural Development, Romanian Association of Pigmeat Employers, veterinary organisms, farmers' associations etc.) and were performed general and detailed analysis by documenting in pigs farms. Finally, using UML methodology, models were developed to manage various aspects necessary for traceability, including the feeding of pigs.

In this research material, are presented UML diagrams of use cases, classes and activities that describe data and processing sequences structures, revealed as necessary for feed management subsystem.

1. FARM INPUTS TRACEABILITY

European regulations define traceability as „the ability to follow a food, feed, food-producing animals or substance incorporated into food or feed in any stage of production, processing or distribution". All food or food producing companies are legally bounded to have traceability systems ([EU 2002], cited in Meisinger et al., 2008).

In Romanian economy, many suppliers exist for agriculture sector. This can be classified in the following groups ([TRA3]):

- Housing (stables, electricity, cooling facilities etc.);
- Equipment (milking machines, tractors, irrigation etc.);
- Feed;
- Medicine;
- Seeds, fertilisers and insecticides;
- Water.

Pigs' feed and medicines can play an important role in the food chain and have implications for the quality composition of agri- food products that people consume.

Consequently, in the feed industry are envisaged the development of programs to ensure feed quality which imply ([TRA3]):

- Creating posibilities to tracking and tracing of pig feed;
- Applicating The Hazard Analysis and Critical Control Point (HACCP) principle into risk evaluation and control;
- Ensuring knowing of the whole feed chain (included raw materials suppliers);
- Implementing and using of an early warning system.

At the pigmeat farms (producers) level, feed traceability represents an „insurance policy" for the organization image, in case of unexpected negative situations, caused by pigmeat qualitative non - compliance form feed or water quality reasons.

Farmers are motivated, not only obligated by the general context, to invest in corresponding facilities at higher quality standards at their farm or lands to produce pork.

In Romanian economic environment, it is the farmers' own responsibility to achieve a healthy economic situation for their business (also in countries like Italy, Germany or Denmark, as the research team remarqued during the documentation visits), but in a transition contry like Romania are required interventions and support financial resources from the State part, to institutional and technical development – basic condition for increasing chances to compete on the international pigmeat market.

Romanian farmers need information about how to implement a Good Agriculture Practice (GAP) and technologies for livestock breeding, feeding, control of animal health status and use of veterinary drugs. Regarding the welfare of animals, the owners are obliged to satisfy the biological needs of each category of animal and provide safety, food and water ([TRA3]).

Starting with tis considerations, we elaborated diagrams to make possible collecting information concerning inputs like pig feed in a feed traceability subsystem.

2. UML DIAGRAMS FOR MODELING FEED TRACEABILITY SYSTEM

In this paper three diagrams were selected as relevant, for depicting a complete image of data processing and treatments of the system of feed traceability at farm level.

Food administration is followed both by lots of animals, locations, and on lots of fodder, fodder categories and raw materials incorporated into feed.

Use cases diagram related to operations designed to record managed farm feed is given in Figure 1.

Classes of objects structure required to manage data related to feed administrated to farm animals is shown in Figure 2.

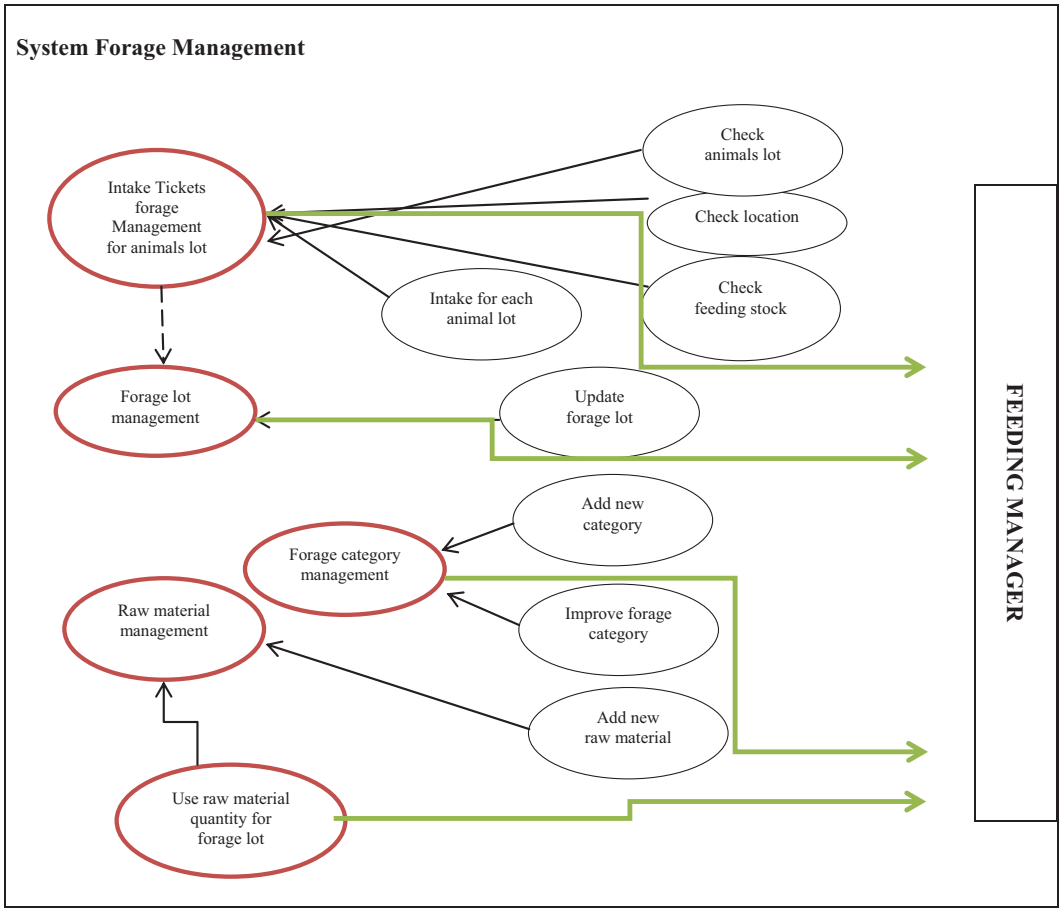

Figure 1. Use cases diagram for fodder traceability system

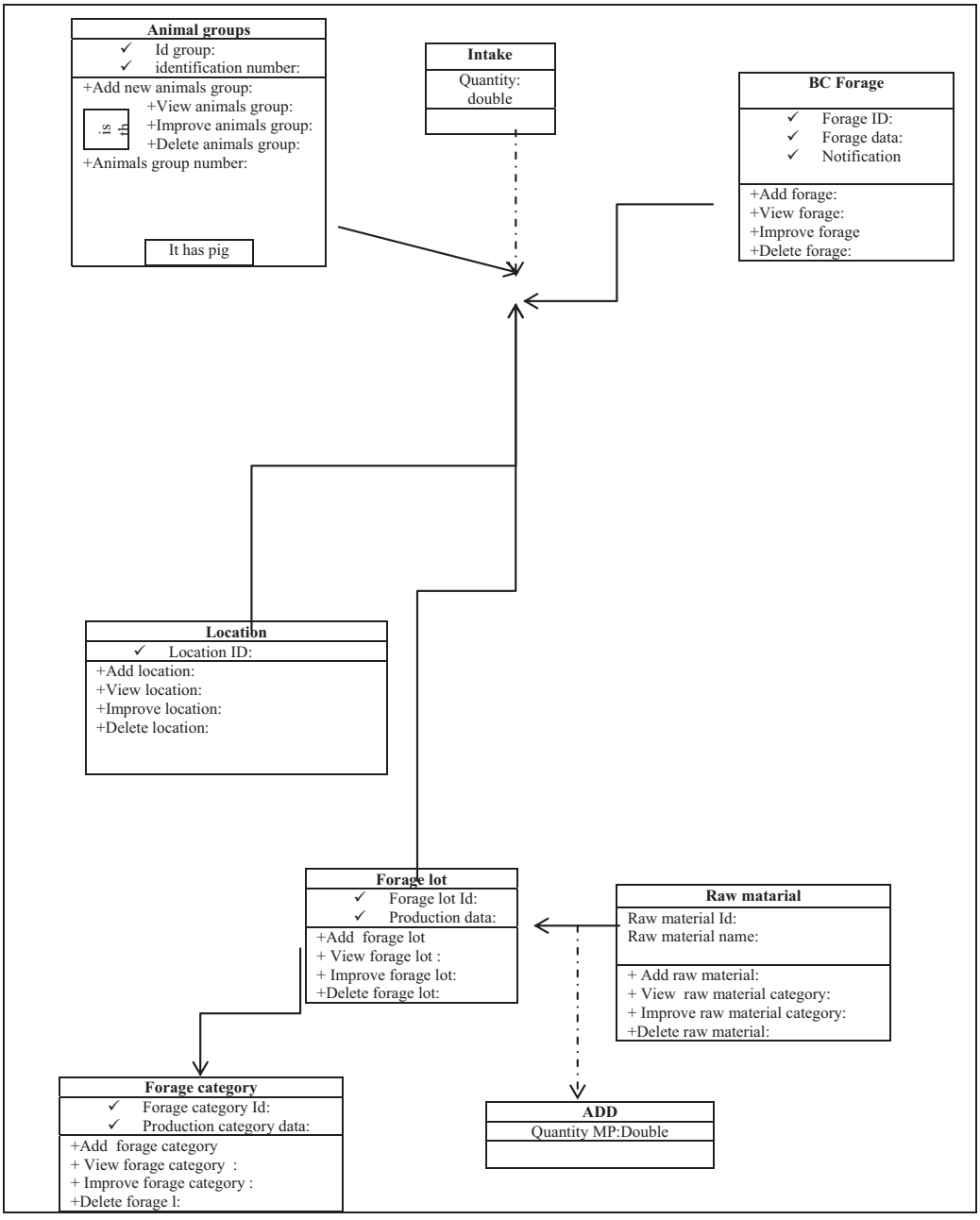

Figure 2. Classes diagram for feed traceability

The system can register the consumption documents, recording feed consumption, the quantities of each feeder element and the date of feeding by lots and locations. Each lot may consist of one or more pigs. For each piglet calved in farm are recorded its parents - sow and boar.

The models propose feed management by lots of feeds, categories and given the quantitative composition of raw materials.

Appropriate data processing operations are illustrated in feeding activity diagram on Figure 3.

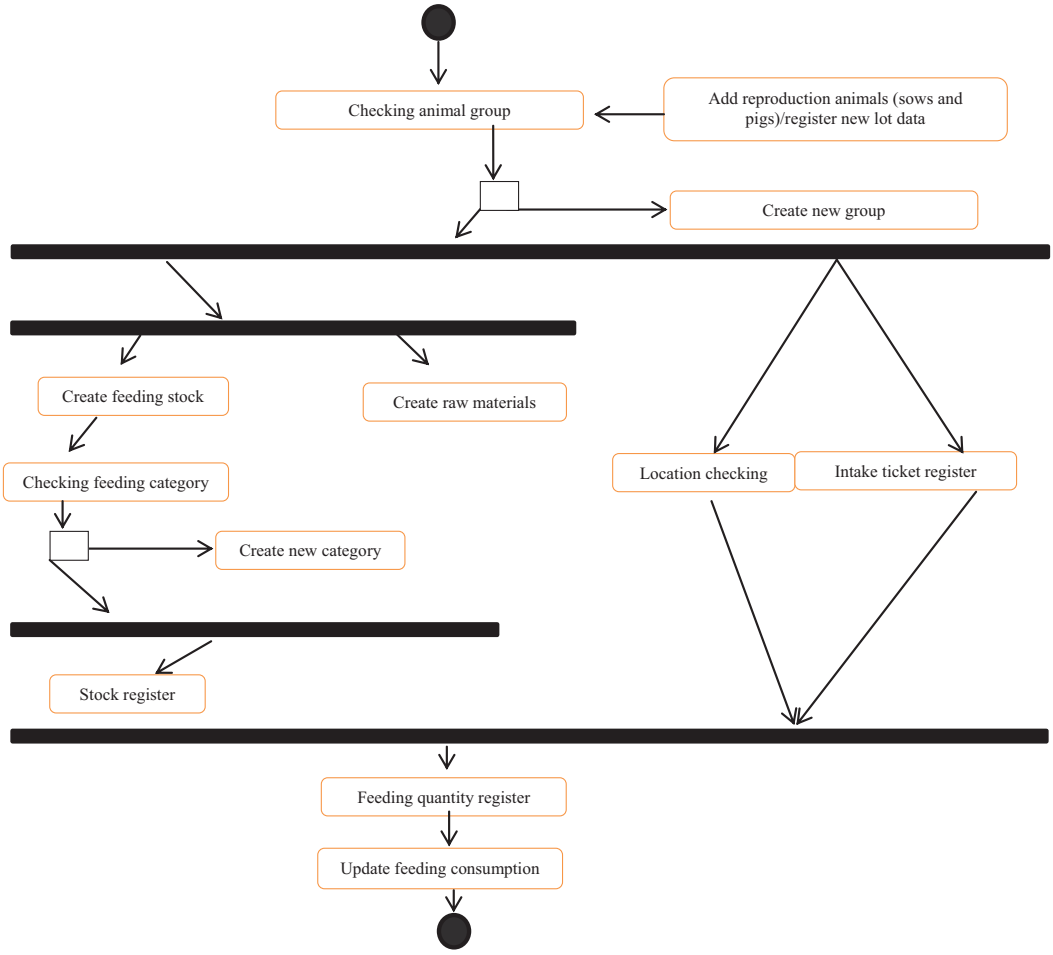

Figure 3. Activities diagram for fodder traceability

3. NEW IC&T

The quality of information tracing to the farms depends on instruments and technologies used for. Traceability systems included databases, datawarehouses, OLAP technology tools, modern technology for intelligent identification, for pork quality monitoring and controlling.

Concerns about the traceability of agri - food product led to the announcement of the completion by IBM in October 2009, of an application for mobile phones (iPhone app) called Breadcrumbs, designed to provide consumers access to information about food, especially groceries. "Breadcrumbs" will be able to scan bar codes and provide a summary of the ingredients, date of manufacture but, in addition, any time of recall of product. Reading barcodes is performed using the iPhone camera, and information offered to the consumer are taken from the Web.

Modern technology has enabled smartphones connection to the Internet objects (convergence of the Internet with real-world objects), devices like the iPhone might become sensor and RFID reader, which allows consumers to interact with real world objects in much more details.

"Breadcrumbs" is a begining for future devices to reach the consumer, providing it with information until now remained inaccessible, like where and when food will be consumed, how long they stayed on the shelf before being

purchased and whether about counterfeit products (MacManus, 2009).

Current information society traverses a revolutionary phase of food and consumer demands. Hence, many challenges arise related to ethics, food availability and accessibility information. Traceability management issues must be efficient so that costs generated by food safety measures do not become increasingly higher.

In the Great Britain case, Graham (2008) reaf-firms the need of taking global trend in farming policies, consumer protection and agri - food products traceability, but in view of existing conditions and national goals. This idea is welcome for a country like Romania too, which should make greater efforts to meet require-ments on multiple levels, imposed by the European Union or various international fora.

CONCLUSIONS

Pig food traceability becomes possible. However, from the literature and practice we can detach difficulties and limitations of this approach, which can affect farms.

Notermans (2003) identifies the possibility that, in case of bulk delivery of ingredients in food preparation, ingredients are used from several batches, in which case the delivery dates, the identification of the prior storage facility and of delivery weight or volume may be the only benchmarks to verify this point.

Then it may be not be possible a clear separation between individual batches of feed - finished goods, because of required to add of ingredients.

Another issue is that, in emergency cases, traceability operations must be performed quickly, which requires the existence of perfor-mant information systems, well designed for the express purpose of ensuring traceability, but can be costly.

Other difficulties related to the organization may be:

- The nature and size of manufacturing plant and storage space of feed and water;
- Exchange of information between different parts of the production chain;
- Level of knowledge of farmers and staff in terms of feed safety and traceability;
- Degree of cooperation within the food industry.

During the next stage of development of the traceability system, the research team must take into account all these observations in the system reengineering approach, for food traceability subsystem functionality can be improved.

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STATE OF THE ART ON NEW PROCESSING TECHNIQUES USED FOR PRESERVATION OF AGRICULTURAL PRODUCTS - A CRITICAL REVIEW

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Abstract

Food processing is the process of transforming raw materials into consumer products and maintaining their quality during storage, transport and sell time. The food processing objectives are focused in particular on adapting the properties of the finished products to the physiological and nutritional requirements. In this framework, the avoidance and reduction of microbiological contamination by using thermal processing treatments, food quality is lost due to enzyme reactions, and limiting these losses is a major concern of the food industry. Alongside conventional methods of thermally treating food, a number of modern methods of treatment are being applied to food processing, such as: PEF-pulsed electric field treatment, irradiation with x-ray, utilization of antimicrobial essential oils and many others. The reasons for developing and applying new, modern methods in food processing are as follows: maintaining and increasing nutritional values; preventing microbiological spoilage; increasing the technological characteristics of raw materials and semi-finished products; increasing product diversity and increasing the value of ready-made products. In this review are shown the newest techniques used in the processing and conservation of agricultural food products.

Key words*: novel, processing techniques, food safety, shelf-life, trends.*

INTRODUCTION

Food products may be internally or externally contaminated with alteration microorganisms that are naturally present in the food product or on the product surface, but also with microorganisms that may appear during the processing stage, storage and transport of the food products, either by direct contamination or by cross-contamination. The main problem in the food industry is the contamination with microorganisms of the food products, and new techniques are developed by researchers and industry every year (Belalia R. et al., 2002).

The internal tissue of a healthy plant is usually free of microorganisms. However, the external surface of plant products is contaminated by soil, air, insects, handling personnel, or packaging. Root plants such as potatoes, beets, or carrots are contaminated at the outside with microorganisms in the soil. Fruits that grow far above the ground can be contaminated by insects and microorganisms in the air. Fruits very rich in sugars and acid can be broken down by yeasts, as in the case of grapes, or by moulds, as in the case of citrus fruits (Siebel et al., 2003).

Animal products are subject to both internal contamination and external environmental contamination. Surface microorganisms cannot penetrate into the depth of muscle tissue until the alteration process has been triggered due to the considerable increase in the number of bacteria (García A. et al., 2014). The delay of penetration of bacteria into the depth of the meat is due to the lack of bacterial proteolytic enzymes, which only form in the logarithmic growth phase. In order to avoid contamination during the storage of the raw materials, the optimal conditions of temperature, humidity, aerobiosis must be kept. The main factors influencing the degradation of physical foods are: light, temperature, humidity, air composition and mechanical injuries that can act independently or in complex (Nabar Y. et al., 2004).

FOOD PROCESSING IMPORTANCE

Food processing techniques are used to transform raw food materials into food products or to transform foods into other forms that can be consumed for human or animal consumption in both the household and food

processors. Most foods are processed to eliminate the risk of microbial alteration and increase availability and conservability (Hui et al., 2006).

The processing method applied to raw materials has a decisive role in the quality and consistency of finished products. Not all processing methods are applied to food for preservation, but some are used to change or stabilize the food texture. Food processing methods can be divided into two main categories: chemical (food with intermediate moisture, water activity, addition of chemicals, pH control) and physical (sterilization, pasteurization, scrubbing, microwave treatment, roasting and freezing).

Currently, most of the processing methods based on the use of conventional heat treatments are widely used to produce foods with a prolonged conservative period. Within these processes the treatment temperature and the retention time play an important role in ensuring the innocuity of the obtained foodstuffs.

NEW TECHNIQUES USED IN THE PROCESSING AND CONSERVATION

1. Food processing through pulsed electric field (PEF)

Pulsed electric field (PEF) treatment is a novel technology which has an electroporation effect on the cells structure and possess a wide range of practical applications, especially in the food industry. (Knorr et al., 2011). Studies have shown that PEF technology enables inactivation of vegetative cells of bacteria and yeasts in various foods. PEF treatment has been applied for the processing of liquid and semi liquid food matrices, as well as the treatment of different solid foods (Barba et al., 2016). PEF can be defined as the application of high-voltage pulses applied to food matrices with the help of two electrodes. A high-voltage direct power current is connected to a capacitor bank in order to store large amount of energy which is going to be discharged as a high-voltage electric pulse on the food product in a treatment chamber. PEF treatment can inactivate microorganisms and in the same time help the treated food products keep their initial characteristics like flavours, nutrients and freshness (Knorr et al., 2011).

The highly effective inactivation on pathogenic microorganisms of the PEF treatment, as well as other advantages when compared to conventional thermal processing, has been widely investigated for food pasteurization and preservation. (Barba et al., 2016). For example, PEF technology with electric field strengths ranging from 20 to 250 kV/cm for short periods of time (ms or μs) has the potential to pasteurize liquid foods at temperatures below 30-40^{o}C, which is much lower than temperatures used in thermal processing (Beebe et al., 2003). Physical methods like high intensity pulses, ultrasound, high preassure carbon dioxide and UV light were combined with the PEF treatment of some food products in order to enhance the effect of the inactivation treatment (Gachovska et al., 2008). Further studies have to be made in order to establish more efficient use of the PEF treatment on food products.

2. Irradiation with X-rays or β-rays of food products

Irradiation or ionizing treatments applied to food products are methods of physical processing for prolonging the shelf-life of these products (Niculiță et al., 2007). The principle of this method involves the production of a controlled amount of beta or X-rays, through the ionized radiation from cobalt or cesium radioactive isotopes or accelerator electrons. The food products treated do not become radioactive.

Research has been made in this area for more than 40 years and the results shown that irradiation of food products can lead to the destruction of insects and parasites in cereals, dried beans, dehydrated fruits and vegetables, meat and seafood. Also there have been cases that showed that the irradiation treatment lead to the prevention of potato or onion sprouting, delaying the maturation of fruits and vegetables and reducing the number of microorganisms in food (Nabar Y. et al., 2004).

The shelf-life of fresh vegetables such as mushrooms, potatoes, tomatoes, onions, mango, papaya, banana, peaches, and strawberries may be prolonged if the irradiation process uses low doses of radiation, so loss of quality does not occur.

3. Ultra High Pressure Technology (UHP) treatment

Ultra High Pressure Technology (UHP) is a new, non-thermal processing technique in which the food products undergo a treatment of high hydrostatic pressures, generally in the range of 100-600 MPa, at room temperature (Niculiţă et al., 2007). Ultra high hydrostatic pressure is a novel food processing technique which opens new opportunities for the food industry to develop new products with superior sensory and nutritional qualities, without unwanted changes in flavor, color, and nutrients value.

When using high pressure processing, microorganisms are destroyed, but covalent bonds do not break and the effect on processed food is minimal. In addition, the positive effect consists of the avoidance of excessive thermal treatments and chemical preservatives (Muntean et al., 2016)

Under the influence of high pressure, the food products molecules alter their behavior according to the Le Chartelier-Braun principle (Hendrick et al., 1998). Due to this principle, at a relatively low temperature (0 to 400ºC) the covalent bonds are almost unaffected by the high pressure, instead the tertiary and quaternary structures of the molecules, which are maintained primarily by the associated hydrophobic and ionic interactions, change after treatment with a high pressure of 1200 MPa (Hendrick et al., 1998). Consequently, high pressure inactivates micro-organisms, while factors that determine food quality, such as nutritional factors or functional characteristics, remain unchanged. High pressure gives the possibility of improved food processing techniques, and the unique features of keeping intact covalent bonds during treatment, selective activation / inactivation of enzymes, bacterial destruction, and sensory quality retention capacity at a much higher rate than in a thermal process some of the benefits of this process, which results in the production of high quality food (Hui et al., 2006).

4. The use of antimicrobial agents to increase the shelf-life of food products

Plants produce a series of secondary metabolites in order to ensure protection against potential predators and pathogenic microorganisms. It is believed that there are approximately 100,000 secondary metabolites that fulfil various beneficial roles in plant life that helped them survive for millions of years (P. Tongnuanchan and S. Benjakul, 2014).

In recent years, interest in the use of herbal extracts and essential oils for the preservation of food products has increased. From a chemical point of view, the essential oils consist of a mixture of esters, aldehydes, ketones and terpenes. The antimicrobial properties of essential oils have been tested in combination with different treatments (edible films and irradiation) against alteration microorganisms present in processed food products, especially against fungi (Chee Hee Y. et al., 2004).

Foods producers are using more often essential oils because of their proven antimicrobial activity and because they are natural compound that are accepted by the end consumers. The most interesting area of use of essential oils is to incorporate them directly into the packaging material covering the surface of the polymer, resulting in an antimicrobial packaging that inhibits the growth or decreases of the number of pathogens in food (Asan-Ozusaglam M. et al., 2016). Tests prove that essential oils are capable of having antimicrobial effect on a large group of food pathogens and several microorganisms found in food (bacteria and fungi) (Elgayyar M. et al., 2001). These antimicrobial effects were demonstrated by two methods: the agar disc diffusion method and the volatilization method using discs. (Packiyasothy et al., 2002).

5. Food processing using ultrasound

Ultrasounds are vibrations similar to sound waves, but at much higher frequencies than those perceived by the human ear (between 18 kHz and 500 MHz). In biological environments, these vibrations produce compression and expansion cycles. The implosion of air bubbles generated at a point with very high pressures and temperatures can break the cellular structures. The lethal effect of ultrasound on some microorganisms has long been known. Ultrasonography has been proposed as a means of sterilizing liquid foods, but inactivation of the most resistant microbial forms, such as bacterial spores, would require ultrasonic treatments so drastic that the degra-

dation of the physico-chemical characteristics of the food would result (Hui et al., 2006).
The intensification of physical, chemical and biochemical operations consists of overlapping over the stationary state of a fluid or solid, or over the normal fluid flow of some oscillatory movements created by the use of ultrasonic vibrations. The oscillation may be of different shapes: sinusoidal or square, and depending on the mode of application it may be: longitudinal when the direction of oscillation is along the axis of the apparatus and transverse, when the direction is perpendicular to or inclined to the axis of the apparatus (Vorobiev, E. et al., 2008).

6. Food processing techniquest by radio waves heating (RF treatment)

Radio frequency (RF) treatment is a form of electric heating process that is applied to food products with a series of electrodes. In contrast to classical thermal treatments, radio wave treatment generates heat mainly inside the treated product by friction of ions induced by the rotation of the dipoles. In radio waves heating, electricity is first transformed into electromagnetic radiation and subsequently applied to food (Brown G. et al., 1947). The practical implications of the use of radio wave treatment consist in the fact that the radiation will go through the classic plastic packages (metal carcasses cannot be used) without the need for direct contact with the electrodes (Yanyun Zhao, et al., 2000).
In principle, an electric field with positive and negative regions is formed in an RF or ohmic heating system. Under these conditions, the positive ions in the product were oriented towards the negative regions of the field and the negative ions were oriented towards the positive regions of the field. The heating that occurs when using the ohmic system occurs because the polarity of the field is constantly changing, the field being static, which usually happens at low frequencies (50Hz in Europe, 60Hz in the U.S.). In the case of RF, the polarity changes to a much higher frequency (27.12 MHz).

7. Food processing by ohmic heating

Ohmic treatment, also known as Joule heating, is defined as the process in which the electric current is passed through food products or other materials with the main purpose of heating them to a certain temperature. Electrical resistance generates internal heat. Ohmic treatment reduces exposure to heat by reducing the time required to achieve a sterilization effect. It also ensures a more even distribution of heat in the product, eliminating the risk of overheating in certain areas, a situation encountered in classical thermal treatment (Shafiur M. et al., 2007).
Ohmic treatment is different from other heating methods due to the presence of electrode-food contact (as opposed to microwave and inductive heat where the electrodes are missing), frequency (unlimited, unlike the radio frequency range or microwaves) and the type. The antimicrobial effect of the ohmic treatment is due to its low frequency (50-60 Hz), which allows the loading of the cell walls with a certain electrical charge forming pore. This is different from high-frequency methods, such as radio frequency or microwaves, where the electric field is reversed before electric charge loading of the cell walls (Sastry S.K. et al., 1998).
The main advantage of ohmic treatment is the ability to heat uniformly and quickly the food products. This can completely reduce thermal stress of the food products compared to conventional heating, where it is necessary to maintain a higher temperature at high temperatures in order to allow heat to penetrate into the thermal centre of the product.

8. Active packaging

Active packaging is an innovative packaging method that allows the product and its protective environment to interact in order to extend the product shelf life and to ensure its microbial safety, while maintaining the quality of the packed food (Ahvenainen, 2003). The most important active packaging systems applied to food products are antimicrobial, antioxidant, and carbon dioxide emitting/generating sachets.
Packaging in modified atmosphere consists of extracting oxygen from inside the packaging and introducing inert gases such as nitrogen and carbon dioxide into its place. The method is indicated for pressure sensitive products and contraindicated for friction-sensitive products.

Nitrogen (N_2) is an inert, odorless and poorly soluble in water gas. Carbon dioxide (CO_2) is a good bacteriostatic and fungistatic agent that reduces the rate of multiplication of aerobic bacteria and the growth of molds (M.C.Villalobos et al., 2018). Although oxygen is generally avoided in the packaging process, there are cases where it is used as a component in the formation of the gaseous mixture. For example, in the packaging of meat, the presence of O_2 keeps the red colour of the product and prevents the appearance of anaerobic pathogens like *Clostridium*. Usage of CO_2 in modified atmosphere packaging (MAP) has been controversial. Low levels <0.4% CO_2 promote redness in red meats which can mask oxidation or spoilage while maintaining a desirable red colour (Michele Perna, 2016).

9. Pulsed light treatments for food preservation

Non-thermal food processing methods have become increasingly popular over the last years, and pulsed light technique is one of the newest processing methods used by the food industry. Pulsed light (PL) is a novel non-thermal processing method used the treatment of food products and food packages, consisting in short high-peak pulses of broad spectrum white light (Gemma Oms-Oliu et al., 2008). The PL method uses high frequency, high intensity pulses of broad-spectrum light in the UV fraction and is capable of inactivating microbial cells and spores (Heinrich V. et al., 2015). The pulsed light (PL) technology makes use of high-power electrical pulses that are subsequently transformed to short-duration, high-power pulses of broad-spectrum (180–1100 nm) electromagnetic radiation (light) via an inert-gas (mainly xenon) flash lamp (Victoria Heinrich et al., 2015). The PL method stands on the theory that the shorter the pulse duration, the higher the delivered energy and consequently the antimicrobial action (Dunn et al., 1989). Three main factors affect the efficiency of a pulsed light treatment and the factors are the treated food product, the degree and nature of microbial contamination and the process parameters (Heinrich V. et al., 2015). The efficiency of the pulsed light treatment is influenced by the state of microbial contamination, its physiological constitution, population density and the growth parameters, growth rate and lag time (Augustin et al., 2011). The antimicrobial effects of UV wavelengths in the PL spectrum are primarily mediated 50 through absorption by highly conjugated carbon-to-carbon double-bond systems in proteins and 51 nucleic acids (Barbosa-Canovas et al., 2000).

CONCLUSIONS

Despite the efforts made in understanding the food contamination process and improving the methods of controlling the microorganisms development, food intoxication remains a major cause of morbidity and mortality in the whole world.

Most food processing methods are based on heat treatment, which ensures the food conservability, but heat treatment also has a less desirable effect on treated food products such as color, taste, flavor and texture changes. In addition, heat treatment can partially or totally affect thermo sensitive nutrients and mainly vitamins (B and C).

With the above in mind, modern non-thermal techniques for reducing the microbiological load of processed food have been developed and used to extend their shelf-life. Among these techniques the most promising are: ionizing treatments, radio frequency (RF) treatment , high pressure treatments (UHP), electric pulse treatments (PEF), photodynamic inactivation of microorganisms, osmotic dehydration and the use of natural antimicrobials in plants.

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MARKETING RESEARCH REGARDING CONSUMER PERCEPTIONS ON USING RADIO FREQUENCY IN BAKERY PRODUCTION

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Abstract

A marketing research using a quantitative method based on a questionnaire was conducted. The structure of the questionnaire was made taking into account the objective that have been pursuing in this study, namely the assessment of consumers' skills and perceptions in connection with the application of RF treatment technology in order to prolong the shelf life of packaged bakery products, in the conditions of eliminating the addition of synthetic additives.
The questionnaire was distributed online via Survey Gizmo platform. The results obtained showed that among the consumers, most young people and especially women from urban areas, innovative technologies are not so well known, especially the products obtained through the use of these technologies.
The paper presents the results of the questionnaire in terms of consumer preferences related to shelf-life, sensory characteristics, price and openness to products treated by innovative methods.

Key words*: unconventional treatments, RF treatment, shelf life, bakery products.*

INTRODUCTION

Radio frequency (RF) heating involves the use of electromagnetic energy at frequencies between 1 and 300 MHz (Datta et al., 2005). Among these, only selected frequencies (13.56, 27.12, and 40.68 MHz) are permitted for domestic, industrial, scientific and medical applications so as not to interfere with communication systems (Marra et al., 2009; Liu et al., 2011). RF generates heat rapidly within food materials due to molecular friction and space charge displacement in response to an externally applied alternating electric field. This technology can deliver thermal energy quickly to every part of the bulk food product in which pathogens may reside. Thus, RF heating could potentially replace conventional heating for solid and semi-solid foods which have low thermal conductivities (Jeong et al., 2017).

Understanding consumer attitudes, knowledge and behaviour is of vital importance to decision-makers in setting food policies, legislation and development-research directions within society. Furthermore, consumers have become more aware of healthy and safe food with low environmental impact (Draghici et al., 2011). Healthy and environmentally-friendly food products such as organic products become competitive on the market only if the ordinary consumer, the "common people" understands the benefits of these products (Niculiţă et al., 2007). Studying how people think about food and their production, how they buy or obtain the necessary food, their own attitude towards diet, and understanding the links between diet and health are entirely parts of multidisciplinary research that intersects both social sciences and natural ones and synthetically represents the consumer's science (Castura, 2018).

The current market context no longer allows the organization to make decisions without prior investigation into the environment in which it operates, as consumers' requirements are evolving in an accelerated way, competition is becoming more and more fierce and macroeconomic and legislative elements can have a decisive influence on success or failure of the company on the market (Steptoe et al., 1995). Any decision on the organization's activity should be based on solid data on the

dimensions and elements of the marketing environment within the organization in question, so that the products and /or services offered by it are in line with market requirements.
The most important dimensions of buying behaviour are: buying or non-buying reasons, buyers' preferences, buying intentions, and the most important dimensions of buying behaviour are: buying or non-buying reasons, buyers' preferences, buying intentions, and purchasing habits. All of these elements have a major influence in the purchasing decision process (Cătoiu and Teodorescu, 2004).
In this study, the consumer's attitude towards bakery products treated with radiofrequency was followed to extend the shelf life without adding synthetic additives.

MATERIALS AND METHODS

The questionnaire was distributed online via the Survey Gizmo platform and includes two sections:

- the first section refers to the consumer's demographic profile;
- the second section refers to consumer attitudes related to the application of RF treatment technology to increase the shelf life of packaged bakery products while eliminating the addition of synthetic additives.

The first section contains 5 questions out of which 4 questions are with answers to your choice and one with an open answer. The second section contains 8 questions, all of which are questions with answers to your choice.
The size of the sample was 321 participants, but only 320 valid questionnaires were selected as result of questionnaire analysis.

RESULTS AND DISCUSSIONS

As a result of the statistical processing of the collected data, a series of graphical representations have been drawn up which highlight a whole series of aspects related to consumer attitudes related to packaged bakery products obtained by applying RF treatment technology under conditions of reduction / elimination the addition of synthetic additives.

From a demographic point of view, it can be seen that the survey participants are mostly women (230 out of 320) in 72% (Figure 1).

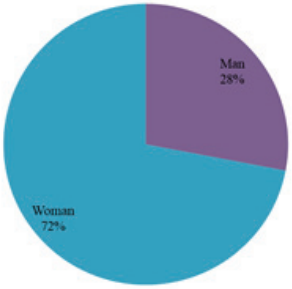

Figure 1. Respondents gender

The urban environment is home for 79% of the survey participants (Figure 2).

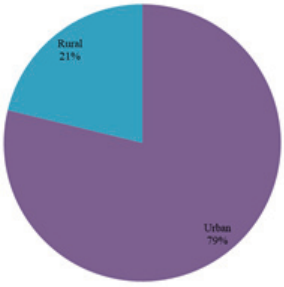

Figure 2. Place of residence of the participants

The intellectual level of the participants in the study results from Figure 3, so the last graduated school is the university for 75% of the participants, high school for 16% and vocational school for 5% of them.

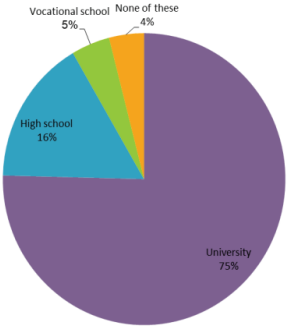

Figure 3. The intellectual level of the participants in the study

Most respondents are young people aged 20 to 35. 19 respondents out of 320 were over 50 years old (Figure 4).

Figure 4. Demographic profile of study participants - age

Of the total number of respondents, 38% have net monthly income for the entire household between 1501 and 2500 lei, while 25% of them have a higher income, ranging from 2501 to 4000 lei (Figure 5).

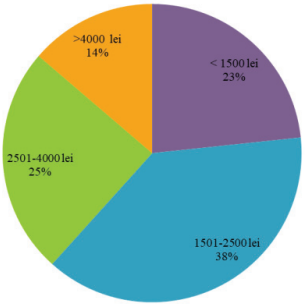

Figure 5. Demographic profile of study participants – age

The first question in the second section of the questionnaire refers to the food that is consumed at least once a month.

The first place was occupied by bread with 87.7%, followed by vegetables and fruits and meat by 85.5%.

Dairy products are placed on the next place, and on the last places the fish and the pasta are only 59.1% and 57.5% (Figure 6).

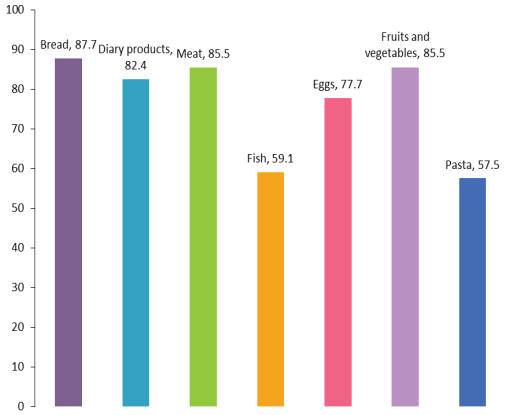

Figure 6. Foods consumed at least once a month

Question 7 was about the consumed type of bread and was answered only by respondents who selected the bread as food consumed so only 278 respondents continued the questionnaire. 52% of consumers said they prefer white bread, while 14% and 15% prefer black bread and whole bread (Figure 7).

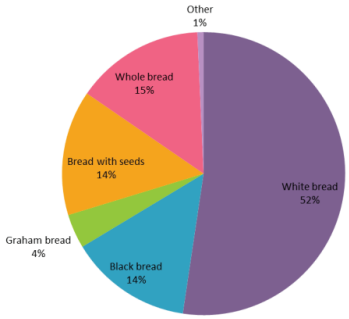

Figure 7. Favourite bread varieties

Regarding the type of bread consumed, the majority of the respondents (53%), preferred the fresh bread (unpackaged), followed by the bread packed with 37% (Figure 8).

For the purchase of bread, neighbourhood stores are preferred by almost 43% of consumers, supermarkets of 30%, and shops specialized in bakery products and hypermarkets of only 17% and 10% of consumers (Figure 9).

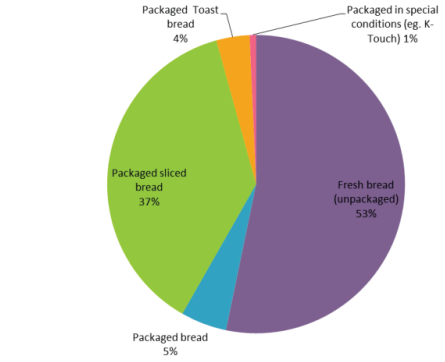

Figure 8. Types of bread consumed

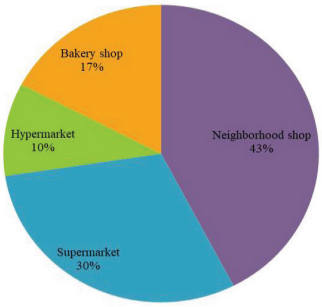

Figure 9. Place of purchase of bread

The next question concerns the importance of the qualities of a loaf when it is purchased. Regarding this issue, 75.8% of the respondents mentioned the freshness of a product as very important when purchasing it, followed by its taste and the validity term for small differences. The packaging of a food is considered very important by 13.4% of the participants, while the producer / mark are an appreciated indicator of 17.6% of them. Appearance, low content of additives, colour and smell are qualities appreciated by 16, 13 or 10% of respondents (Table 1).

The emphasis was on radiofrequency as an innovative method of treating food products with positive effects on quality in terms of increasing their shelf life. Radio frequency (RF) is part of a group of innovative techniques based on electromagnetic heating (eg infrared or microwave) and which has the potential to deliver high-quality foods from the point of view of food safety and with a deadline higher validity (Kim et al., 2012; Orsat et al., 2014; Trujilo et al., 2014).

Asked how openly would be to try bread without preservatives treated with radio frequency, 95% of respondents said they were very open (Table 2).

Tabel 1. The importance of quality parameters when buying bread

Importance / Characteristics	1 - Very little important		2 - Less important		3 -Medium		4 - Important		5- Very important		Responses
	Count	Row %	Count	Row %	Count	Row %	Count	Row %	Count	Row %	Count
Content of preservative additives	32	0.119	27	0.101	42	0.157	64	0.239	103	0.384	268
Shelf life	14	0.051	9	0.033	16	0.058	61	0.221	176	0.638	276
Assortment	9	0.033	11	0.04	53	0.195	99	0.364	100	0.368	272
Aspect	6	0.022	9	0.033	36	0.132	116	0.426	105	0.386	272
Package	28	0.104	57	0.212	72	0.268	76	0.283	36	0.134	269
Freshness	4	0.015	1	0.004	4	0.015	56	0.207	205	0.759	270
Taste	2	0.007	1	0.004	12	0.043	76	0.275	185	0.67	276
Smell	7	0.026	5	0.019	25	0.093	96	0.356	137	0.507	270
Colour	2	0.007	9	0.033	47	0.173	114	0.419	100	0.368	272
Producer/ mark	25	0.091	50	0.182	73	0.266	78	0.285	48	0.175	274
Ingredients	7	0.025	11	0.04	49	0.178	78	0.283	131	0.475	276

Tabel 2. Level of acceptability

	1- not open at all		2		3		4		5- very open		Responses
	Count	Row %	Count	Row %	Count	Row %	Count	Row %	Count	Row %	Count
Level of acceptability	28	0.101	26	0.094	62	0.224	66	0.238	95	0.343	277

Taking into account the benefits of innovative products, 53% of survey participants would not pay a higher price for radio frequency treated products, while 18% of them would not buy the products (Figure 10).

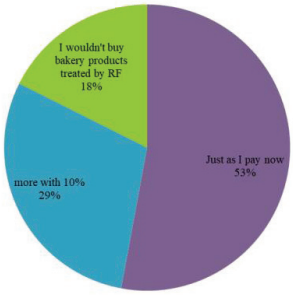

Figure 10. Availability of higher price payment for RF treated bakery products

On the last question, "Under what conditions would you be more open to buy products that have been used in innovative processing methods?", 55% of respondents say there should be more information on such methods, while 20% want to be recommended by specialists (doctors, nutritionists, researchers) (Figure 11).

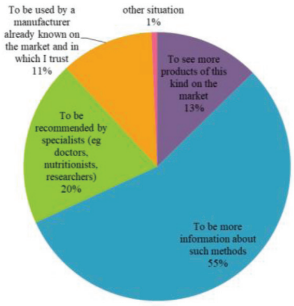

Figure 11. Conditions of acceptability for products treated with innovative methods

CONCLUSIONS

The socio-demographic profile of the respondents was as follows: more than half of the participants in the interview were women (72%); 79% of the respondents were from urban areas, most of them being young people aged between 20 and 35 years old and 75% of them have higher education.

The most frequently consumed food product was bread with 87.7% of the responses, followed by vegetables, fruits and meat (85.5%), dairy products (82.4%), eggs (77.7%), pasta (57.5%) and fish (59.1%). For the purchase of bread, neighbourhood stores are preferred by 43% of consumers, followed by supermarkets (30%), specialized stores for bakery products (17%) and hypermarkets (10%).

52% of the respondents prefer white bread, while black bread and whole bread is preferred by 14% and 15% respectively. 53% of the respondents prefers fresh bread (unpacked), while 37% prefers packed bread.

Taking into account the benefits of innovative products, benefits presented in the survey, 53% of the participants would buy radiofrequency treated products if they cost the same, 29% would pay more with 10% and 18% of them would not buy such products. Furthermore, 55% of the respondents say there should be more information about such methods, while 20% would like such products to be recommended by specialists (doctors, nutritionists, researchers).

The overall conclusion of this study is that innovative technologies and the products obtained using these technologies are not that well known. Consumers are susceptible to the use of innovative technologies to obtain food, mainly being concerned by the impact on their

health. These fears are often justified, especially due to the lack of information at the level of the average consumer.

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A POTENTIAL CHOLERA EPIDEMIC SOURCE: SOME FRESH VEGETABLES IN GOMBE

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Abstract

Most of the reported outbreaks of gastrointestinal disease are linked to the consumption of fresh products contaminated by bacteria. In view of these problems, this research wishes to determine the presence of Vibrio cholerae, in fresh vegetables sold in some Gombe markets, by isolating and identifying the biotypes. A total of 184 vegetable samples consisting of 3 vegetable types: Cabbage (Brassica oleracea L.), Lettuce (Lactuca sativa L.) and Tomato (Solanum lycopersicum Mill.) were collected and analyzed, during the month of August, 2016. Samples were inoculated on Thiosulfate Citrate Bile-salt Sucrose Agar and subjected to biochemical tests.Of the 184 cultured samples,73.39% had yellow colonial growth, out of which 16.25% were confirmed to be V. cholera. Further screenings demonstrated that 23.08% are of each O139 and O1 Eltor biotypes, and other Vibrios were represented by 53.85%. Isolates from cabbage were 50% of each O139 and O1 Eltor biotypes. There were different biotypes observed among the sampled vegetables, thus indicating close association of contamination source to the vegetables, and collectively possess the risk of cholera not only at sporadic cases but of epidemics capacity to consumers.

Key words*: biotypes, classical, Eltor, Vibrio cholerae O139.*

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INTRODUCTION

Cholera is one of the oldest and best understood of the epidemic-prone diseases. The ancestral home of cholera is thought to be the Ganges delta on the Indian subcontinent, where epidemic of cholera as disease was described as far back as the 16th century (Kindhauser, 2003). *V. cholerae*, is classified into two serotypes: O1 and non O1 (Bidinost et al., 2014).

The O1 serogroup of *V. cholerae* is further classified into two biotypes, namely, the classical and Eltor biotypes. The classical cause of epidemic cholera possesses the O1 antigen, and is known as *V. cholera* O1.

Strains of the *V. cholera* O1 are further subdivided on the basis of their O antigens into subtypes Ogawa and Inaba; some strains possess determinants of both of these subtypes and are known as subtype Hikojima.

Cholera is a diarrheal disease caused by infection of the intestine with the bacterium *V. cholerae*, either type O1 or O139. Both children and adults can be infected (WHO, 2004).

Most of the reported outbreaks of gastrointestinal disease linked to the fresh products have been associated with bacterial contamination, the consumption of „four range" vegetables, a term that refers to packaged, cleaned, possibly chopped and mixed vegetables ready to be seasoned and eaten, have gained popularity among consumers (Falomir et al., 2010).

This type of meal is consumed by many individuals in Gombe metropolis where they are obtained in readymade form street hawkers and other vegetables traders. Okafo et al. (2003) reported the presence of *Escherichia coli*, *Vibrio* spp. and *Salmonella* spp. in raw vegetables harvested from soils irrigated with contaminated streams in Nigeria.

The introduction of pathogens into soils via these agricultural inputs can result in both environmental persistence and contamination of product growing in such environments (Steele and Odumeru, 2004).

Vegetables, particularly those eaten raw and without peeling, have been demonstrated to be the vehicle for transmission of a range of microorganisms (Erdogrul and Sener, 2005). In view of these, the research wishes to determine the presence of *V. cholerae* in raw vegetables sold in Gombe metropolis, Nigeria.

MATERIALS AND METHODS

Description of area of study

The study area comprises of Gombe metropolis in Gombe State, which is located on longitude 110^0 10'E and 100^0 15'N and Kashere town, in Akko local government area of Gombe State Nigeria. Kashere is located between longitude 10^0 55^{II} 34^{I} and latitude 9^0 48^{II} 50^{I} of green witch meridian above sea level, with the Sudan savannah ecological zone of Nigeria. With a mean annual rainfall ranges of 600 mm-1200 mm and the maximum and minimum temperature of 22.7°C and 33.5°C, respectively. The vegetation cover is open savannah wood land with trees up to six meters or more. It is few kilometers drive from Pindiga district, the town occupies more than 20 kilometers square (Tanimu, 2014).

Sample collection

Three (3) vegetable types: Cabbage (*Brassica oleracea* L.), Lettuce (*Lactuca sativa* L.) and Tomato (*Solanum lycopersicum* Mill.) were collected for the study from three different markets (Shongo market, Gombe main market and Gombe old market) in August, 2016. These markets were selected because they serve as a major source of vegetables to Gombe metropolitan at retail and individual level. Total of 83 vegetable samples were purchased random from the three markets for this study (number of vegetable type depends on availability in the market during purchase). Each sample was placed in a separate polythene bag; samples were transported to laboratory and processed in less than 3 hours from collection.

Sample processing

Vegetable samples collected were processed based on the method described by Farjana and Rashed (2012) in which 1g of vegetable was homogenized in 9 ml of peptone water and incubated for 8 hrs at 37°C for resuscitation of weak *Vibrio* cells.

Isolation and identification

Thiosulfate Citrate Bile-salt Sucrose (TCBS) Agar was prepared according to the manufacturer **(**Oxoid, Hamsphire, England). Gram staining was done according to the method described by Nester et al. (2001). Other biochemical tests carried out include Triple Sugar Iron (TSI), which was prepared as recommended by the manufacturer and results were read after incubation at 37°C for 24 h. Cytochrome oxidase test was prepared using 0.5% tetramethyl-*p*-phenylenediamine hydrochloride (BBL Co.). The method used for the arginine dihydrolase, Lysine and ornithine decarboxylase assays were performed by using Moeller decarboxylase base medium (Difco) amended with an amino acid at a concentration of 1% (wt/vol) and adjusted to pH 6.8. String test was conducted to differentiate between *Vibrio* and *Aeromonas* species by preparing 0.5% solution of sodium deoxycholate by dissolving 0.5 g in 100 ml distilled water. After inoculation, the medium was covered with mineral oil and incubated at 37°C for 24 h. Cells grown in the presence of 0, 6, 8 and 10 % (wt/vol) NaCl in nutrient broth were used to determine the requirement for $NaCl^-$.

The medium was inoculated and incubated at 30°C or 37°C for up to 7 days, and positive results were determined by examining the turbidity, as described by Ottaviani et al., (2003) and Kaysner et al. (2004). Acid production from 1% Arabinose and Lactose fermentation were determined by using peptone water with bromocresol indicator (pH 6.8), and the results were read after 24 h of incubation at 37°C.

The methyl red reaction was tested by using MR-VP medium (Koneman et al., 1994) incubated at 37°C for 48 h after inoculation and Voges-Proskauer test assay was also performed by using a culture grown in MR-VP medium at 37°C for 48 h. *V. cholerae* O1 El-tor is differentiated from *V. cholerae* Classical and other *V. cholerae* members by its ability to give positive result to Voges-proskaeur biochemical test in accordance with the method described by Grace (2014).

Capsule stain was performed in accordance with the method describe by Roxana and Ann (2007) using milk broth culture. *V. cholerae* serogroups O139 are capsulated members as such detection of capsule in *V. cholerae* can be used to identify *V. cholerae* O139 in the absence of O139 polyvalent anti-sera. Anthony capsule stain protocol was conducted to observe encapsulated *V. cholerae* as describe by Roxana and Ann (2007).

Cholera red test and Citrate utilization were also performed in accordance with the method described by Koneman et al., 1994.

RESULTS AND DISCUSSION

From the 484 samples of vegetables cultured, 73.39% had yellow colonial growth typical of *V. cholerae*. Of the yellow colonies 73.39% were confirmed as *V. cholerae,* an equivalent to 7.06% of the total number sampled. The relatively low percentage occurrence of *Vibrio* could be attributed to the conclusion drawn by Falomir et al. (2010), that fresh vegetables normally carry natural non-pathogenic epiphytic microorganisms, but during growth, harvest, transportation and further handling the products can be contaminated with pathogens from animal and human sources (Falomir et al., 2010). From this result, it is obvious that vegetables like lettuce, tomatoes and cabbage could serve as a potential source or reservoir of *Vibrio cholerae*. Binsztein et al. (2004) suggested that in the interim period between cholera epidemics, *V. cholerae* is still present in the environment in a Viable But Non Culturable (VBNC) state, thus, the occurrence of culturable *V. cholerae* of 16.25% is of public health concern, since there could still be viable but non-culturable (VBNC) once still presence in these samples as a result of adverse environmental conditions.

The high percentage of isolation observed in Gombe new market relative to other sources, could be attributed to a possibility of on-farm contamination due to the use of manure from animal or human faeces.

Other possible sources may include contamination from birds' faeces, since no peculiar environmental condition was observed at the time of collection.

Further screening revealed 23.08% of each O139 and O1 Eltor biotypes respectively, while other *Vibrio* were 53.85% (non O139 and non O1 Eltor) biotypes as shown in Table 1.

Table 1: Level of microbial contamination of *V. cholerae* in some selected vegetables in Gombe Metropolis

Sample type	Total no. of samples	No. of growth on TCBS (%)	Yellow colonies (%)	No. of *V. cholerae* identified (%)	No. of *V. cholerae* O139 (%)	No. of *V. cholerae* O1 Eltor (%)	No. of other *V. cholerae* (%)
Lettuce	61	46 (75.4)	41 (89.13)	6 (14.63)	1 (16.67)	1 (16.67)	4 (66.67)
Tomatoes	75	36 (48.00)	22 (61.11)	5 (22.73)	1 (20.00)	1 (20.00)	3 (60.00)
Cabbages	48	27 (56.25)	17 (62.96)	2 (11.76)	1 (50.00)	1 (50.00)	0 (0.00)
	184	109 (59.24)	80 (73.39)	13 (16.25)	3 (23.18)	3 (23.08)	7 (53.85)

Table 2: Summary of total number of *Vibrio* spp. isolated from different sources

Samples type	Total no.	No. of growth on TCBS(%)	Yellow colonies (%)	No. of *V. cholerae* identified (%)	No. of *V. cholerae* O139(%)	No. of *V. cholerae* O1 Eltor (%)	No. of other *V. cholerae* (%)	Sources
Lettuce	20	18 (90.00)	18 (90.00)	4 (22.22)	1 (25.00)	1 (25.00)	2 (50.00)	Gombe
Tomatoes	25	20 (80.00)	13(65.00)	4 (30.78)	1 (25.00)	1 (25.00)	2 (50.00)	New
Cabbages	18	16 (18.89)	15 (93.75)	2 (13.33)	1 (50.00)	1 (50.00)	0 (0.00)	Market
1st Total	63	54 (85.71)	46 (85.19)	10 (21.74)	3 (30.00)	3 (30.00)	4 (40.00)	
Lettuce	22	19 (86.36)	14 (73.70)	1 (7.14)	0 (0.00)	0 (0.00)	1 (100)	Gombe
Tomatoes	25	11 (44.00)	9 (81.81)	1 (11.11)	0 (0.00)	0 (0.00)	1 (100)	Old
Cabbages	20	9 (45.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	Market
2nd Total	67	39 (58.21)	23 (58.97)	2 (8.69)	0 (0.00)	0 (0.00)	2 (100)	
Lettuce	19	9 (4.74)	9 (100)	1 (11.11)	0 (0.00)	0 (0.00)	1 (100)	Shongo
Tomatoes	25	5 (20.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	Market
Cabbages	10	2 (20.00)	2 (100)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
3rd Total	54	16 (29.63)	11 (68.75)	1 (9.09)	0 (0.00)	0 (0.00)	1 (100)	

The presence of *V. cholerae* O139, O1 Eltor biotypes and other biotypes in this research is in agreement with WHO report, that *V. cholerae* O1 El Tor has gradually spread to most of the continent (WHO, 2010). Similarly, the isolation of three different serogroups is also another

threat, which needs public health surveillance and strategies in anticipation of possible cholera epidemic in Gombe state.

Among the sample types, tomato samples harbor about 22.73% *V. cholerae*, while lettuce and cabbage had 14.63% and 11.76%, respectively, of *V. cholerae*. In the serotypes identified, cabbage had 50% of each O139 and O1 Eltor biotypes respectively, while isolates from lettuce and tomatoes had 66.67% and 60.00% as non O139 and non O1 Eltor biotypes as indicated in Table 1.

Most of the vegetables that are brought to Gombe Metropolis Markets are usually from nearby irrigation sites, whose main source of water for irrigation is from contaminated river that carries wastewater from domestic sources. It is also a usual practice to use sheep or cow dung and the addition of poultry litter is cheap, easy and increases the soil fertility, as observed by local farmers, thus most prefers it. This practice could lead to contamination as Hamilton et al. (2006) reported, that contamination can arise as a consequence of treating soil with organic fertilizers, such as sewage sludge and manure, and from the irrigation water, as well as from the ability of pathogens to persist and proliferate in vegetables (Hamilton et al., 2006). Norma and his colleagues reported isolation of tomatine and tomatidine, flavonoids, chlorophyll, carotenoids, and phenolics antioxidant and inhibited the growth of pathogens such as *E. coli* O157:H7, *Salmonella typhimurium, Staphylococcus aureus* and *Listeria ivanovii* (Norma et al., 2015).

Similar observations were reported in the antimicrobial activity of red cabbage whose constituent includes phenolic substances, flavonoids and glucosinolates. Dorantes et al., (2000) reported antimicrobial activity of *Capsicum* due to the phenolic compound and 3-hydroxycinnamic acid (coumaric acid). Lee et al., (2003) reported antibacterial activity of a group of fruits and vegetables including: bell pepper, carrot, cucumber, garlic, ginger, grape, red onion, red cabbage, spinach and strawberry and suggested that all green vegetables have no antibacterial activity on *Staphylococcus epidermidis* and *Klebsiella pneumoniae* whereas all purple and red vegetable and fruit juices have showed antibacterial activities, like tomatoes and red cabbage. Glucosinolates are secondary metabolites that occur in many species of plants including cabbage (Kusznierewicz et al., 2012), and it was reported to exhibit significant antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and fungi with direct or synergistic effect in combination with other compounds. These could probably explain the low isolation rate that was observed among cabbage samples. It is however important to note that, though cabbage was reported to have contained some constituents that were found to exhibit antimicrobial properties, *V. cholerae* of epidemic potential was still observed in this work, as colonizers on cabbage.

CONCLUSIONS

Cholera is primarily known as a water-borne disease in the endemic regions, contamination of food can also be an imperative mode for cholera transmission (Glass et al., 1992). Samples collected from Gombe new market (GNM) had the highest *Vibrio* occurrence (21%) compared to Gombe Old market (GOM) and Shongo market (SM) (8.69% and 9.09% respectively). It is also pertinent to note, although the percentage distribution of *V. cholerae* was observed to be higher in samples collected from SM, yet the number is higher in GOM compared to SM. There were 30.00% of each O139 and O1 Eltor biotypes observed in samples collected from Gombe new market, while no isolate was identified from GOM and SM as shown in Table 2. Isolation of *V. cholerae* O139 and other serogroups in this study signifies a risk of cholera epidemics and other enteric diseases in Gombe which was not recorded since 2010.

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POTENTIAL OF BACTERIOCIN-LIKE SUBSTANCES PRODUCED BY *Lactobacillus plantarum* UTNCys5-4 TO INHIBIT FOOD PATHOGENS IN RAW MEAT

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Abstract

In Ecuador the mild climate, the inappropriate storage condition and food manipulation are suitable environments for spoilage/pathogens growth, therefore, to satisfy with the consumer demands on food quality, searching for novel natural preservative is of interest. Lactic acid bacteria are very attractive to be exploited from the biotechnological point of view. In this research, the effectiveness of bacteriocin-like substances produced by Lactobacillus plantarum UTNCys5-4 (Genbank No KY041686.1) isolated from native wild-type tropical fruits to control the spoilage bacterial growth in raw meat was evaluated. The microbiological analysis indicated that the beef meat filets purchased from local market exhibit contamination. A 2.3-fold reduction of total coliforms viability was registered when treated the raw meat with crude-extract containing Cys5-4 bacteriocin up to day 12 of storage with refrigeration. A significant change of meat pH was observed in the non-bacteriocin treated samples along with the increase in the concentration of released ammonia indicating the degradation of protein. A visible change in color of meat was observed only in the samples treated with nitrite. The study revealed the high potential of bacteriocin Cys5-4 to inhibit the growth of spoilage bacteria, indicating its promising approach to be use in preservation.

Key words*: bacteriocin, spoilage bacteria, preservation.*

INTRODUCTION

To guaranty the food safety and security of the products, the proliferation of the spoilage and bacterial strains should be controlled. During the last two decades the research have been centered on identification of new natural antimicrobial compounds to replace the food chemicals for the purpose of conservation (Deegan et al., 2006; Arena et al., 2016). Biopreservation is a robust and natural tool to extend shelf life and to enhance the safety of foods by application of naturally occurring microorganisms or their antimicrobial compounds (i.e. bacteriocins). For many years, lactic acid bacteria (LAB) attracted significant attention for food industry due to their GRAS status (Generally Considered as Safe) (Biscola et al., 2013). Bacteriocins, known as antimicrobial compounds produced at ribosomal level, are not considered antibiotics and does not produce illicit allergic reaction in humans or animals, therefore are investigated for their potential as natural preservatives in food. Despite of many LAB bacteriocin producers, nisin remains the only component commercialized as food additive (Delves-Broughton, 2005; Gálvez et al., 2007; Hartmann et al., 2011; Nath et al., 2014). The potential *ex vitro* of some bacteriocin was reported (Fiorentini et al., 2001; Banerjee et al., 2013; Fangio and Fritz, 2014; Zamfir et al., 2014). Ecuador is known for its biodiversity in either plants, birds and animals with regions across the mountains from land to the ocean where the climate is very changeable, and the temperature varies with the altitude. Among undeveloped natural areas, Amazon was included in the governmental policy as important biological resources to be investigated. Globally, the metropolitan life grew and eating outside is becoming habitual.

In this context, Ecuador is not an exception since a substantial proportion of the ready-to-eat food is sold on the streets. Despite the growth of alimentary sector and low regulation for food consumer, there is no effective improvement on the food manipulation or

hygienic control. Most of artisanal minimally processed foods, typical dishes (i.e. mote), natural fruit or cereals fermented drinks (i.e. chicha morada), meat and fish, appears to contain a significant number of spoilage bacteria. This must be related with the inappropriate manipulation and storage, inadequate cooking, contaminated equipment or poor personal hygiene, therefore the risk of developing diseases is elevated. In spite of, considerable human illness related to food contaminants have been reported by the Ministry of Public Health (2014). Consequently, an attention was assumed to increase the consumer protection by preventing contamination, improving communication about safety by facilitating relevant research on food preservation. In this context our research proposed the exploration of the native wild-type microbiota of tropical fruits to identify newly antimicrobial substances (Benavidez et al., 2016; Tenea and Yepez, 2016). It is believed that the microorganisms from this region might provide a newly source of functional compounds to be examined. Thus, a large-scale study selection of native bacteriocinogenic LAB strains of native fruits was early reported (Garzon et al., 2017). Among several strains showing highly antimicrobial potential towards food pathogens founded often in artisanal products the *Lactobacillus plantarum* UTNCys5-4 demonstrated elevated antimicrobial potential *in vitro* against several pathogenic bacteria. Recently, we showed that this bacteriocin exerted activity *ex vitro* in artisanal drinks controlling the pathogen growth overtime in combination with refrigeration (Tenea and Barrigas, accepted manuscript). In this study, the effect of the crude-extract containing bacteriocin Cys5-4 was evaluated in beef raw meat to monitor and control the pathogen growth as overall good manufacturing practice to ensure an adequate safety and quality of meat-based food products.

MATERIALS AND METHODS

Bacterial strains and crude-extract preparation

The *L. plantarum* UTNCys5-4 (Genbank No. KY041686.1) isolated from tropical wild-type fruits of *Malus* sp. (Sucumbíos Provence) was used. To prepare the crude-extract (CE), the bacteria was grown in broth MRS (Merck) at 32°C for 24 hours and the supernatant was collected by centrifugation at 13,000 x *g* for 20 minutes, 4°C, following by the filtration using 0.22µm porosity syringe filter and storage with refrigeration before use. As standard CE from *Lactobacillus plantarum* ATCC8014 (LP) was used.

Meat microbiological evaluation

The meat samples consisting of bovine muscle were purchased from an ambulatory local vender and microbiologically analyzed in concordance with the Ecuadorian Normative, NTE INEN 1529-15 and 1529-8. Briefly, 5g meat were inoculated in peptone (1%), homogenized and incubated for 24 hours at 37°C. Moreover, decimal dilutions made on sterile water were plated on Plate Count Agar (Difco) to determine the growth of mesophilic aerobic and psychrotrophic aerobic bacteria (35 ± 0.5°C, 48 hours); moreover, aliquots were plated on SS (*Shigella-Salmonella*, Difco), incubated for 48 hours at 37-40°C to determine the presence of *Salmonella* and *Shigella*; moreover aliquots were placed on chromocult agar (Merck) to determine the total coliforms and eosin methylene blue (Difco), to determine the presence of *E. coli*; furthermore DRBC agar plates (Difco), for the enumeration of yeasts and molds (incubation at 25°C for 7 days) were used.

Effect of Cys5-4 crude-extract on raw meat

The meat filets (75g / each treatment) were divided for different assays: a) treatment with CE of Cys5-4 at the final concentration of 18 AU/g; b) treatment with the same concentration of CE of LP. c) treatment with nitrite at 200 ppm/kg according with the INEN Normative for meat preservation (1338. 2012); d) meat control (untreated). Briefly, the samples were immersed for 10 min in individual sterile trays containing the crude-extract of each bacteria, as described above and nitrite and maintained for 10 min to dry under the laminar bench to avoid any cross contamination. The experiments were performed in triplicate starting with different batch of meat and kept in refrigeration for 12 days in polystyrene food delivery boxes wrapped with sterile plastic bag (Ziploc).

pH determination

Determination of pH at different intervals of 0, 1, 3, 6, 9 and 12 days was performed. Five g of meat sample was homogenized with 50 mL distilled water cooled at 25°C.

The mixture was stirred for 30 minutes and decanted. The pH value was measured in the supernatant, using a pH meter (RoSH, Balance Instrument Co., Ltd).

Microbiological evaluation

The meat filets treated with crude-extract of Cys5-4, LP, nitrite and control (untreated) were microbiologically analysed at different intervals of time (0, 1, 3, 6, 9 and 12 days) using agar plate assay method to determine the number of total viable cell counts (Pratush et al., 2012).

Determination of ammonia

Determination of ammonia in filtrate samples of meat was performed with the addition of Nessler reagent (0.09 mol/L solution of potassium tetraiodomercurate (II) in 2.5 mol/L potassium hydroxide and EDTA (20 mM).

The addition of the Nessler reagent will produce a yellow to brown color dependent on the concentration of ammonia found in the sample.

By monitoring the color change the concentration of released ammonia can be determined by spectrophotometry at the wavelength of 400-450 nm.

The EDTA solution was used to avoid precipitation. Briefly, 5g from each meat sample treated with crude-extract, nitrite and control, was placed in the beakers, treated with solution of NaOH 6N for 15 minutes, aliquots of 300 µl were transferred in the 10 ml balloons and 100µl of EDTA was added.

Moreover, the samples were treated with 100µl of the Nessler reagent for 10 minutes followed by calibration with sterile distilled water, and immediately determine the absorbance at 450 nm using the spectrophotometer (Nova60, Millipore, Merck) with previously determined ammonia standard curve (90-200 ppm).

A value greater than 120 ppm being associated with spoilage contamination (Hijaz et al., 2007)**.** To determine the minimum value an uncontaminated meat muscle was used.

RESULTS AND DISCUSSIONS

The contamination of ready-to-eat and fresh food products with pathogenic/ spoilage microorganisms and its persistence, growth, multiplication and/ or toxin production has emerged as an important public health concern. Food-borne illness is a major international problem and the worthy cause of the reduced economic growth. Thus, several synthetic additives to preserve food or enhance their organoleptic characteristics are used (Castellano et al., 2017). To assure the quality and safety of the food several natural methods were proposed including the use of bacteriocin secreted by lactic acid bacteria (Arena et al., 2016). In this study, the effect of crude-bacteriocin produced by the native strain *L. plantarum* Cys5-4 was evaluated in raw beef meat. Although the meat muscle is a sterile microenvironment (Mach et al., 2008) the microbiological analysis indicated that the purchased meat does not comply with the quality standards as *Salmonella*/ *Shigella* and *E. coli* were identified (Table 1). The thee meat batches purchased at two weeks intervals showed the presence of a larger number of coliforms (9.57-9.82 log CFU/g) which, according with the legislation are considered no acceptable for consuming. Nonetheless, the meat is sold out without any restriction.

Table 1. Presence of contamination in meat

Meat Samples	Coliforms (log CFU/g)	*Salmonella/ Shigella*	*E. coli*	Yeasts/ molds	pH
Batch # 1	9.57	+/+	+	-/ -	5.83
Batch # 2	9.80	+/+	+	+/+	6.10
Batch # 3	9.82	-/+	+	+/-	6.15

We suggested that a possible contamination pathway might be after the animal slaughtering. Is possible that due to the inappropriate manipulation or storage the surface of meat might exposed at the spoilage bacteria. The molds were presented in batch #2, while yeasts were presented in batch #2 and #3. The presence of spoilage/ pathogenic bacteria in meat juices for example, is a question of cross contamination of other food products. Bacteria also can be founded on equipment, hands, and the air. The Imbabura Province as other regions of the country has the established inspection

programs applicable for meat produced and sold in the market, but seems they are susceptible to application for the small local venders. However, the state inspection program must enforce requirements for all producers. For the safety concerns and concordance with the normative, no *Salmonella* / *Shigella* are accepted in food products. The analysis of the three meat batches showed the contrary (Table 1). Food may act as a vector for the transfer of antimicrobial resistant bacteria and antimicrobial resistance genes to humans. When examined the antibiotic resistance of the pathogens found in meat using diffusion agar assay with antibiotic dicks, a tetracycline, ampicillin and cefuroxime resistance was detected for *E. coli* and *Shigella* (data not shown). Moreover, to evaluate the effect of the incorporation of bioactive substances to control the pathogens growth in fresh meat, independent samples immersed in solution containing CE-Cys5-4, CE-LP, nitrite stored for 12 days under refrigeration were microbiologically analyzed at different intervals of incubation time. In control samples, the total population of mesophilic aerobic bacteria were slightly reduced overtime due to the refrigeration. The meat samples treated with CE-Cys5-4 showed about 2.3 log CFU/g decrease in each individual batch of treated meat, indicating an inhibitory action associated to the presence of antimicrobial substances contained in the CE (Table 2).

Table 2. Viability of spoilage bacteria in meat

Incubation time (day)	Viability (log CFU/g)			
	Control	Cys5-4	LP	Nitrite
0	9.57	9.57	9.57	9.57
1	9.60	9.57	9.54	9.58
3	8.53	6.46	6.40	6.34
6	7.25	5.84	5.46	5.74
9	6.76	4.47	5.90	5.39
12	6.99	5.90	6.14	6.13

Control: untreated meat; Cys5-4: meat + crude-extract Cys5-4; meat + crude-extract LP; meat+ nitrite

The overall reduction of the cell counts was observed in the samples treated with LP up to day 6 (5.46 log CFU/ml), while at the day 9 a slightly increase was registered, indicating that the crude extract of LP was no longer active at that point. A reduction in cell counts was observed in the samples treated with nitrite, but the meat showed a browned color at day 6 compared with the meat samples treated with CE-Cys5-4 or CE-LP maintaining the same color as purchasing. Figure 1 displayed the cell growth in plates after 9 days of refrigeration with visible decrease in the treatments. Previous research showed the potential of cell free supernatant containing a bacteriocin produced by *Bacillus cereus* P9 in beef meat (Fangio and Fritz, 2014). A similar reduction in the total populations of mesophilic bacteria, when meat samples are packed with materials containing bacteriocins was observed (Dawson et al., 2005). At Day 12 the total cell counts increases indicating that activity of the active component might be inactivated.

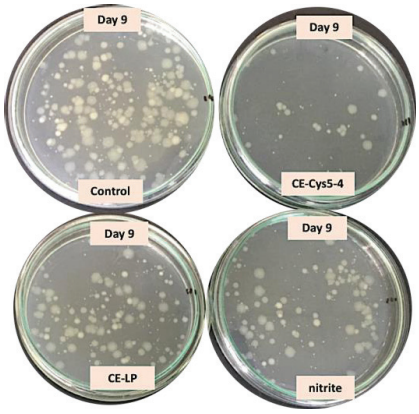

Figure 1. The presence of viable cells in meat treated or no with bacteriocins at day 9 of storage. CE: crude-extract of Cys5-4 and LP; nitrite: meat with nitrite

Other research using nisin adsorbed on the cellophane for packaging the meat, indicated that the final level of total bacterial count was considerably reduced after 12 days of incubation that the total count of bacteria in the initial level (Ercolini et al., 2010). The pH may affect the color, tenderness and quality (Jelenikova et al., 2008; Luning et al., 2011). In this research, the initial pH values for each batch seems to be acceptable, but an increase was recorded with the incubation time in the non-treated samples. If at Day 0 the values the pH varied from 5.83 to 6.15, at the Day 12 increased up to 6.44 log CFU/g indicated that the presence of contaminant induced protein degradation which possible determine the release of amino acids and alkaline compounds as ammonia. Table 3 showed the overall variation of pH in meat with and without treatment.

Table 3. Changes of pH in beef meat batch #1 during refrigeration with and without bacteriocin

pH	Day 1	Day 3	Day 6	Day 9	Day 12
Control	5.83	5.83	5.95	6.02	6.44
LP	5.72	5.77	5.80	5.92	6.00
Cys5-4	5.69	5.77	5.80	5.81	5.93
Nitrite	5.79	5.8	5.89	6.10	6.24

Control: untreated meat; Cys5-4: meat + crude-extract Cys5-4; meat + crude-extract LP; meat+ nitrite

In the samples treated with crude-extract of Cys5-4 the pH was maintained from 5.69 to 5.93 indicating that the addition of bacteriocin might protect for the proliferation of spoilage bacteria. This data correlated well with the reduction of the total coliforms in meat batch inoculated with bacteriocin. The ammonia concentration varied overtime indicating that a degradation of meat occurred due to the contamination (Figure 2). In the sample treated with bacteriocin was lower that its control counterpart. For example, in batch #3 the control overhead 170 ppm. Previous study indicated that the ammonia background levels varied little between the different meat muscles as well as varied with the aging and the increase of contamination (Pivarnik et al., 2001).

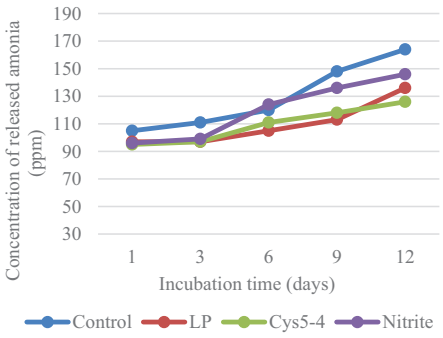

Figure 2. Variation of ammonia released in contaminated meat overtime. Control: meat untreated; LP, Cys5-4: meat + Cys5-4; LP meat with CE-LP, Nitrite: meat treated with 200 ppm/kg nitrite

These backgrounds levels of ammonia are important as FDA recommends analysis of similar non-ammonia-exposed foods to determine the normal background levels. The product can be released if the ammonia content does not exceed the normal value corresponding to the meat type used or origin (Hijaz et al., 2007). In case of beef filets the minimum value are about 90 ppm. In the present study the minimum ammonia value determined in the meat sample free of contamination varied from 86 to 90 ppm. Contrary with other studies where the effect of certain bacteriocins was monitored in food artificial added contaminants (Amin, 2012; Fango and Fritz, 2014) in the present investigation all batches of meat purchased from the local vender were founded contaminated, however the registered reduction of cell counts was of already existing pathogens being significant when bacteriocin added compared with nitrite.

CONCLUSIONS

Taken together, the results indicated the potential of crude-extract containing bacteriocin Cys5-4 to control the growth of pathogens in contaminated beef meat. The reduction of pathogenic microorganisms on meat filets treated with bacteriocin Cys5-4 in combination with refrigeration is a promising approach for maintaining the product safety. Thus, its application as natural preservative in meat as part of overall good manufacturing practice program might protect the meat for further contamination during manipulation, transportation, storage and released product for the consumer.

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ASSESSING BIOAVAILABILITY OF METALS IN BIOFUEL FEEDSTOCKS, AND IMPLICATIONS FOR CONTAMINATED LAND USE STRATEGIES

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Abstract

The production of energy crops such as Camelina sativa on contaminated land offers the possibility of a high-value low-cost long-term remediation strategy and a potential counterbalance to land abandonment. This shift in agrarian practice offers a potentially viable source of income to primary stakeholders and brings the land back into useful production. We report on the development of methodologies for charting the traceability of potentially toxic elements in camelina cultivated on contaminated land from soil to plant material and raw oil. Translocation factors for Cd and Zn suggest camelina has the potential to act as accumulator, offering potential phytoremediation benefits. However careful consideration of the use and value of the co-products is needed to determine an accurate business case scenario.

Key words: *biofuel, contaminated land, bioavailability, potential toxic elements, camelina, translocation factors.*

INTRODUCTION

Land is a finite resource upon which the human race is entirely dependent for its well-being (Bridges and van Baren, 1997; Louwagie et al., 2011). The common perception of land and soil as an infinitely exploitable resource is ultimately unsustainable (Eswaren et al., 2001; Gobin et al., 2004). On a European scale, there are many areas where land has become degraded or contaminated by human activities such as poor farming practice, mining, industry and waste disposal. Whilst estimates of contaminated land widely vary from region to region and are localised in nature, a very large fraction (>70%) of the currently identified three million sites affected by chemical land degradation can be attributed to anthropogenic pollution with predominant contaminants being potentially toxic elements (PTE) such as Pb, Cd and Zn, and mineral oils (EEA, 2007; EEA, 2010).

Contaminated land represents a particular problem in that it is often abandoned and left as unsightly wasteland that can have a detrimental effect on health and the social-economics of the area. Concerns that PTEs may detrimentally enter the food chain if crop production is undertaken and issues relating to receptors, end-points and overall fitness often restrict the usage of such land.

Remedial treatment of these sites is often costly or unsustainable. However, in some cases the production of energy crops such as *Camelina sativa* (camelina) offers a possible high-value-low-cost long-term remediation strategy and a potential counterbalance to land abandonment (Keenleyside and Tucker, 2010). This shift in agrarian practice offers an attractive alternative that allows the primary stakeholders a potentially viable source of income and brings the land back into useful production (Hoogwijk et al., 2003; Campbell et al., 2008; Gallagher, 2008; Fargioneet et al., 2010; Cai et al., 2011); whilst allowing the demands of the Common Agricultural Policy (CAP) and key imperatives of the Renewal Energy Directive to be met (EEA 2007b; Directive 2009/28/EC).

Nevertheless, concerns are raised that camelina feedstock grown on contaminated land or irrigated with contaminated water, may have trace metals present within the plant matter and the economic viability of the crop may be compromised.

As part of EU FP7 project: Initiative Towards sustainable Kerosene for Aviation (ITAKA) a methodology for traceability of the PTEs in the value chain to assess impacts of cultivating camelina on contaminated land was developed using camelina crops grown on four metal contaminated field sites in Romania. This paper

presents some of the initial findings from the case-study site at Rovinari, Gorj County, an overburden-dump site from the local lignite mines.

MATERIALS AND METHODS

The current study focussed on the development of methodologies for the traceability of PTEs in camelina cultivated on contaminated land. Such methodologies were needed as the cultivation of energy crops on contaminated land is an innovative approach, and there are few standard methods to draw upon. The methodology has been devised to consider the key three compartments: soil, plant material and raw oil.
Given the proposed end use of the camelina oil in aviation biofuel and the challenges posed in developing an effective methodology, the assessment of the methodology focussed on a small subset of key metals identified in Def-Stan 91-91 and by industrial stakeholders to be of greatest concern with respect to thermal instability and turbine rotor degradation.
In the field cultivation trials, camelina was cultivated at selected field sites in Romania (Figure 1). With all field study trials, these crops are subject to a much wider range of unregulated environmental parameters than in greenhouse trials, therefore rigorous and standardised sampling protocols should be adhered to in the analysis to minimise experimental uncertainty.

Figure 1. Locations of the four Romanian contaminated field sites

A comprehensive elemental analysis of the soil at each of the Romanian field cultivation trial sites was conducted, pre-cultivation and pre-harvest as a preliminary assessment for camelina's remediative potential. The soil characterisation methodology employed a random stratified sampling protocol using a minimum of 25 samples/ha in accordance with ISO 10382-1:2002 to allow for the inherent uncertainties in the distribution of metals in contaminated soils. Partial digestion of the bulk base compost was carried out using *aqua regia* (AnalaR quality), HCl (aq) (32.25%) and HNO_3 (aq) (69%), (in ratio 3:1 v/v)), with quantities compliant with ISO 11466:1995. Elemental concentration of 20 elements (Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Ti, V and Zn) of digestion analytes were determined using ICP-OES analysis. Dutch Target and Intervention Values for soil remediation (DTIV) (VROM, 2000, VROM, 2009) and the Romanian Reference Values (RRV) have been employed to evaluate the degree of contamination in the soil samples analysed, for the 11 target-metals As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V, and Zn, the presence of which have been highlighted to be of concern in bio-kerosene (Def Stan 91-91).
Analysis of plant material was undertaken the 11 target-metals using trace element analysis reagents and microwave-assisted digestion, with the methodology incorporating key elements of EN13804:2013; EN13805:2002 and EN14084:2003. For the determination of trace metals in oil, a number of industry standard methods (including UOP 389 and ASTM D7771-15 / ASTM D5185-09) were trialled and assessed with regard to reliability and reproducibility, and two indirect methods using EDTA extraction followed by ICP-OES analysis, and microwave-assisted digestion and subsequent ICP-OES analysis. Overall, the indirect method of acid microwave-assisted digestion was considered the most reliable technique.

Study site

The case study site is located at Rovinari, Gorj County. Rovinari (44°55' 17.9"N; 23°10' 51"E) is a mining town in Gorj County, Oltenia, Romania, located on the E79 and next to the River Jiu; it is approximately 288 km west-northwest of Bucharest and 24 km south-west of the county seat, Târgu Jiu.
Rovinari and its environs form one the largest open cast lignite mines in Romania. The extrac-

ted lignite is used to power the Complexul Energetic Rovinari thermo-electrical power plant situated close to the town.

The field study site is surrounded by the Carrier Gala lignite quarry located to east the town of Rovinari. The Sterile site is an area where the overburden material of the lignite quarry has been deposited. The soil matrix is composed of yellow-brown (2.5Y68) clays with pebble-cobble sized rock fragments and lignite fragments. The total carbon and total nitrogen range of the soil is determined to be between 2.6-8.9% and 0.10-0.35%, respectively, with a C:N ratio of 21-33. The soil displays a degree of alkalinity, pH 8.2-8.4, similar to pH values have reported for other sterile dumps within the locality (Cărăbis et al., 2011). The soil had been previously conditioned with lignite-based fertilizer and cropped with maize, with a crop yield estimated at 5.5 tonne/ha. The study site was ploughed to a depth of 300 mm, cultivated and was sown to a crop of *Camelina sativa*, cultivar GP202, in the autumn of 2012. During the growing season (May 2013) a crop survey was carried out; 12 randomly selected 1 metre squares being sampled for plant density, height and branching to assess crop viability.

RESULTS AND DISCUSSION

Soil

The geochemical characterisation of the metals within the soil for the case study site, Rovinari-Sterile showed pre-cultivation concentrations of the 11 target -metals As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V, and Zn, to be 7.7 mg kg^{-1}; 0.80 mg kg^{-1};11mg kg^{-1}; 35 mg kg^{-1}; 19 mg kg^{-1}; 2%; 520 mg kg^{-1}; 43 mg kg^{-1};10 mg kg^{-1}; 30 mg kg^{-1} and 58 mg kg^{-1} respectively. As, Cr and Ni concentrations were found to be in excess of the DITV and RRV, although they do not exceed the Target / Threshold (sensitive areas) of the DITV and RRV, respectively. Comparison of the data of the current research with concentrations reported by other authors (Dodocioiu and Susinski, 2010; Bălăceanu et al.,2011; Gămăneci and Căpăţînă, 2011) for such metals in soils within 7.5 km radius of the thermal power plant at Rovinari showed the concentrations of Cd, Cu, Mn, Ni, Pb and Zn determined for this study site to be predominantly within the range determined in the earlier studies; whilst the soil concentration of Co at the Rovinari Sterile site was found to below the lower limit. The comparative differences in the concentrations of Cd, Co, Cu, Mn, Pb and Zn, may arise in part due to the location of the study site to the northeast of the Rovinari power complex. It is reported, that the predominant wind direction is from the north and northeast (Bălăceanu et al.,2011). This may have resulted in a lower degree of enrichment of the soil by atmospheric deposition from power plant stack emissions than experienced by areas to the south and south-west of the power complex (Lazar et al., 2008; Bălăceanu et al., 2011). Kruskal-Wallis one-way analysis of variance by ranks was used to identify any significant differences in the measure of central tendency for the determined soil metal concentrations from the pre-harvesting and pre-cultivation sampling. Significant differences ($P<0.05$) between the median values for the two samplings was observed for the metals, Al, Ba, Ca, Cd, Fe, K, Mg, Mo, S and Ti, of these Cd, Mg and S were the differences were found to be strongly significant ($P < 0.001$). The lack of any apparent significant difference between the pre-harvesting and pre-cultivation median soil concentration of As, Co, Cr, Cu, Ni, Pb V and Zn suggests that in terms of remediative potential, suggests that the uptake in to the camelina crop is likely to be insufficient for such metals to have a measurable remediative affect in a single cropping year.

Crop Survey and Production

There appeared to be delineation in crop morphology between the southern and northern half of the field. In the southern part vigorous crop growth is observed with crop heights of 30-82 cm, > 60% of plants displaying branched flower inflorescences and crop emergence commonly between 60-100%, with plant counts of 60-200/m^2. By contrast, the northern section of the study site show higher plant counts/m^2 (260-450) but plants are characteristically smaller (20-70 cm) and exhibit spindly growth with lower incidence of branched flower inflorescences (< 60%). Foliar effects similar to those seen at other study sites (details discussed elsewhere in ITAKA deliverable D5.17 suggesting underlying causative factors. Red

margins and red-orange mottling, along with necrotic lesions and interveinal chlorotic areas on leaves (Figure 2) were also noted.

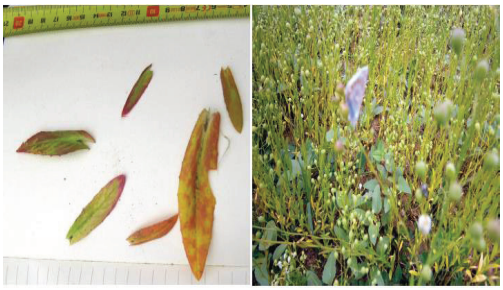

Figure 2. Stress induced variations in foliar chlorosis and discolouration at Rovinari study site

To determine the viability of contaminated land for production of camelina, comparison with camelina crops grown on uncontaminated land in similar climatic and pedological conditions is desirable. Cultivation of camelina in the agronomic trial plots have been undertaken at the didactic farm Moara Domnească (SDE Belciugatele - USAVMB), Ilfov County, to determine optimal agronomy for the camelina crop varieties including GP202 and GP204 (Dobre et al., 2014). Potential yields in excess of 1400 kg/ha were achievable but were dependent on cultivar. The best potential yields were found to occur with the camelina cultivar GP202.Consideration of the production (330 kg/ha) from the Romanian contaminated land field site, Rovinari-Sterile finds that the production was 60% of that of achieved in the agronomic trials Moara Domnească, Ilfov when no chemical fertilizer is applied. The disparities in the yield of camelina between the agronomic trials and the contaminated study sites may not arise solely from the adverse influence of contaminant levels on nutrient uptake but may also reflect differences in soil parameters such as soil OM, soil mineralogy and soil acidity.

Metal transference

Typically, other research has primarily focussed on the uptake of metals into the roots, shoots and leaves (Ebbs and Kochian, 1997; Baryla et al., 2001; Chatterjee and Chatterjee, 2000; Shanker et al., 2005; Yoon et al., 2006; Ben Ghnaya et al., 2009; John et al., 2009; Sinha et al., 2010; Pourrut et al., 2011; Tian et al., 2014). However due to need to assess whether of the uptake of the metals As, Cd, Co, Cr, Cu, Fe, Ni, Pb, V and Zn into the co-products, such as oil meal and silicles (seed-pods), are potentially detrimental to the business case for camelina, the concentrations of metals in the four compartments, roots, shoots, silicles and seed, were determined. The results of the analysis for the pre-harvest plant material from the 2012/2013 camelina crop cultivated at the Rovinari-Sterile site for the metals of concern to the aviation industry (Cd, Co, Cu, Fe, V and Zn) and metals of concern to the food chain (As, Cr, Ni and Pb) are presented in Figure 3.

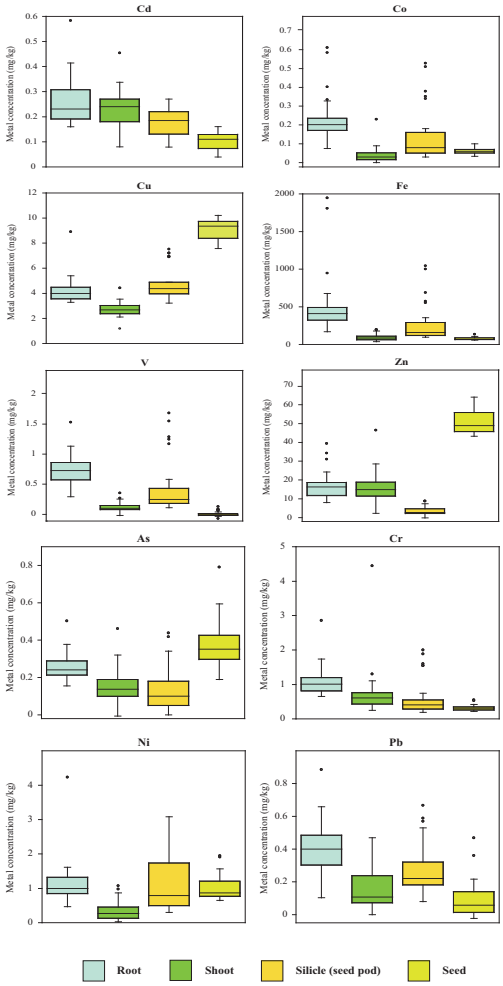

Figure 3. Concentrations of the specific target-metals in roots, shoots, silicles and seeds from *Camelina sativa* plant material harvested from the 2012-2013 cultivation at the Rovinari field site

Elemental analysis of the four plant components of roots, shoots, silicles, and seed from the Rovinari-Sterile case-study site, found that As, Cu and Zn were present in the highest concentrations within the seed. Given the well-established relationship between As and P in both soil and plant material, this is not unexpected and the management of available soil P may be key to the future successful of camelina production on As contaminated sites.

Consideration of the near parity noted between the root and shoot concentration of Cd and Zn, suggests not only a higher degree of transference of such metals from the roots into the aerial parts of the plants compared with As, Cr, Cu, Fe, Ni, Pb and V, but also possible similarities in the uptake mechanism into the plant, for example, the uptake of Cd via Zn^{2+} channels of low specificity (Clemens, 2006). Inspection of the subsequent differences in the partitioning of Cd and Zn in the silicles and seed (Figure 3) further suggest that in agreement with the findings for *Brassica napus* the storage of Cd in camelina is likely to be in the leaf and stem organelles rather than in the fruit of camelina as inferred for Zn (Baryla et al., 2001; Carrier et al., 2003; Verbruggen et al., 2009).

The phytoremediation potential in terms of the ability of *Camelina sativa* to tolerate and accumulate the metals As, Cd, Co, Cr, Cu, Fe, Ni, Pb, V and Zn can be estimated using bioconcentration factors (BCF) and translocation factors (TF).

Bioconcentration factors are used to assess the ability of a plant to accumulate metals from the soil and are defined as the ratio of metal concentration in the plant compartment (root, shoot, silicles, and seed) to that in the soil. A plant's ability to tolerate or accumulate through the translocation of metals in the first instance from the roots to the shoots is assessed using TFs, which are defined as the ratios of metal concentration in the shoots to the roots. To examine the extent to which metals are transported to the silicles and seed after transference from the root to the aerial parts of the plant TFs for the movement of metals from the shoot to silicles and the shoot to seed were also calculated. The BCFs for the four plant compartments analysed for the Rovinari-Sterile camelina crop along with the corresponding TFs for the metals, As, Cd, Co, Cr, Cu, Fe, Ni, Pb, V and Zn are summarized in Table 1.

Scrutiny of the BCFs determined that the camelina roots from the Rovinari-Sterile site were most efficient in taking up Cd, Cu and Zn (BCF: 0.39; 0.23; 0.26, respectively). Bioconcentration factors lower than 0.2 are expected where plants are grown on contaminated soil (McGrath and Zhao, 2003; Brunetti et al., 2010). Similarly the TF values suggest that camelina is most efficient at translocating the same three metals Cd (TF: 0.91), Cu (TF: 0.68) and Zn (TF: 1.0).

Table 1. Bioconcentration factors and translocation factors for the specific target-metals in *Camelina sativa* grown at the Rovinari site

		Elemental concentration (mg kg^{-1} dry weight)									
		As	Cd	Co	Cr	Cu	Fe	Ni	Pb	V	Zn
BCF											
Root/soil	Median	0.030	0.39	0.017	0.026	0.23	0.019	0.024	0.039	0.023	0.26
	IQR	0.0091	0.198	0.0091	0.0123	0.0365	0.00755	0.0119	0.0210	0.00964	0.0955
Shoot/soil	Median	0.018	0.38	0.0025	0.016	0.15	0.0040	0.0068	0.012	0.0037	0.24
	IQR	0.0105	0.158	0.0318	0.00821	0.0311	0.00199	0.00673	0.0173	0.00238	0.131
Silicles/soil	Median	0.012	0.28	0.0072	0.011	0.25	0.0070	0.020	0.023	0.0087	0.047
	IQR	0.0228	0.116	0.00965	0.00596	0.0745	0.00849	0.0322	0.0150	0.00877	0.0475
Seed/soil	Median	0.045	0.17	0.050	0.0077	0.53	0.0031	0.0021	0.0067	0.00018	0.81
	IQR	0.0185	0.120	0.0170	0.00233	0.148	0.00150	0.00994	0.0138	0.00126	0.236
TF											
Shoot/root	Median	0.63	0.91	0.15	0.59	0.68	0.24	0.23	0.35	0.17	1.0
	IQR	0.376	0.294	0.086	0.291	0.140	0.103	0.29	0.255	0.0957	0.417
Silicles/shoots	Median	0.57	0.77	2.6	0.81	1.7	2.4	3.5	2.1	2.4	0.18
	IQR	0.980	0.273	4.38	0.775	0.293	2.2	6.95	1.74	2.77	0.194
Seed/shoot	Median	2.3	0.45	1.8	0.53	3.3	0.90	3.2	0.38	0.058	3.6
	IQR	1.50	0.186	2.22	0.374	0.729	0.703	3.84	0.474	0.320	1.4

The transfer of metal contaminants to the shoot, silicles and seeds are found to decrease with the exception of the transfer of As, Cu and Zn from the soil to the seed. It may be anticipated that as Cu and Zn are essential plant micronutrients that the uptake and translocation in the plant would be enhanced. By contrast, the BCF for Cd is an order of magnitude higher than other non-essential metals such as As, Co, Cr, Ni, Pb and V considered. Other authors have suggested that TF values less than unity were indicative of tolerance to a given metal in the plant (Brunetti et al., 2010), and where TFs < 0.60 this may be suggestive of restricted uptake and possible exclusion mechanisms being operational in the plant (Baker and Brooks, 1989; Yoon et al., 2006). Brunetti et al. (2010) further suggested that TF values greater than

one are indicative of accumulator plants, therefore it is possible that *Camelina sativa* has the potential to act as accumulator for Zn and Cd. Although it is known that other Brassica species act as hyper accumulators for Cd and Zn (Carrier et al., 2003; Verbruggen et al., 2009) further work is needed to verify the accumulator status of camelina with respect to these two metals. Consideration of TFs calculated to assess the efficiency with which metals are transferred from the shoot into the silicles and seeds, suggest that whilst the movement of Co and Ni into the shoots from the roots may be restricted, once in the aerial part of the plant such metals are readily mobilized to the silicles and seeds. Further work is needed to assess the impact of such mobility on the food chain, should the any exclusion mechanisms that are operational be negated. The degree of variability displayed by the TF data suggests that there may be genetic variation in the ability to tolerate or accumulate metals within the plant population of the camelina crop (Yang et al., 2005).

Oil

Analysis of the oil extracted from the camelina grown on the four contaminated sites indicated that concentrations of Cd, Co and V were below the limit of detection. By contrast, Cu and Fe were present in all oil samples whilst there was no consistency in the occurrence of Zn in the oil samples.

Consideration of the analysis of the oil extracted from the camelina grown on Rovinari-Sterile site indicated that concentrations of Cd, Co, Ni, V and Zn were below the limit of detection. Whilst the concentration of As (0.36 mg kg^{-1}), Cu (0.66 mg kg^{-1})and Fe (0.27 mg kg^{-1}) present in the oil samples were at least an order of magnitude greater than those discerned for Cr (0.007 mg kg^{-1}) and Pb (0.03 mg kg^{-1}).

Oil from camelina grown on a nominal non-polluted site (Moara Domnească) was also analysed for comparative purposes. Analysis of the trace element concentrations in the composite oil samples for all the Romanian study sites, focused on the six metals Cd, Co, Cu, Fe, V and Zn, previously identified as problematic within aviation fuel and in keeping with the developed methodology. Scrutiny of the oil data sets suggest that in particular, for the concentration of Cu in the oil is influenced by the cultivar of camelina grown as well as soil characteristics such as pH and external crop nutrient inputs.

CONCLUSIONS

The use of the camelina co-products (oil-meal, silicles and straw) as a valuable component to animal feeds, in particular in the poultry industry, has been fundamental to the business case scenario for camelina biofuel production. Consideration is given to whether co-products from camelina crops produced on contaminated is likely to have the same import.

Translocation factors for Cd and Zn suggest *Camelina sativa* has the potential to act as accumulator. Careful consideration of the use and value of the co-products from camelina grown on certain contaminated lands is therefore recommended: The fractional distribution of metals in the oil and seed is non-uniform, so the oil extraction process may affect a multiplicative increase in the concentration of metals in the crushed seed. Which when given the potential for direct access to the food chain from the use of meal for livestock fodder, and the use of shoot material as bedding material could be of concern. To minimize such concerns it is suggested that the use camelina straw from Cd contaminated sites is restricted to use as a soil conditioner, where appreciable cost benefits may be achieved in terms carbon sequestration and agronomic improvements in soil organic matter.

Comparison of the oil metal concentrations determined for the four contaminated sites with that of oil from the nominally unpolluted site at Moara Domnească, highlight the need for further work to determine the effect of external crop inputs, such as nitrate fertilizers, on the uptake, translocation and storage of metals in the camelina crop grown on contaminated land. This study has developed an effective methodology for the measurement of metals in the camelina value chain, and by evaluating and defining some of the specific vulnerabilities of camelina physiology, gone some way to answering the broad and rather imprecise question of ‘Can a biofuel such as camelina be

grown on contaminated land?' The equally imprecise answer is 'Yes, but site specific characteristics must be duly considered'.

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REVIEW ON SOME CURRENT SKIN ANTISEPTICS

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Abstract

This article will review some current antiseptics used for skin disinfection: povidone iodine (PVP-I), chlorhexidine digluconate (CHG) and octenidine dihydrochloride (OCTD). These antiseptics are used in both healthcare and household products, for different types of applications preoperative skin preparation, surgical hand scrub, skin wound cleanser, skin wound protectant, hand hygiene, oral care and body wash. In the past years more and more concerns have been raised due to increased resistance to antiseptics and antibiotics of pathogens over the years. Some studies support the cross-resistance to antibiotics triggered by antiseptics. Increased resistance and cross-resistance may result from inadequate skin disinfection, over dilution of the antiseptics and environmental residues of antiseptics. More studies are required to assess the mechanisms of resistance and cross-resistance to antiseptics and antibiotics and also the impact of sub-lethal antiseptic concentration on clinical isolates. Usage guidelines of chlorhexidine were proposed recently, but usage guidelines should address all valuable skin antiseptics in order to be used only in cases where there is a clear benefit.

Key words*: resistance, cross-resistance, povidone iodine, chlorhexidine digluconate, octenidine dihydrochloride.*

INTRODUCTION

Antiseptics are antimicrobial substances that are applied to living tissues or human skin. Both antibiotics and antiseptics use had led to the emergence of new mechanisms of microbial resistance. Over the past years, the problem of nosocomial infections and increased antiseptics and antibiotics resistance and cross-resistance is worrisome in many countries and solutions are see ked. This article will review three of the most used antiseptics: povidone iodine, octenidine dihydrochloride and chlorhexidine digluconate.

1. POVIDONE IODINE

Povidone iodine (PVP-I) was discovered in 1952 by Shelanski . PVP-I is a complex of iodine and polyvinylpyrrolidone (povidone). Povidone is a polymer without biocidal activity, but having affinity to cell membranes it delivers the elemental iodine to the target. PVP-I has a better chemical stability and it is less reactive compared to previous elemental iodine formulations, showing increased safety and tolerability on the skin and mucous membranes. PVP-I also brings better solubility of iodine and a sustained way of releasing free inorganic iodine in solution. The antimicrobial activity is given by the quantity of free iodine released in the solution (Gottardi, 2001; Ripa et al., 2003). PVP-I has broad-spectrum antimicrobial properties and efficacy against biofilms (Bigliardi et al., 2017).

Different concentrations of PVP-I are used. Formulations of 10% PVP-I contain 1% available iodine and yield free iodine concentrations of 1 ppm (Anderson, 1989). 10% PVP-I is used as preoperative skin preparation, catheter site disinfection, wound antisepsis, anogenital antisepsis. 7.5% PVP-I is used as preoperative skin preparation, surgical hand scrub and antiseptic handwash. 5% PVP-I is used for preoperative skin preparation, preoperative ocular antisepsis, wound antisepsis, catheter site disinfection. 0.5% PVP-I is used for mouth/throat antisepsis. 0.5% PVP-I is used for vaginal antisepsis (Ripa et al., 2003; Kıvanç et al., 2016; Firanek et al., 2016; Kieran et al., 2017; Silas et al., 2017). A recent *in vitro* study showed that a single application of PVP-I at concentrations equal and higher

than 2.5% and three applications of 30 seconds of PVP-I at 0.7% concentrations produced a 3-$\log_{10}$ reduction of *Staphylococcus epidermidis* colonies (Silas et al., 2017).

Iodine rapidly penetrates the cell wall of microorganisms and inactivates cells by forming complexes with amino acids and unsaturated fatty acids, resulting in impaired protein synthesis and alteration of cell membranes (WHO, 2009). The antimicrobial effect of PVP-I can be strongly affected by temperature, exposure time, concentration of total available iodine, the amount and type of organic and inorganic compounds (e.g. alcohols and detergents) and rising pH (WHO, 2009; Wiegand et al., 2015).

An exploratory clinical study showed excellent antibacterial efficacy with no detectable microorganisms by the tenth day and rapid reepithelialization of 10% PVP-I ointment (Vogt et al., 2017).

In a recent randomized controlled trial (RCT), 5% PVP-I was significantly more effective in decolonizing *Staphylococcus aureus* at 4 hours post-application compared to saline, PVP-I being associated with only 21% *S. aureus* positive patients compared with 59% for saline (Rezapoor et al., 2017). Another study showed that 5% PVP-I significantly reduced the resident *S. aureus* from the anterior nares of human subjects compared to saline at 1, 6 and 12 h after swabbing application (Anderson et al., 2015). Also 5% PVP-I showed >2.0 Log_{10} CFU reduction in methicillin-resistant *S. aureus* (MRSA) regardless of mupirocin sensitivity in an *in vitro* test (Anderson et al., 2015).

During an *in vitro* evaluation, PVP-I was effective against all 81 *Acinetobacter baumannii* isolates tested, and their logarithmic reduction ≥ 5 were observed in 100% of the isolates in their undiluted form (Lanjri et al., 2017).

In two recent *in vitro* studies, PVP-I was effective against *Candida auris*, when tested according to EN 13624:2013 (Moore et al., 2017) and inhibited *C. auris* isolates at concentrations 0.07%-1.25% (Abdolrasouli et al., 2017).

Studies have shown that PVP-I exhibits antibacterial activity, particularly against the *Pseudomonas* species and *S. aureus* that are prevalent in biofilms (Kunisada et al., 1997; Drosou et al., 2003).

A PVP-I ointment tested against biofilms of *Pseudomonas aeruginosa*, multi-species biofilms of *Candida albicans* and MRSA, resulted in no viable *P. aeruginosa*/ *C. albicans*/ MRSA biofilm material recovered after 4 and 24 hours from the treatment (Hoekstra et al., 2016).

Yamasaki et al. (2017) reported at least a 7 Log_{10} reduction in viability of bacteria produced by PVP-I in biofilm studies. A few recent RCTs suggest that CHG based antiseptics are superior to PVP-I. In a large RCT performed on 2349 randomly assigned patients undergoing catheter insertions, compared 2% CHG - 70% IPA (isopropyl alcohol) (1181 patients) with 5% PVP-I - 69% ethanol (EtOH) (1168 patients), demonstrated that CHG-IPA was associated with lower incidence of catheter-related infections compared with PVP-I - EtOH, 0.28 and 1.77 per 1000 catheter-days, respectively; hazard ratio 0·15, 95% CI 0·05-0·41; p=0·0002 (Mimoz et al., 2015). In another large RCT involving 1132 randomized (796 included in the analysis set) patients undergoing catheter insertions, Yasuda et al. (2017) compared 0.5% and 1.0% alcohol based CHG with 10% aqueous PVP-I and found that the incidence of catheter colonization was 3.7, 3.9 and 10.5 events per 1000 catheter-days in 0.5% CHG, 1% CHG and PVI groups, respectively ($p = 0.03$), confirming that 0.5% and 1.0% alcohol based CHG are superior to 10% aqueous PVP-I for the prevention of intravascular catheter colonization.

In a retrospective cohort study including a total of 4,259 patients undergoing abdominal hysterectomy, 70.5% (n=3,005) of the patients were assigned to CHG and 29.5% (n=1,254) were assigned to PVP-I. The unadjusted rate of SSI (surgical site infection) was 2.6% (95% CI 2.1-3.3; n=79) for CHG and 3.6% (95% CI 2.7-4.8; n=45; P=.09) for the PVP-I group, but for the matched groups the rate of SSI was 1.5% (95% CI 0.8-2.6; n=12) for the CHG-alcohol group and 4.7% (95% CI 3.5-6.4; n=40) for the PVP-I group (P<.001). This study suggested that CHG based skin antisepsis was associated with overall lower odds of SSI compared with PVP-I (Uppal et al., 2017).

The results of Privitera et al. (2017) meta-analysis confirmed that CHG is superior to PVP-I for both SSI incidence (risk ratio, 0.70; 95% confidence interval, 0.52-0.92) and bacterial skin colonization (risk ratio, 0.45; 95% confidence interval, 0.36-0.55).

WHO (WHO, 2016) strongly recommended the use of an alcohol-based antiseptic solution preferably based on CHG for surgical site preparation on intact skin, based on a meta-analysis of available studies showing alcohol-based CHG is beneficial in reducing SSI rates compared to alcohol-based PVP-I.

In a RCT involving 388 patients undergoing clean or clean contaminated surgeries, 220 patients were treated with 10% PVP-I and 186 patients were treated with 2% CHG – 70% IPA, Bibi et al. (2015) showed lower infection rates in CHG group (7.1% infection rate) compared with PVP-I group (10% infection rate), but it was not statistically significant (p=0.324). In this study *P. aeruginosa* (23.5%) was the predominant pathogen associated with SSI followed by *S. aureus* (17.6%) (Bibi et al., 2015).

Kieran et al. (2017), in a RCT involving 304 neonates, found no statistically significant differences (p=0.631) in preventing catheter-related blood stream infection between 2% CHG – 70% IPA group and 10% aqueous PVP-I group, but more infants treated with PVP-I had thyroid dysfunction. Ghobrial et al. (2017), in prospective study performed on 6959 spinal surgery patients (0.992% SSI), showed that there is no significant difference (p = 0.728) in the incidence of SSI between 7.5% PVP-I group (33 [1.036%] of 3185) and 2% CHG – 70% IPA group (36 [0.954%] of 3774).

Concurrent use of CHG and PVP-I could be an option to concerns regarding development of acquired bacterial resistance. Following a prospective SSI database analysis, Davies and Patel (2016) suggested that the use of both CHG and PVP-I significantly reduced SSI rates compared to CHG or PVP-I alone. In a two arm RCT enrolling 407 patients, the detection of viable bacteria in the samples taken after disinfection was significantly lower (p = 0.009) in the group assigned to a sequential application of PVP-I and CHG (59 [29.1%]) compared to the group treated only with PVP-I (85 [41.7%]) (Patrick, 2017).

2. OCTENIDINE DIHYDROCHLORIDE

Octenidine dihydrochloride (OCTD) (N,N'-(1,10-Decandiyldi-1(4-H)-pyridynyl-4yliden) bis(1-octanamine)-dihydrochloride) is a bispyridine antimicrobial compound exhibiting antiseptic activity against a wide range of bacteria (Gram-positive and negative), some fungus and dental plaque agents (Patters et al., 1983; Harke, 1989). OCTD was introduced as a topical antiseptic agent more than 25 years ago and now it is used as an antiseptic in many applications and represents an alternative to older substances such as CHG, PVP-I or triclosan (Hübner et al., 2010). In a pH range 5-9 the bactericidal activity of OCTD is not affected (Wiegand et al., 2015). By present no clinically significant local and systemic side effects were associated with OCTD (Willy et al., 2017).

OCTD at concentrations of 0.1% and lower has bactericidal and fungicidal effect (Harke, 1989). 0.05% OCTD is preferred as a chronic wound preparation and for decolonization of multi drug resistant organisms from wounds a 0.1% OCTD/phenoxyethanol solution is preferred (Kramer et al., 2017). As an antimicrobial wash and coating OCTD at concentrations of 0.01, 0.05 and 0.1 produced >5 Log_{10} CFU/cm^2 reductions of *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli* O157:H7, within 2 minutes (Upadhyay et al., 2016).

OCTD demonstrated a significant >6 Log_{10} bacterial reduction factor within 30 seconds contact time in the presence of organic load (0.6% albumin), against six MRSA isolates and two methicillin-sensitive *S. aureus* (MSSA) isolates (Conceição et al., 2016). Another recent in vitro study, performed according to standard method EN 13727, demonstrated the effectiveness (>5 Log_{10} bacterial reduction factor within 1 minute contact time) of OCTD at both concentrations, 0.01% and 0.05%, against all five different tested species: *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Acinetobacter baumannii* and *P. aeruginosa* (for each species using five clonally unrelated isolates, including a susceptible wild type strain, and four multidrug-resistant Gram-negative isolates) (Alvarez-Marian, 2017).

Kung et al. (2016) showed in a *in vitro* study that OCTD is effective against *Trichomonas.*

vaginalis, the 50% effective concentration (EC50) values ranged from 5.7 to 21.37µg/mL after 5 min, from 6.48 to 10.82µg/mL after 15 min and from 0.68 to 2.11µg/mL after 30 min of treatment, suggesting that OCTD could be a promising treatment of bacterial and fungal vaginal infections.

In a study which included 36 MRSA-positive patients, octenidine achieved complete decontamination of 24 patients (67%) (Danilevicius et al., 2015).

OCTD demonstrated a significant reduction of skin flora from the arm and showed a long-term effect after 24 h, when tested according to Deutschen Gesellschaft für Mikrobiologie und Hygiene standard method 13 (Brill et al., 2015).

Similar results in reduction of MRSA acquisition, after five-day cycles of routine bathing of intensive care patients, between CHG and OCTD were reported (Spencer et al., 2013). A large prospective study performed on MRSA-colonized patients showed that daily OCTD body washes was not associated with either a significant reduction in MRSA transmission, MRSA infections, or MRSA bacteremia (Harris et al., 2015).

OCTD based mouth rinses showed comparable results with CHG based mouth rinses, regarding antibacterial and antiplaque efficacy in the human oral cavity (Welk et al., 2016; Decker et al., 2017).

In clinical study involving patients undergoing elective isolated coronary artery bypass graft, Reiser et al. (2017) showed that there was no significant difference ($p=0.39$) in SSI between the control group (15.4% SSI rate) and the group treated with OCTD (13.3%), which consisted in OCTD ointment three times daily and showering the night before and on the day of the surgery with OCTD soap.

OCTD proved *in vitro* antibiofilm activity against *S. aures* and *E. coli*, but not on *Candida tropicalis* (Slobodnikova et al., 2014).

In an *in vitro* study, Narayanan et al. (2016) showed that 0.3%, 0.6% and 0.9% concentrations of OCTD were effective in significantly ($p<0.05$) inactivating *A. baumannii* isolates, either multidrug resistant or drug susceptible isolate, when tested on different surfaces (polystyrene, stainless steel and catheters).

Amalaradjou et al. (2009) showed that OCTD can be effective in preventing the establishment of Listeria monocytogenes biofilms and also to eliminate the preformed biofilms.

OCTD (94 ± 1% reduction rate) and CHG (91 ± 1% reduction rate) demonstrated good efficacy in disinfection of MRSA-biofilms (Gunther et al., 2017). Another *in vitro* study showed that 0.1% OCTD is effective against the following biofilm forming microrganisms: *Enterococcus faecalis, S. aureus, Candida albicans*, within 30 seconds exposure (Ghivari et al., 2017).

OCTD was the most effective when compared, at minimal effective concentration, with CHG and PVP-I, according to standard test method EN 1040 and 1275 against *S. aureus, P. aeruginosa* and *C. albicans* (Koburger et al., 2010).

According to Brill et al. (2015) CHG and OCTD showed comparable efficacy in reducing the resident skin from the arm.

Cherian et al. (2016) showed the 0.1% OCTD was more effective than 2% CHG against *E. faecalis*.

3. CHLORHEXIDINE DIGLUCONATE

Chlorhexidine digluconate (CHG) was first synthesized in the 1950s in United Kingdom and introduced into the USA in the 1970s (Davies et al., 1954; Denton, 2001). CHG is probably one of the most widely used skin antiseptic, due to its broad-spectrum efficacy and low skin irritation. CHG is a cationic bisguanide, water-soluble is generally compatible with other cationic molecules, such as quaternary ammonium compounds. CHG is used as skin antiseptic prior to surgical, catheter insertion or other clinical procedures, in dressings, in healthcare personnel hand wash, in patients bathing (Davies et al., 1954, Paulson, 2003; Denton, 2001; McDonnell and Russell, 1999; Vali et al., 2017).

The antimicrobial activity of CHG is reduced in the presence of anionic detergents (sodium lauryl sulfate) found in natural soaps and mouth rinsing products, various inorganic anions, non-ionic surfactants, and hand creams containing anionic emulsifying agents; CHG forms salts of low solubility with anions (Paulson, 2003; Denton, 2001; Barkvoll et al., 1989; Larson, 1995; Walsh et al., 1987).

Aqueous and alcohol based CHG preparations are available in different concentrations ranging between 0.4% to 4% (WHO, 2009; Vali et al., 2017).

The CHG mechanism of action is known to be related to the binding to negatively charged bacterial and attachment to cytoplasmic membranes and subsequent disruption of this membranes resulting in precipitation of cellular contents, thus affecting the osmotic equilibrium of the cell (Rotter, 1999; Larson, 1995; Vali et al., 2017).

Antimicrobial activity of CHG is influenced by pH, but in a pH range 5-9 the bactericidal activity of CHG is optimal, however pH influence varies with each microorganism (Paulson, 2003; Wiegand et al., 2015).

Double application of CHG (first application: standard hand rub and rinse of the 4% CHG; second application: aqueous solution of 5% CHG applied with no further rinsing of the hands) under the conditions of EN 12791 test method, was superior to single CHG application ($p<0.01$) and n-propanol ($p<0.05$) and surpassed EN12791 (Herruzo and Vizcaino, 2017).

In recent *in vitro* studies, CHG was effective against MRSA obtained from Canary black pigs (Espigares et al., 2017), and also against *Acinetobacter* spp. including international clone II at a concentration of 1000mg/L and exposure time for at least 30s (Hayashi et al., 2017).

Hennig et al. (2017) demonstrated in a study performed according to EN 12791 (2016) that alcohol based CHG formulation (mean log reduction factors of 1.42 ± 0.79 and 1.24 ± 0.90 immediately and after 6 h) achieved significantly lower ($p \leq 0.025$ immediately after application and $p \leq 0.01$ after 6 h) mean log reduction factors compared with alcohol-only formulation (mean log reduction factors of 1.96 ± 1.06 immediately after application and 1.67 ± 0.71 after 6 h) in either immediately or after 6 h.

Previous studies reported no impact of CHG bathing on healthcare associated infections (HAI) (Climo et al., 2013; Bass et al., 2013), while others claim to reduce the HAIs (Climo et al., 2009; Vernon et al., 2006).

Recent studies conducted over long period of time claim that CHG bath reduces most of the HAIs. In quasi-experimental interventional, 9years study, Mendez et al. (2016) showed that CHG bathing significantly reduced the vancomycin-resistant *Enterococcus* (VRE) colonization and infection rates ($p=0.001$), but not for multidrug-resistant (MDR) Gram-negative pathogens. Another recent prospective study conducted in 4 medical units from Canada for over 7 months, confirmed that daily bathing with CHG significantly decreased the hospital-associated infections with VRE by 36% (23.2 vs. 36.0 cases *per* 10,000 patients, $p=0.03$) and also with MRSA by 55% (5.1 vs. 11.4 cases per 10,000 patients, $p=0.04$) compared with control cohorts which were assigned to non-medicated soap and water bathing (Lowe et al., 2017), although skin CHG concentrations were lower when rinsing with water after CHG solution bath compared with CHG solution without rinsing (Alserehi et al., 2017). During an over 18 months study performed in a hospital from Mexico, which included 158 isolates, 2% CHG bath was associated with a reduction in antibiotic resistance and favored the reduction of MRSA isolates and a temporary reduction of ST5-MRSA-II (New York/Japan) clone (Velázquez-Meza et al., 2017). Also, in a prospective, quasi-experimental, single-center study, performed on 237 patients, 4% CHG bath significantly reduced the percentage of contaminated blood cultures in treated group (6.3%) compared with daily cleaning with common soap (15.0%) (Garrido-Benedicto, 2017).

In an *in vitro* study, Wang and Ren (2017) reported significant log reductions of biofilm cells of *S. mutans* and *S. aureus* achived by CHG combined with low-level direct current.

4. RESISTANCE AND CROSS-RESISTANCE

Resistance has been defined as the temporary or permanent ability of an organism and its progeny to remain viable and/or multiply under conditions that would destroy or inhibit other members of the strain (Cloete, 2003; Oancea and Stoia, 2010). Antiseptic-resistance gene expression can be induced by exposure to subinhibitory concentrations of antiseptics (Smith et al., 2008).

Among mechanisms of resistance that have been reported we can mention: efflux pumps (Paulsen et al., 1996; Levy, 2002; DeMarco et al., 2007; Jaglic et al., 2012), inactivation of the active ingredient (Kaulfers, 1995), changes in the cell wall structure (Kaulfers, 1995), changes in membrane fluidity (Gadea et al., 2017).

The most frequent antiseptic antiseptic-resistance genes are listed in Table 1 along with the corresponding primers.

The qacA/B is found in the chromosome and on plasmids of *S. aureus* (Liu et al., 2009; Reich et al., 2016). qacA and qacB are part of the largest known protein family of secondary transporter systems called "Major Facilitator Superfamily" (Wassenaar et al., 2015). The qacA gene confers resistance to ethidium bromide and CHG (Mayer et al., 2001; Shamsudin et al., 2012; Reich et al., 2016). The qacB gene confers resistance to monovalent organic cations and some bivalent compounds (Mayer et al., 2001; Shamsudin et al., 2012; Opacic, 2010).

The smr gene is part of the "Small Multidrug Resistance" protein family and it is identical with qacC gene. The smr gene is regarded as an antiseptic resistance gene (Grinius et al., 1992; Mayer et al., 2001; Shamsudin et al., 2012; Vali et al., 2017; Liu et al., 2015; Shi et al., 2015).

The qacE and qacΔE1 (functional deletion variant of qacE) genes can be found in Gram-negative species and they are corelated with reduced susceptibility to CHG and other biocides (Wassenaar et al., 2015; Guo et al., 2015).

Efflux genes and specific antibiotic resistance genes are located together on mobile genetic elements and antiseptic-resistance and cross-resistance to antibiotics may be aquired together (Yamamoto et al., 1988; Wales and Davies, 2015). Resistance to several antibiotics in association with qac genes has been reported in different clinical isolates (Zhang et al., 2011; Babaei et al., 2015).

Gomaa et al. (2017) reported a significant correlation between the presence of qac genes and MBL-encoding bla_{VIM} (Verona integron-encoded metallo-β-lactamases) (p=0.0064; coexistence detected in 68.1% of the isolates) and bla_{NDM-1} (New-Delhi-metallo- β - lactamase) (p=0.0467; coexistence detected in 68.1% of the isolates) genes and a significant correlation between qac genes carriage and increased MICs of CHG (p = 0.0096).

Until now, no development of resistance to PVP-I or OCTD has been reported (Al-Doori et al., 2007; Willy et al., 2017; Bigliardi et al., 2017).

On the other hand, many studies reported resistance and cross-resistance to CHG. Gomaa et al. (2017) reported higher MIC of CHG than the concentrations recommended for disinfection in 54.5% of all metallo-β-lactamase producing *Acinetobacter baumannii* clinical isolates. Wu et al. (2016) reported cross-resistance of 14 clinical isolates of *S. aureus* to at least one antibiotic following exposure to CHG at. sublethal doses for up to 14 days, sugesting that antibiotics and antiseptics could have similar antimicrobial mechanisms.

Table 1: Antiseptic-resistance genes

Gene name	Primer	Sequence	Reference
qacA/B	qacAB-Forward qacAB-Reverse	GCAGAAAGTGCAGAGTTCG CCAGTCCAATCATGCCTG	Noguchi et al., 2005
qacE	qacE-Forward qacE-Reverse	ATGAAAGGCTGGCTT TCACCATGGCGTCGG	Kucken et al., 2000; Mahzounieh et al., 2014
qacΔE1	qacΔE1-Forward qacΔE1-Reverse	TAGCGAGGGCTTTACTAAGC ATTCGAAATGCCGAACACCG	Wang et al., 2007 Mahzounieh et al., 2014
smr	smr-Forward smr-Reverse	GCCATAAGTACTGAAGTTATTGGA GACTACGGTTGTTAAGACTAAACCT	Noguchi et al., 2005
cepA	cepA- Forward cepA-Reverse	CAACTCCTTCGCCTATCCCG TCAGGTCAGACCAAACGGCG	Fang et al., 2002

The main mechanisms of reduced susceptibility to CHG are CHG efflux and changes in outer membrane content (Wand et al., 2016; Hassan et al., 2013; Costa et al., 2013; Rajamohan et al., 2010; Russell, 2002; Tattawasart et al., 2000). The use of efflux pump inhibitors together with CHG may be an option for preventing the efflux of the CHG from *P. aeruginosa* resulting in a increased efficacy of CHG (Mombeshora et al., 2017). Bhardwaj et al. (2017) identified adaptive changes in genes with predicted or experimentally confirmed roles in CHG susceptibility (efrE), global nutritional stress response (relA), nucleotide metabolism (cmk), phosphate acquisition (phoU), and glycolipid biosynthesis (bgsB). Also Bhardwaj identified a link between serial sub-inhibitory CHG exposure and reduced daptomycin susceptibility.

Guzmán Prieto et al. (2017) concluded that increased CHG resistance may have been selected by microorganisms exposure to antiseptics.

CONCLUSIONS

Povidone iodine remains an effective antiseptic due to its antimicrobial properties and lack of resistance.

Octenidine dihydrochloride is a relatively recent antiseptic, compared with povidone iodine and chlorhexidine digluconate, but with promising antimicrobial results and with no resistance reported until now.

Chlorhexidine digluconate is a valuable antiseptic, but it should be used as a targeted antiseptic in applications where there are proven benefits in order to avoid new bacterial resistance cases.

There is clinical evidence that chlorhexidine digluconate based antiseptics are superior to povidone iodine based antiseptics.

New antiseptic treatments such as successive multiple-antiseptics application (chlorhexidine digluconate and povidone iodine) or antiseptic application along with low level direct current could help in fighting against resistant organisms and biofilm-producing organisms.

There is an increased reduced susceptibility to both antibiotics and antiseptics. Reduced susceptibility to some antiseptics and the potential for cross resistance to some antibiotics highlights the need to restrict the use of antiseptics. Hand hygiene could have a good impact in reduction and transmission of resistant organisms.

Antimicrobial effectiveness of antiseptics should be assessed periodically to overcome dissemination of resistant organisms.

The effect of prolonged exposures at low level concentrations of antiseptics on clinical pathogens should be assessed. Standardized methods could be developed to test and to oversee if antiseptics can trigger resistance and cross-resistance, in order to comply with the UE and USA regulations regarding the biocides marketing.

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PROBIOTICS AS ANTIFUNGAL AGENTS: AN ANTI-*Candida* REVIEW

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Abstract

In recent years, microbial infections have become increasingly difficult to treat. Classical treatments, which involve the use of antibiotics in various pharmaceutical compounds with antimicrobial activity, shows the problems difficult to be solved. For this purpose, researchers are looking for a solution and therefore possible alternative treatments to treat microbial infections. Favorable results are obtained from the use of probiotics, a lot of in vitro and in vivo studies demonstrating the ability of certain probiotics, in particular lactic acid bacteria, to inhibit the growth of pathogenic microorganisms. Probiotics are a class of bacteria similar to those existing in specific human microflora, with beneficial role on human health. Real problems came from endemic fungal infections of humans who, in their favorable conditions, proliferate and lead to the appearance of serious diseases. An example and also a cause of many diseases is the genus Candida. The review presents the main findings related to the response of different Candida species to in vitro and in vivo treatments with different probiotics.

Key words*: Candida sp., candidiasis, probiotics.*

INTRODUCTION

Human microbiota is complex and includes both commensal microorganisms, and pathogenic or facultative pathogenic microorganisms. *Candida* species is an example of microorganisms present in human microbiota which, in terms of balance of microflora of the human individual, it doesn't pose any health problems. However, in cases of proliferation, the genus *Candida* can produce some mucous infections, such as oral or vaginal infections, but also spread infections throughout the body, called candidemia. The main trigger of candidiasis is *Candida albicans* (Silva M. P. et al., 2016), followed by *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei*, representing over 90% of cases of invasive infections caused by the genus *Candida* (Sardi J. C. O. et al., 2013; Pappas P. G. et al., 2015). According to the CDC (Centers for Diseases Control and Prevention) and the National Health Care Safety Network, genus *Candida* is in the fifth place in the above hospital-acquired and in fourth place in the case of bloodstream infections (Yapar N., 2014). However, a high percentage of 36.5% was reported in community-acquired candidemia, within North America (USA and Latin America) percentage rising to 68.8% of reported cases of candidemia and in Europe the percentage it was 22.4% (Pfaller M. A., 2011). According to the clinical data of the 2019 patients with candidemia between July 1, 2004 and March 5, 2008 from Prospective Antifungal Therapy (PATH) Alliance database, presented in 2009 by Horn D. L. et al, the main organism in the incidence of candidemia is *Candida albicans*, with a rate of 45.6%. After 12 weeks of monitoring, 711 of the 2019 patients died (35.2%), 704 were alive (34.9%), while in the case of the 604 patients monitoring was lost. The highest mortality was in the case of *Candida krusei* infections (52.9%), and lowest in the case of *Candida parapsilosis* (23.7%). Other more recent sources indicate a mortality of up to 50% in the case of systemic *Candida* infections acquired in U. S. A. hospitals (Silva M. P. et al., 2016) or, according to the European Society of Anesthesia (ESA) Intensive scientific subcommittee, up to 70% (Pamela Oleary R.-A. et al., 2017). Classic treatments and most used as well in treatment of candidiasis are antifungal medicines, such as type of azoles fluconazole or ketoconazole, the type, such as echinocandins micafungin, caspofungin or type polyenes, like amphotericin B or nucleoside analogues, such as type flucytosine (Spampinato C. et Leonardi D., 2013). But in

recent years, genus *Candida* acquires resistance to antifungal therapy, resulting in a real problem for treating different types of candidiasis (Sanguinetti M. et al., 2015). For this reason, it is absolute necessity to search and develop alternative therapies for the treatment of candidiasis. **Probiotics** are part of the new trends in world medicine, and their successful use as alternative treatments would be a real gain in human battle with microorganisms, in this case the most dangerous species of *Candida*.

In 2001, probiotics have been defined by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) as "live Microorganisms Which When Administered in Adequate trace confer a health benefit on the host", definition accepted and adopted almost completely globally (Hill C. et al., 2014). There are numerous studies in the literature describing the benefits, not few in number, of probiotics on human health. Of these, should be mentioned the following: treatment of infections caused by microorganisms or viruses, combating diarrhea caused by antibiotic treatment, alleviating inflammatory chronic bowel disease, decreased risk of developing allergies, restoring the balance of intestinal microflora, strengthening the immune system, decreased cancer risk colon (Saad N. et al., 2013) or their use in treatments against other cancers (Chen K. et Khismatullin D.B., 2014). Moreover, they can increase nutrient uptake and due to their antimicrobial properties indirectly decrease the need to administer antibiotics (X.-H. Guo et al., 2010; Angmo K. et al., 2016). Another remarkable effect is that probiotics lower the serum cholesterol (X.-H. Guo et al., 2010; C.-F. Guo et al., 2015; Lee N.-K. et al., 2015; Angmo K. et al., 2016), and their use in the treatment of diabetes, is also important (G. Giraffa, 2012; Chen P. et al., 2014; Lee N.-K. et al., 2015). Microorganisms used as probiotics are worthily mentioned *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp., *Saccharomyces* sp. (Silva M.P. et al., 2016) and genetically modified bacteria which naturally are facultative pathogen or pathogen, for example *Escherichia coli* (Silva M.P. et al., 2016; Hwang I.Y. et al., 2017).

In the presented context the use of probiotics in fighting candidiasis may be aviable solution in current medical conditions.

DATA COLLECTION

Online research was conducted using PubMed, ScienceDirect, Cochrane, and Embase data. Also, were included several studies published in InTech and Hindawi databases. *In vitro* and *in vivo* tests were generally selected, as well as some reviews published between 2009 and 2017, in which promising results of probiotics were presented in the treatment of various types of candidiasis.

The words or clusters of keywords that were searched for in this research were: "Candida genus", "Candida species", "candidiasis", "candidemia", " oral candidiasis", "vaginal candidiasis", "invasive candidiasis", "esophageal candidiasis", "cutaneous candidiasis", "probiotic and candidiasis", "probiotic anti-Candida", "candidiasis treatment", "candida treatment", "*Lactobacillus Candida*", "*Bifidobacterium Candida*", "*Bacillus Candida*", "clinical trial candidiasis", "*in vivo* candidiasis", "*in vitro* candidiasis".

DATA FINDINGS

The collected data has approached both *in vivo* and *in vitro* studies related to main *Candida* species to different probiotic groups (Table1).

(1) *In vitro studies and possible mechanisms by which probiotics act against Candida*

The first step in studying the properties and effects of probiotics against various types of candidiasis is the analysis of the data from *in vitro* tests, which can analyze both the mechanisms of probiotics acting against the *Candida* genus, but also the actual effect of some probiotic strains against *Candida* strains.

Some **active probiotic compounds**, such as capric acid of *Saccharomyces boulardii*, may reduce filamentous growth and biofilm formation or *Candida albicans* adhesion (Murzyn A. et al., 2010). The effect on *Candida* filamentous growth, biofilm formation and adhesion to the cellular substrate and the immune response of *C. albicans* to the production of cytokines has been studied; promising results were obtained using *L. rhamnosus*, *L. reuteri* and *L. crispatus*

(Martinez R. C. R. et al., 2009; Rizzo A. et al., 2013). There are reported cases in which strains of *L. casei* and *L. rhamnosus* can produce antifungal peptides as a viable alternative to the prevention and treatment of *Candida* stomatitis (Song Y.-G. and Lee S.H., 2017). They are not excluded from use for prevention or against *Candida* infections neither combinations of probiotics with prebiotics such as arabinose, xylose and xylitol. Combinations of *Lactobacillus species* with prebiotics had an inhibitory effect on the growth of *Candida albicans* and *Porphyromonas gingivalis*, but also on the production of insoluble glucan by *Streptococcus mutans* (Kojima Y. et al., 2015).

Decreasing pH by producing lactic acid by the *Lactobacillus* genus can also inhibit the growth of the *Candida* species (Köhler GA etal., 2012; Chew SY et al., 2015), while the production of H_2O_2 could inhibit *C. albicans* (Köhler GA et al., 2012), but not *C. glabrata* (Chew SY et al., 2015). The inhibitory effect on *Candida sp*. by lowering the pH by the accumulation of organic acids such as lactic or acetic, secreted by lactic species in the gastrointestinal tract was also evidenced by Shokryazdan P. and collaborators in 2014.

The effectiveness of the *Lactobacillus* genus against *Candida* was also analyzed by the action of a filtered culture, LAB supernatant, demonstrating the anti-*Candida* activity of *L. acidophilus* (Vilela S. F. et al., 2015). Seneviratne C.J. and al. succeeded in 2016 fractionation and purification of the cell-free LAB supernatant. Of the 41 fractions, 8 completely inhibited the growth of *Candida albicans* after a 24 h incubation, 4 of the 8 inclusive after 48 h, and 2 fractions had a total inhibitory effect after 72 h of incubation.

In a study published in 2010, Hasslöf et al. have studied the antifungal activity of 8 strains of *Lactobacillus* (*L. plantarum* 299v, *L. plantarum* 931, *L. rhamnosus* GG ATCC 53103, *L. rhamnosus* LB21, *L. reuteri* PTA 5289, *L. reuteri* ATCC 55730, *L. acidophilus* La5 and *L. paracasei*) against 2 *Candida albicans* reference strains (ATCC 28366 and ATCC 10231) and 3 clinically isolated strains (*C. albicans* 1957, *C. albicans* 3339 and *C. albicans* GDM8). The test included 4 probiotic concentrations (10^9, 10^7, 10^5 and 10^3 CFU / ml) and overlay interference tests. Depending on the concentration, most of *Lactobacillus* strains inhibited the growth of *Candida* strains. Antimicrobial activity can also be enhanced by enriching cell cultures of probiotics with some minerals. If the use of supernatant probiotics such as *L. plantarum* and *L. johnsonii* does not influence the growth of *C. albicans*, the enrichment of supernatants with selenium nanoparticles strongly inhibits the growth of *C. albicans* cells (Kheradmand E. et al., 2014).

Studies published in 2014 (Coman MM et al., Verdenelli MC et al., Jiang Q. et al.) have analyzed the inhibition of *Candida* strains growth (*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*) on culture media or vaginal epithelial cells. The lactic species included in the research were *L. rhamnosus*, *L. paracasei*, *L. brevis*, *L. casei*, *L. plantarum* or *L. fermentum*. All lactobacilli strains have had the ability to inhibit all *Candida* strains but to varying degrees. A very strong inhibition effect was obtained by combination of two strains, *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502, called SYNBIO®.

The inhibitory action of probiotics against *Candida albicans* may also be due to the proliferation of probiotics by nutrient depletion in the culture medium (Ujaoney S. et al., 2014). There have also been reports of non-probiotic bacteria without direct fungicidal activity (*Streptococcus salivarius*) inhibiting *Candida* substrate adhesion (Ishijima S.A. et al., 2012).

(2) *In vivo studies on animals*

The general way in which both the human body and the animal respond to *Candida* infections is the immune system. Improving the immune response to infections caused by *C. albicans* has been successfully obtained following treatments with probiotics in animals. The *L. casei* diet of malnourished mice resulted in the proliferation of pro-inflammatory cytokines, the activation of phagocytic cells, and the increase in IL-10 levels that could help prevent damage caused by the inflammatory response to *Candida* infections (Villena J. et al., 2011).

Stimulation of the immune system with probiotics had promising results from the data of two recent studies in which *Galleria mellonella* was treated with *L. rhamnosus* (Ribeiro F. de C. et al., 2016) and *L. paracasei* (Rossoni R.D. et al., 2017).

Table 1. **Reported probiotics with anti- *Candida* effects**

Pathogen	Probiotic (Genera/Species)	Reference
Candida albicans	***Lactobacillus* sp.** *L. rhamnosus, L. paracasei, L. plantarum , L. acidophilus, L. reuteri, L. casei, L. brevis, L. bulgaricus, L. johnsonii, L. animalis, L. salivarius, L. murinus, L. fermentum, L. gasseri, L. crispatus, L. delbrueckii* ssp. *bulgaricus*	Coman M.M. et al., 2014; Hasslöf P. et al., 2010; Kheradmand E. et al., 2014; Köhler G.A. et al., 2012; Kojima Y. et al., 2015; Kovachev S.M. & Vatcheva-Dobrevska R.S., 2014; Kumar S. et al., 2013; Jiang Q. et al., 2014; Martinez R.C.R et al., 2009; Matsubara V.H. et al., 2012; Mendonça F.H.B.P. et al., 2012; Ribeiro F.de C. et al., 2016; Rizzo A. et al., 2013; Romeo M. J. et al., 2011; Rossoni R.D. et al., 2017; Roy A. et al., 2014; Song Y.-G. & Lee S.-H., 2016; Ujaoney S. et al., 2014; Verdenelli M.C. et al., 2014; Vicariotto F. et al., 2012; Vilela S.F. et al., 2015; Villena J. et al., 2011;
	***Streptococcus* sp.** *S. salivarius, S. thermophiles*	Kovachev S.M. & Vatcheva-Dobrevska R.S., 2014; Ujaoney S. et al., 2014; Song Y.-G. and Lee S.-H., 2016; Ishijima S.A. et al., 2012
	***Bifidobacterium* sp.** *B. longum, B. bifidum, B. breve, B.lactis, B. infantis*	Kumar S. et al., 2013; Mendonça F.H.B.P. et al., 2012; Roy A. et al., 2014; Song Y.-G. &bLee S.-H., 2016; Ujaoney S. et al., 2014;
	***Saccharomyces* sp.** *S.boulardi, S.thermophilus*	Murzyn A. et al., 2010; Kumar S. et al., 2013
	***Bacillus* sp.** *B. coagulans*	Ujaoney S. et al., 2014
Candida glabrata	***Lactobacillus* sp.** *L. rhamnosus; L. reuteri, L. paracasei, L. fermentum, L. plantarum, L. casei, L. brevis, L. bulgaricus, L. acidophilus*	Chew S.Y. et al., 2015; Coman M.M. et al., 2014; Jiang Q. et al., 2014; Mendonça F.H.B.P. et al., 2012; Roy A. et al., 2014; Verdenelli M.C. et al., 2014; Vicariotto F. et al., 2012;
	***Bifidobacterium* sp.** *B. breve, B. longum, B. bifidum, B. lactis*	Mendonça F.H.B.P. et al., 2012;Roy A. et al., 2014
Candida krusei	***Lactobacillus* sp.** *L. rhamnosus, L paracasei, L. casei, L. reuteri, L. brevis, L. casei, L. reuteri, L. brevis, L.bulgaricus, L. acidophilus, L. fermentum, L. plantarum*	Coman M.M. et al., 2014; Jiang Q. et al., 2014; Mendonça F.H.B.P. et al., 2012; Roy A. et al., 2014; Verdenelli M.C. et al., 2014; Vicariotto F. et al., 2012;
	***Bifidobacterium* sp.** *B. breve, B. bifidum, B. longum, B. lactis*	Mendonça F.H.B.P. et al., 2012; Roy A. et al., 2014
Candida parapsilosis	***Lactobacillus* sp.** *L. rhamnosus, L. paracasei, L.casei, L.reuteri, L. plantarum, L. acidophilus, L. fermentum*	Romeo M. J. et al., 2011; Coman M.M. et al., 2014; Mendonça F.H.B.P. et al., 2012; Roy A. et al., 2014; Verdenelli M.C. et al., 2014; Vicariotto F. et al., 2012;
	***Bifidobacterium* sp.** *B. breve, B. longum, B. bifidum, B. lactis*	Mendonça F.H.B.P. et al., 2012; Roy A. et al., 2014
Candida tropicalis	***Lactobacillus* sp.** *L. rhamnosus, L paracasei. L. acidophilus, L. casei, L. plantarum, L. fermentum*	Kumar S. et al., 2013; Coman M.M. et al., 2014; Mendonça F.H.B.P. et al., 2012; Verdenelli M.C. et al., 2014
	***Bifidobacterium* sp.** *B. longum, B. bifidum, B. breve*	Kumar S. et al., 2013; Mendonça F.H.B.P. et al., 2012
	***Saccharomyces* sp.** *S. boulardi, S. thermophilus*	Kumar S. et al., 2013
Candida kefyr	*Lactobacillus casei,*	Mendonça F.H.B.P. et al., 2012
Candida lipolytica	*Bifidobacterium breve*	Mendonça F.H.B.P. et al., 2012
Candida guillermondii	***Lactobacillus* sp.** *L. casei, L. rhamnosus, L. reuteri*	Mendonça F.H.B.P. et al., 2012; Romeo M. J. et al., 2011
	Bifidobacterium breve	Mendonça F.H.B.P. et al., 2012

In both cases, treatment with lactobacilli resulted in a significant increase in hemocytes and, implicitly, in a much stronger immune response against *C. albicans*.

The efficacy of probiotics was also analyzed in relation to classical treatments against oral candidiasis.

In 2012, Matsubara VH and colleagues tested two strains of lactobacilli (*L. acidophilus* and *L. rhamnosus*) and nystatin, a widely used drug for treating oral candidiasis, to reduce *C. albicans* infection in the oral mucosa. In this case, the subjects were groups of mice infected with *C. albicans* on which nystatin treatment

did not have the expected effect, but the use of probiotics, particularly *L. rhamnosus*, significantly reduced colonization of *C. albicans*. In the treatment of oral candidiasis, probiotics that are not fungicidal, but still prevent the adhesion of *Candida* to the substrate can also be used. In a study published in 2012, Ishijima S.A. and coworkers, working on mice, concluded that *Streptococcus salivarius*, although not having antifungal properties, can be used to treat oral candidiasis because of its properties to compete with *Candida albicans* for oral mucosal adhesion.

(3) *Human clinical trials*

Due to the major problems in recent years of treating human patients against candidiasis, the efficacy of probiotics as alternative treatments is impetuously needed to be proven in clinical trials.

A major and extremely common problem, especially of the elderly, is **oral candidiasis**. There are numerous clinical studies in which probiotics have been tested in treating this candidiasis. A simple way to prevent or treat oral candidiasis could be to consume food containing probiotics. The products of a dairy company have been tested several times. In the case of the product containing only *Lactobacillus casei* Shirota, there were no obvious reductions in *Candida* colonization at the oral level after a 28 days daily consumption (Sutula J. et al., 2012; Sutula J. et al., 2013). However, in the variant of *Bifidobacterium breve* and *Lactobacillus casei*, reductions in the presence of various *Candida* strains at the buccal level were reported. In 2009, a study by Dos Santos A. L. and collaborators was published in which 111 people were tested. The results concluded that daily consumption, over a 20-day period, significantly reduces the *Candida* population. Consumption-friendly results of this product were reconfirmed in 2012 with the onset of a clinical trial by Mendonça F. and colleagues.

If treating candida with food enriched with probiotics does not always have the expected results, formulating probiotics in the form of tablets, capsules or even paste and administering them for a while may yield optimal results. Applying a paste containing *Lactobacillus bulgaricus*, *Bifidobacterium longum* and *Streptococcus thermophilus* at the buccal level 3 times a day for 4 weeks can significantly reduce the population of various types of *Candida* in the elderly (Li D. et al., 2014). Similar results were obtained by daily administration of capsules containing *L. rhamnosus*, *L. acidophilus* and *B. bifidum* for 5 weeks (Ishikawa KH et al., 2014) or by treatment with tablets containing *L. reuteri* strains (Kraft- Bodi E. et al., 2015).

Urogenital candidiasis is also very common, especially among women. Treating them with classical agents, such as fluconazole, is still the primary way to treat vaginal candidiasis. However, supplementing classical treatment with probiotics (*Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*) can reduce, besides *Candida* species, the symptoms associated with this type of candidiasis, such as vaginal discharge, burning vaginal feeling, dyspareunia or dysuria (Martinez R.C.R. et al., 2009), or even to reduce the negative local changes caused by *Candida*-enhanced population on vaginal fluorine, vaginal tissue changes and pH (Kovachev S.M. and Vatcheva-Dobrevska R.S., 2015).

Also, consumption of yogurt enriched with *Bifido bacterium* and *Lactobacillus* strains can reduce colonization of the urogenital tract in HIV-infected individuals (Hu H. et al., 2013). The effects of vaginal candidiasis and its relapse can be drastically reduced by treatment with products of latobacillus (*L. acidophilus* and *L. fermentum*) and prebiotics (arabinogalactan and fructooligosaccharides). After 28 days, the symptoms associated with candidiasis disappeared in 86.6% of patients, and 11.5% of patients were recurrent in the following month (Vicariotto F. et al., 2012).

In cases of **gastrointestinal candidiasis**, there are numerous deficiencies in treating them due to some factors present in these cases: the presence of diseases such as ulcerative colitis or Crohn's disease or the need to treat infants or very young children. In case of treatment for newborns or children, milk products enriched with probiotics or other probiotic preparations as a powder may be used. These preparations contain strains of *L. reuteri*, *L. rhamnosus*, *L.*

acidophilus, *S. boulardii*, *S. thermophilus*, *B. longum*, *B. bifidum*, *B. lactis*, and prebiotics (fructooligosaccharides) which, after administration, reduce the *Candida* populations, even in cases of candidemia (Romeo MG et al., 2011, Demirel G. et al., 2013, Kumar S. et al., 2013, Roy A. et al., 2014). The administration of probiotics together with a prebiotic complex can improve the health of patients diagnosed with Crohn's disease by improving the immune system (Steed H. et al., 2010). In the case of remission or moderate ulcerative colitis, after the administration of probiotics and prebiotics, the improvement of the patient's health status was revealed, a major role in the improvement of the immune system being due to the growth of microflora of lactobacilli and bifidobacteria and the increase of IL- 10 (Miele E. et al., 2009; Fujimori S. et al., 2009; Hegazy S. K. and El-Bedewy M. M., 2010; Tursi A. et al., 2010; Wildt S. et al., 2011; D'Incà R. et al., 2011; Ishikawa H. et al., 2011; Oliva S. et al., 2012).

CONCLUSION AND FUTURE PERSPECTIVES

Data from clinical trials, from animal studies or from *in vitro* studies indicates that the probiotics can be used with favorable outcomes in treating different types of candidiasis. Of course, it is still necessary to test the efficacy of probiotics in the treatment of candidiasis on different age groups or different states of severity of the disease. It is necessary to understand the mechanisms whereby probiotics work, the long-term effects of their administration and sought solutions for optimal treatment, fast and without adverse effects.

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IN VITRO STUDY OF THE USE OF SOME MEDICINAL PLANTS AGAINST THE FISH PATHOGEN *Aeromonas hydrophila*

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Abstract

In current study the antibacterial activity of different medicinal herbs against fish pathogen Aeromonas hydrophila was evaluated. The water extract of medicinal herbs (Glycyrrhiza glabra, Rhodiola rosea, Althaea officinalis, Sambucus nigra, Inula helenium, Pinus sylvestris, Ocimum basilicum, Salvia officinalis) were prepared by the following method: the plants were extracted in water solution at proportion 1:10. The received homogeny solutions were filtered and centrifuged at 7000 rpm for 30 minutes. Afterwards the extracts were filtered with sterile syringe filters with the size 0.2 μm. The bacterial strain of Aeromonas hydrophila (ATCC 7965) was used in current research. The bacterial activity of plant extract against Aeromonas hydrophila were tested with disk diffusion method. The highest antibacterial effect against Aeromonas hydrophila were determined in water extract of Salvia officinalis and its inhibition zone was higher with 33.34% compared with the size of zone measured for the control variant.

Key words: *Aeromonas hydrophila, antagonistic activity, medicinal plants.*

INTRODUCTION

The aquaculture is one of the fast developing agriculture sectors (Subasinghe et al., 2009). The fast growth in aquaculture production is also associated with the increase of the vulnerability and perceptivity of fish to diseases in aquafarms.

One very important in economics aspect disease which causes high economic losses in aquafarm is hemorrhagic disease. The pathogen associated with this disease is *A. hydrophila* (Maiti et al., 2009; Beaz-Hidalgo et al., 2010). For the treatment of this fish disease usually antibiotics were distributed to fish together with the feed.

Unfortunately it was found that the usage of antibiotics increases the resistance of microorganisms (Sánchez-Romero and Casadesús, 2014). From other side the carcinogen effect of microbiological dyes vastly applied in aquaculture as disinfectants in the past was proved (Srivastava et al., 2004).

These reasons led to necessity alternative therapeutants to be found for the treatment of pathogens in aquaculture. One possible decision for preventing the fish in farms from different diseases is a probiotics (Balcázar et al., 2006; Kesarcodi-Watson et al., 2008). Other possible variant for the counteraction to diseases in aquaculture are essential fatty acids (Randrianarivelo et al., 2010).

Different extracts from medicinal plants were successfully used for the treatment of different pathogens in aquaculture in the last decade (Bansemir et al., 2006; Turker et al., 2009).

Bulgaria is reach of medicinal plants which are used from Bulgarian traditional medicine for treatment of human diseases from hundreds of years. These medicinal plants could possibly be used for the treatment of different fish pathogens including *Aeromonas hydrophila*.

The inhibition effect of extracts of different medicinal plants against fish pathogen *Aeromonas hydrophila* was tested *in vitro* in current study.

MATERIALS AND METHODS

Preparation of water extracts from different medicinal plants

Water extract of medicinal plants were made according to Dellavalle et al. (2011). The plants were bought from herbal pharmacy and were extracted in water solution at proportion 1:10. The received homogeny solutions were filtered

and centrifuged at 7000 rpm for 30 minutes. Afterwards the extracts were filtered with sterile syringe filters with the size 0.2 µm (Minisart, Sartorius Stedim Biotech GmbH, Germany). The medical plants and the morphological parts which were used in current experiments were shown in Table 1.

Table 1. The morphological parts of medicinal plants used in experiments

Medicinal plants	Morphological parts of medicinal herbs
Golden root (*Rhodiola rosea*)	Leaf, stem and blossom
Mashmallow (*Althaea officinalis*)	Root
Elderberry (*Sambucus nigra*)	Blossom
Elecampane (*Inula helenium*)	Root
Scots pine (*Pinus sylvestris*)	Tips
Basil (Ocimum basilicum)	Leaf and stem
Sage (Salvia officinalis)	Leaf and stem

The strain of fish pathogen *Aeromonas hydrophila* ATCC 7965 was used in tests.

In vitro* tests of extracts from medicinal plants against fish pathogen *Aeromonas hydrophila

The suspension of bacteria *Aeromonas hydrophila* with concentration $1.2x10^6$ CFU/ml was spread on Petri dishes contained Mueller-Hinton agar. Disc diffusion method was used for determination of inhibition of fish pathogen *Aeromonas hydrophila* from extracts of different medicinal plants. Prior *in vitro* tests sterile discs (Himedia) with diameter 6 mm were impregnated with different plant extracts and were placed with sterile pincers on sterile media. The Petri dishes were incubated for 48 hours at 28°C. Antibacterial activity of extracts was determinate by measurement the diameter of inhibition zones. The disc impregnated with distilled water was used as a control variant.

Data analysis

The statistical differences in received data were checked by ANOVA (MS Office, 2010).

RESULTS AND DISCUSSIONS

Furmanowa et al. (2002), found antimicrobial effect of *Rhodiola rosea* roots and callus extract on some strain of *Staphylococcus aureus*. *In vitro* tests with water extract of golden root, against fish did not show inhibition effect against fish pathogen *A. hydrophila* and the diameter of its inhibition zone was 6 mm (Figure 1).

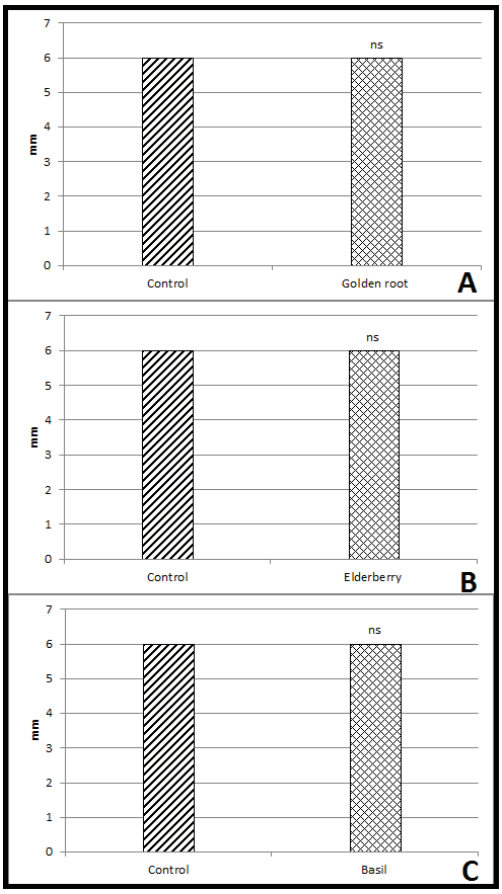

Figure 1. Diameter of inhibition zone in control and experimental variants: extract from golden root (*Rhodiola rosea)* - A; extract from elderberry (*Sambucus nigra*) – B; extract from basil (*Ocimum basilicum*) - C

Cioch et al. (2017) stated that aqueous extracts of elderberry showed antimicrobial activity in higher concentration (> 4 mg/ml). Tested from us concentration of elderberry did not show inhibition effect against fish pathogen *A. hydrophila* (Figure 1).

In vitro tests with water extract of basil (*Ocimum basilicum*) against *A. hydrophila* did not show inhibition effect against this fish pathogen, and the diameter of its inhibition zone was 6 mm. Runyoro et al. (2010) found

that *Ocimum basilicum* oil showed weak antibacterial activity.

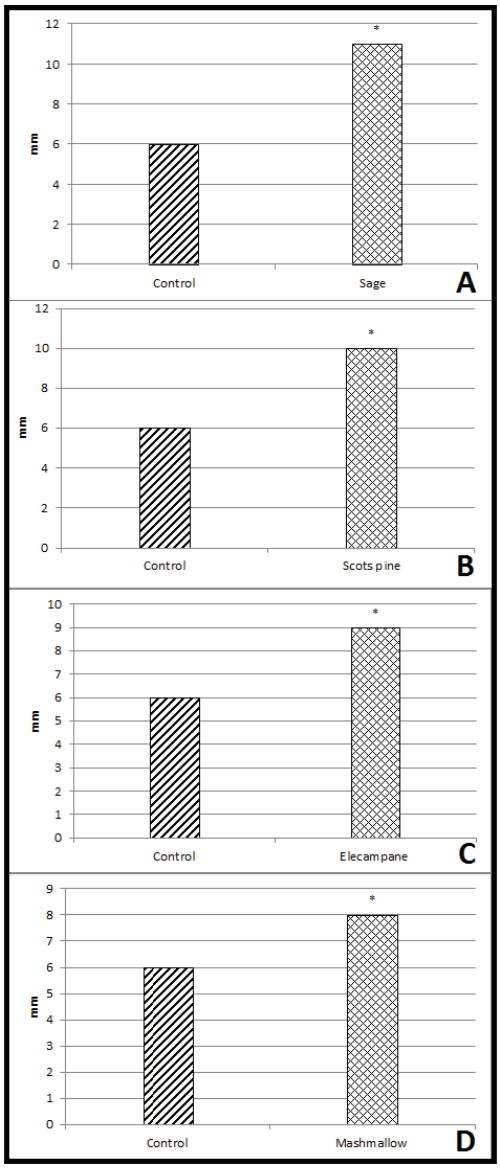

Figure 2. Diameter of inhibition zone in control and experimental variants: extract from sage (*Salvia officinalis*) - A; extract from scots pine (*Pinus sylvestris*) - B; extract from elecampane (*Inula helenium*) - C; extract from mashmallow (*Althaea officinalis*) - D

The aqueous extracts of other four tested medicinal plants showed inhibition effect against *A. hydrophila*. The inhibition effect of sage against fish pathogen was the highest, followed by this found in the extract from *Pinus sylvestris* tips and the weakly inhibition effect was found for the extract from elecampane and mashmallow (Figure 2). The inhibition zone of sage was higher with 33.34% compared with the diameter of zone measured for the control variant. Our results are in line with the results found from Delamare et al. (2007), who stated that the oil of sage possess remarkable bacteriostatic and bactericide activity against different pathogens - *Bacillus cereus*, *Bacillus*, *Aeromonas hydrophila*, *Aeromonas sobria,* and *Klebsiella oxytoca*. Maruzzella and Lichtenstein (1956) found that the oil from scots pine had bactericide activity against different Gram-positive and Gram-negative bacteria.

CONCLUSIONS

The inhibition effect of sage against fish pathogen *Aeromonas hydrophila* was the highest, followed by this found in the extract from *Pinus sylvestris* tips and the weakly inhibition effect was found for the extract from elecampane and mashmallow. *In vitro* tests conducted from us did not find inhibition effect of extracts from golden root, elderberry and basil against fish pathogen *Aeromonas hydrophila* ATCC 7965.

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DEPOLYMERIZATION OF KRAFT LIGNIN WITH LACCASE AND PEROXIDASE: A REVIEW

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Abstract
Lignin is a complex aromatic polymer of phenyl propene units non-linear and randomly linked. The main building blocks are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is the third most abundant biopolymer on earth and usually accounts for 15-35% of the lignocellulose biomass. The degradation of lignin is extremely difficult due to the complexity of the chemical structure (variable upon the source) and the high molecular weight. Two major types of enzymes involved in the depolymerization of lignin are oxidoreductase: laccase and peroxidase, the main microbial producers being fungi and some bacteria. Due to its highly branched structure, lignin is considered to be the most recalcitrant component of lignocellulose, most of it not being recovered. Therefore, there's a demand for more effective methods for depolymerization of lignin in order to obtain value-added products. This review underlines the importance of valorization of lignin through enzymatic depolymerization with laccase and peroxidase.

Key words*: kraft, laccase, lignin, peroxidase.*

INTRODUCTION

Lignocellulose presents a special interest due to its structure and composition, consisting of complex biopolymers, such as: cellulose, hemicellulose and lignin, materials with potential applicability for energy production (Mitache et al., 2015).
Although cellulose and hemicellulose are relatively easy to hydrolyse to obtain their subunits, lignin depolymerization is difficult mainly because of its amorphic and complex three-dimensional structure and of its characteristics that make itself a binding polymer of the cells, fibres and vessels in wood or lignified parts of the plant.
Lignin plays an important role in plant's resistance, providing defence against pathogen attack, mechanical support, stress response and water transport (Li et al., 2016; Boerjan et al., 2003; Kilpeläinen et al., 2007).
Therefore, there's an imperative need to remove lignin from the biomass in order to have access to cellulose and hemicellulose. Initially, lignocellulose biomass was delignified through chemical pathways, but since lignin accounts for 15 – 30% of the biomass it wasn't an economical process, so scientists started to search for ways to valorise lignin with enzymatic degrading systems that will not affect cellulose and hemicellulose.
This review is focused on the importance of degradation of kraft lignin and highlights the main enzymes involved in the depolymerisation: laccase, lignin peroxidase, and manganese peroxidase.

LIGNIN

Native lignins have certain variations in their chemical composition based upon their source, thereby making it difficult to define the precise structure of lignin.
Generally, lignin is considered to be a network polymer containing building blocks of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (as observed in Figure 1), that are non-linear and randomly linked.

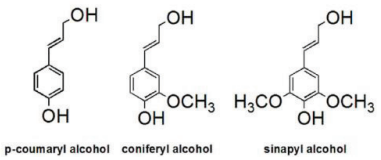

Figure 1. Lignin precursors

Kraft-lignin is a by-product obtained in the pulp and paper industries, through a Kraft

process. Kraft lignin differs considerably from natural lignin in its structure and chemical composition, but given the fact that native lignin's structure is so variable and complex, kraft lignin is often used as a substitute model for native lignin.

The content of lignin in the cell wall structure is the most significant contributor for the biomass recalcitrance to microbial and enzymatic deconstruction (Li et al., 2016). Over the years, several biological and chemical methods were conducted in order to convert lignin in value added products. One of the major challenge in using chemical methods for lignin degradation is related to catalyst selectivity (Xu et al., 2014), being difficult to develop a general catalyst that can specifically work on native lignins structures that are so randomly variable. On the other hand, biological methods cannot compete with chemical processes involved in lignin depolymerization because in the end they can't produce the desired products more economically. But, biological methods are still preferred due to their selectivity and mild reaction conditions. Therefore, enzymatic deconstruction of lignin is somewhat a less studied field that requires more research.

Being a large heterogeneous polymer and not containing hydrolysable linkages, lignin degradation requires the action of extracellular enzymes and more important oxidative enzymes (Hatakka, 2005).

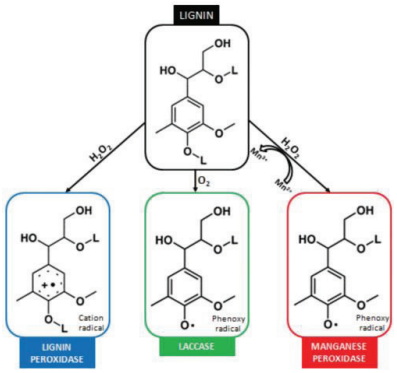

Figure 2. Main enzymes used for lignin depolymerization
Source: de Cassia Pereira et al., 2017

The main enzymes involved in lignin depolymerization are: lignin peroxidase, manganese peroxidase and laccase (Figure2). Other enzymes include: horseradish peroxidase and dioxygenases (protocatechuate 3,4-dioxygenase; 1,2,3-trihydroxybenzene 1,2-dioxygenase and catechol 1,2-dioxygenase) (Octavio et al., 2006).

These ligninolytic enzymes have an immense potential for several industrial and biotechnological processes, such as: food industry, textiles, pulp and paper industry, bioremediation, medical, pharmaceutical cosmetic applications etc. (Maciel and Ribeiro, 2010; dos Santos Barbosa et al., 2008; Kunamneni et al., 2008; Maijala et al., 2007).

LACCASE

Laccase (benzenediol: oxygen oxidoreductases, E.C. 1.10.3.2) is one of the most studied enzyme (Desai and Nityanand, 2011).

Laccases are multi-copper containing enzymes belonging to blue oxidases group and are able to catalyse one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water (Gochev and Krastanov, 2007).

Unlike most enzymes, laccases have the ability to display their activity on a wide range of substrates like monophenols, diphenols, polyphenols, methoxyphenols, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Desai and Nityanand, 2011; Ai et al., 2015).

Laccases from fungi are identified by their capacity to oxidize different substrates such as guaiacol, remazol brilliant Blue R, tannic acid, Poly R-478 etc. to specific coloured products (Desai and Nityanand, 2011).

Even though laccases have a broad substrate specificity on phenolic compounds, they cannot work on non-phenolic sub-units, unless in the presence of mediators - low molecular - weight organic compounds that act as „electron shuttles" (Desai and Nityanand, 2011).

When laccase, cannot oxidize alone a substrate, it will first oxidize a mediator that will form highly reactive and unstable cationic radicals, which will diffuse away from the enzymatic pocket and will oxidize more complex substrates that could not enter into the active site due to their size. After that, the co-mediator (oxidized mediator) will return to its original state and the electrons taken by laccases are

finally transferred back to oxygen to form water (as shown in Figure 3) (Desai and Nityanand, 2011; Gochev and Krastanov, 2007).

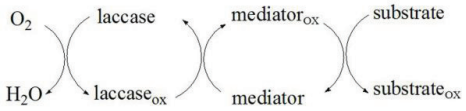

Figure 3. Mechanism of substrate oxidation by laccase with a mediator
Source: Christopher et al., 2014

The most often used mediators are: 2,2`-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), 1-hydroxybenzotriazole (HBT), benzotriazole (BT), remazol brilliant blue (RBB), chlorpromazine (CPZ), promazine (PZ), 1-nitroso-2-naphtol-3,6-disulfonic acid (NNDS), N-hydroxyphtalimide (NHPI), 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS), 3-hydroxyanthranilic acid, N-hydroxyacetanilide (NHA), violuric acid (Octavio et al., 2006; Desai and Nityanand, 2011; Gochev and Krastanov, 2007; Lange et al., 2013; Brijwani et al., 2010).
Laccases can display their activity on a wide range of temperatures and pH. Optimum pH value is variable based on the reactions caused by the substrate employed, molecular oxygen or the enzyme itself (Desai and Nityanand, 2011). Laccases have a significant biotechnological potential due to their broad substrate specificity being used in: environmental bioremediation (removal of pollutants, such as alkenes, chlorophenols, dyes, herbicides, polycyclic aromatic hydrocarbons and benzopyrene) (Gochev and Krastanov, 2007), food and beverages, biosensors, pulp and paper industry (pulp delignification), transformation of antibiotics and steroids, detergent manufacturing bioethanol (Octavio et al., 2006; Brijwani et al., 2010).

Mechanism of action

Laccases contain 4 copper atoms, that are classified into three types, referred to as type 1 (T1), type 2 (T2) and type 3 (T3). The copper atoms differ from each other in their electron paramagnetic resonance (EPR) signals (Gochev and Krastanov, 2007).
The type 1 Cu is responsible for the blue colour of the protein, type 2 Cu does not confer colour and the type 3 Cu atoms consists of a pair of Cu atoms in a binuclear conformation (Desai and Nityanand, 2011).
Type 2 and Type 3 copper sites forms a trinuclear centre responsible for the catalytic mechanism of the enzyme (Desai and Nityanand, 2011).
Laccase catalysis pathway implies three major steps: the type 1 Cu is reduced by a reducing substrate, the electron is transferred from the type 1 Cu to the trinuclear cluster of type 2 Cu and type 3 Cu and at the trinuclear centre will take place the reduction of oxygen to water (Brijwani et al., 2010).
Laccase is considered to act as a battery, that stores electrons from individual oxidation reactions in order to reduce molecular oxygen. Therefore, there are required four molecules of reducing substrate for the complete reduction of molecular oxygen to water (Desai and Nityanand, 2011).

Sources

Amongst all of the large blue copper containing proteins, laccases are the most widely distributed in sources such as: bacteria, fungi, higher plants and insects (Desai and Nityanand, 2011; Gochev and Krastanov, 2007).
Laccase was first characterized when it was extracted from the Japanese lacquer tree *Rhus vernicifera* in 1883. Later, in 1896, it was demonstrated that laccases were also present in fungi (Desai and Nityanand, 2011).
In higher plants, laccases can be found in *Rhus vernicifera*, *Rhus succedanea*, *Lactarius piperatus*, *Prunus persica* (Octavio et al., 2006), *Acer pseudoplatanus, Chaetomiaceae* sp. (Christopher et al., 2014).
Laccase activity has been reported only in a few bacteria such as: *Azospirillum lipoferum, Marinomonas mediterranea, Streptomyces griseus*, *Bacillus subtilis* (Octavio et al., 2006), *Streptomyces lavendulae*, *Streptomyces maltophilia, Streptomyces coelicolor*, *Bacillus licheniformis* (Desai and Nityanand, 2011; Christopher et al., 2014).
The most studied laccases are the ones from fungal sources, including genera of *Ascomycetes*, *Deuteromycetes*, *Basidiomycetes* and cellulolytic fungi (Christopher et al., 2014). Amongst these, the most frequently described were the laccases from the white-rot

basidiomycetes such as: *Trametes versicolor, T. hirsuta, T. ochracea, T. villosa, T. gallica, Phlebia radiata, Coriolopsis polyzona, Lentinus edodes, Pleurotus ostreatus* (Desai and Nityanand, 2011; Brijwani et al., 2010), *Pycnoporus cinnabarinus, Coprinus cinereus* (Christopher et al., 2014). Other fungal strains include: *Agaricus blazei, Melanocarpus albomycea* (Christopher et al., 2014), *Stereum ostrea, Lentinus tigrinus, Ganoderma* spp., *Polyporus versicolor, Pholiata* spp., *Podospora anserine, Neurospora crassa, Aspergillus nidulans, Pyricularia oryzae* (Octavio et al., 2006), *Trichoderma harzianum, Trichoderma atroviride, Trichoderma longibrachiatum, Aspergillus niger, Phanerochaete chrysosporium, Theliophora terristrus, Stereum ostrea* (Gochev and Krastanov, 2007). Marine derived fungi that display laccase activity were: *Coriolopsis byrsina, Cerrena unicolor, Diaporthe phaseolorum, Pestalotiopsis uvicola* (Desai and Nityanand, 2011).

Bacterial laccases are more stable to high pH and temperature compared with the fungal ones (acidic optimum pH). The optimal temperature for laccases is usually between 50 - 70°C (Christopher et al., 2014).

Depolymerization of Kraft lignin

At first, some genera of basidiomycetes involved in lignin depolymerisation were found to lack lignin peroxidases, indicating that different enzymes were responsible for the degradation. After some research, it was suggested that laccases could play a key role in lignin depolymerisation (Gochev and Krastanov, 2007).

Laccases importance in this degradation is due to their capacity to work on both phenolic and non-phenolic compounds (Desai and Nityanand, 2011).

Regarding the depolymerization of lignin, laccase will first attack the phenolic lignin moiety (<20% of total lignin), releasing phenolic residues (as shown in Figure 4) with oxidized side chains (phenolic aldehydes, ketones and acids). After that, through a mediator facilitated process, laccase will oxidize the non-phenolic benzylic structures (Christopher et al., 2014). The phenolic fragments resulted in the first oxidation are able to infiltrate in the bulk lignin polymer and act as a natural mediator, thus helping the enzyme to oxidize more recalcitrant non-phenolic lignin (Christopher et al., 2014; Reddy et al., 2003).

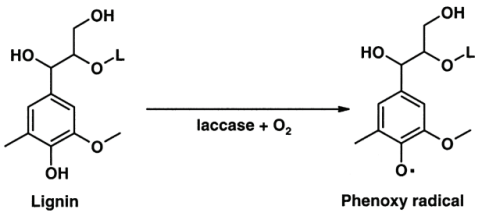

Figure 4. Simplified reaction of lignin depolymerisation with laccase
Source: Hatakka, 2005

An important factor in lignin degradation with laccase is the molecular weight of lignin and its phenolic content (Niku-Paavola et al., 2002). It was implied that during laccase action, both polymerization and depolymerization reactions can take place, as the phenoxy radicals produced can either lead to oxidation or polymerization (Tamminen et al., 2003).

The most common products obtained after lignin depolymerization with laccase and mediators are: 2,6-dimethoxy-4-methylbenzaldehyde, 4-ethyl-2,6-dimethoxybenzaldehyde and 2,6-dimethoxy-4-((E)-prop-1-enyl) benzaldehyde) (Du et al., 2013).

White rot fungi are the main microbial strains involved in the lignin degradation due to their extracellular high laccase activity (Christopher et al., 2014).

In comparison to peroxidases, laccases have a broad substrate specificity and are able to display their activity using only atmospheric oxygen as electron donor, instead of hydrogen peroxide used by peroxidases (Christopher et al., 2014). By not using hydrogen peroxide, laccases show a greater stability which allows them to be used more efficiently in an immobilised way (Octavio et al., 2006).

The ability of laccase to work with mediators in order to oxidize both phenolic and non-phenolic compound of lignin is considered to be a significant participant in lignin valorisetion (Christopher et al., 2014).

PEROXIDASE

Peroxidases (E.C. 1.11.1.7) involved in lignin degradation are heme-containing enzymes that

can oxidize a variety of organic and inorganic substrates in the presence of hydrogen peroxide as electron acceptor (O'Brien, 2000; Falade et al., 2017).

These enzymes are a group of oxidoreductases that catalyses the reduction of peroxides such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds. Heme-peroxidases are extracellular enzymes associated with lignin depolymerization. They include three types of enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP).

Lignin-peroxidase (diaryl propane oxygenase; LiP, E.C. 1.11.1.14) is a monomeric hemo-protein. This enzyme has the capacity to catalyse hydrogen peroxide dependent oxidative depolymerization of lignin (Falade et al., 2017).

Manganese peroxidases (Manganese-dependent peroxidases; MnP; E.C. 1.11.1.13) are extracellular glycoproteins that are considered to be the most common ligninolytic enzymes produced by white-rot fungi (Falade et al., 2017). The production of these enzymes is regulated by nutrients availability and environmental factors (Silva, 2014).

Versatile peroxidases (VP; E.C. 1.11.1.16) are a group of enzymes belonging to the class II subfamily of peroxidases. Its name derives from its versatile nature, being able to oxidize directly diverse substrates from hydroquinones, substituted phenols to bulky lignin, without redox mediators (Ravichandran and Sridhar, 2016).

VP was first mistaken with MnP in 1996 by Martínez et al. and in 2000 by Giardina et al. (Ruiz-Dueñas et al., 2009).

VP is also known as a hybrid peroxidase or manganese-lignin peroxidase, because of its ability to combine the catalytic properties of both MnP and LiP, being able to oxidize both phenolic and non-phenolic compounds.

These three types of peroxidases can work together on lignin degradation if they are produced by the same organism. While LiP oxidize the non-phenolic components of lignin and MnP targets the phenolic ones, VP has the ability to oxidize both phenolic and non-phenolic structures (Falade et al., 2017).

Ligninolytic peroxidases are used in a variety of applications such as: removal of contaminants, organic and polymer synthesis, pulp and paper industry, biosensors, analysis and diagnostic kits, enzyme immunoassays, biofuel production (Hamid, 2009).

Mechanism of action

Peroxidases catalyse the oxidation of a wide variety of substrates, using H_2O_2 or other peroxides. In general, the peroxidase catalytic cycle involves distinct intermediate enzyme forms and the activation of molecular oxygen is achieved in a two-step process. First, the native ferric enzyme is oxidised by hydrogen peroxide to form an unstable intermediate called compound I (Co I), which has a heme structure of Fe IV=O-porphyrin p-cation radical (Hamid, 2009), with consequent reduction of peroxide to water. Then Co I oxidise electron donor substrate to give compound II (Co II), releasing a free radical. Co II is further reduced by a second substrate molecule, regenerating the iron (III) state and producing another free radical.

Lignin peroxidase oxidize various non-phenolic structures of lignin including β-O-4 linkage-type arylglycerol-aryl ethers. The oxidation mechanism involves the formation of radical cation through one electron oxidation and this action leads to side-chain cleavage, demethyla-tion, intramolecular addition and nonetheless rearrangements (Falade et al., 2017).

Although, LiP mainly oxidize non-phenolic structures, it can also act on a variety of phenolic compounds such as: guaiacol, acetosyringone, catechol, vanillyl alcohol, syringic acid etc. (Falade et al., 2017).

An important redox mediator for LiP activity in lignin depolymerization is veratryl alcohol, a non-phenolic metabolite and a high redox potential substrate (Falade et al., 2017).

The catalytic capacity of LiP has been attributed to its exposed tryptophan residues, that forms a tryptophanyl radical on the surface of the enzyme through long-range electron transfer to the heme (Falade et al., 2017).

The variation in the tryptophan environment can influence the enzyme activity, stability and substrate specificity (Falade et al., 2017).

The mechanism of action of LiP involves three steps: oxidation of the resting ferric enzyme by hydrogen peroxide resulting in formation of compound I (oxo-ferryl intermediate), transfer

of one electron from the substrate to the compound I, to form compound II and subsequent donation of a second electron from the reduced substrate to compound II (Abdel-Hamid et al., 2013).
Similar to laccase, manganese peroxidase can have the capacity to oxidize non-phenolic compounds in the presence of mediators such as thiol or lipid radicals.

Sources
Peroxidases are widely distributed in nature in plants, animals and microorganisms.
LiP was first discovered in the extracellular medium of white-rot fungus *Phanerochaete chrysosporium* in 1983 (Falade et al., 2017). After that, several sources have been reported such as: *Trametes versicolor, Phanerochaete sordida, Phlebia radiata* (Falade et al., 2017), *Trametes villosa, Trametes trogii, Phlebia tremellosa, Phlebia ochraceofulva, Junghuhnia separabilima* (Hatakka, 2005).
Several white-rot basidiomycetes have displayed exclusively MnP activity as extracellular peroxidase, such as: *Ceriporiopsis subvermispora, Dichomitus squalens, Lentinula (Lentinus) edodes, Phanerochaete sordida, Pleurotus ostreatus* (Gasser et al., 2012), *Pleurotus eryngii* (Camarero et al., 1999), *Abortiporus biennis, Agaricus bisporus, Bjerkandera* sp., *Cyathis stercoreus, Heterobasidion annosum, Nematoloma frowardii, Panus tigrinus, Rigidoporus lignosus* (Hatakka, 2005).
Pleurotus eryngii is considered to be a model organism for studies regarding biodegradation of lignin, due to its selectivity in removing lignin when cultivated on natural substrates (Camarero et al., 1999).
Some reports indicate that some white-rot fungi can produce both LiP and MnP: *Phanerochaete chryosporium, Phanerochaete flavido-alba, Phlebia radiata, Bjerkandera adusta* and *Trametes versicolor* (Gasser et al., 2012; Hatakka, 2005).
VPs have been detected mainly in *Pleurotus* and *Bjerkandera* species (Gasser et al., 2012). Some reports suggest that VP's can also be found in *Panus, Calocybe, Trametes, Lepista, Dichomitous* and *Spongipellis* species (Ravichandran and Sridhar, 2016).

Depolymerization of Kraft lignin
In nature, lignin is efficiently mineralized by multiple enzymes produced mainly by white-rot fungi, which are found in forest litter and fallen trees (Gasser et al., 2012).
Initially, LiP was considered as the main enzyme connected to the oxidative breakdown of lignin due to the highly amount of non-phenolic units in lignin structure (Camarero et al., 1999).
The discovery of lignin peroxidase was a major step in understanding the mechanism of lignin depolymerization (O'Brien, 2000).
Lignin peroxidase has a high redox potential for the oxidation of non-phenolic structures which represent up to 90% of lignin (Figure 5) (Martinez, 2005).

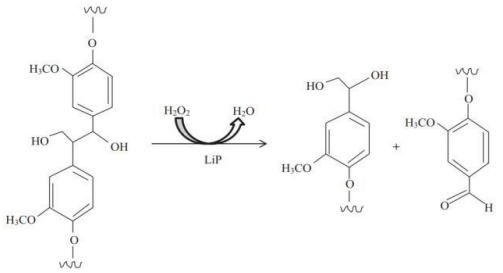

Figure 5. Simplified lignin depolymerization with lignin peroxidase
Source: Falade et al., 2017

Besides the involvement in degradation of non-phenolic structures of lignin, LiP can also oxidize the aromatic rings of lignin via long-range electron transfer (Gasser et al., 2012), which results in formation of unstable cation radicals, that will undertake different non-enzymatic reactions (Falade et al., 2017).
In comparison with laccase, LiP doesn't require mediators to degrade high redox potential compounds, but it does need hydrogen peroxide to initiate the catalysis (Maciel and Ribeiro, 2010).
There are many reports on depolymerisation of both native and synthetic lignins with manganese peroxidase (Falade et al., 2017). The action of manganese peroxidase is started by the activation of hydrogen peroxide with the iron protoporphyrin IX, that in return will oxidize the manganese co-factor from Mn^{2+} to the highly reactive Mn^{3+}. The Mn^{3+} centre is chelated by carboxylic acid anions that will

produce small, freely diffusible species, which will act as redox mediators, oxidizing phenolic lignin structures, as seen in Figure 6 (Falade et al., 2017; Gasser et al., 2012; Lange et al., 2013).

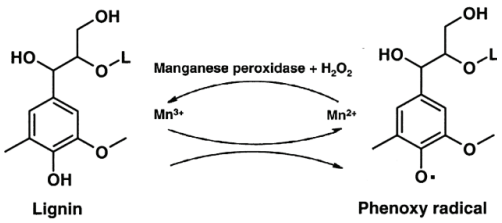

Figure 6. Simplified lignin depolymerisation with MnP
Source: Hatakka, 2005

Additionally, it was reported that lipid peroxidation by MnP might be an important factor in degradation of non-phenolic structures of lignin, due to the fact that the formed peroxyl radicals (Figure 6) can act as agents that will promote the oxidation of non-phenolic β-O-4-linked lignin compounds (Gasser et al., 2012).

In comparison with LiP, MnP has displayed a preference to oxidize *in vitro* phenolic substrates, due to its lower redox potential. On the other hand, some reports suggest that unlike LiP, MnP may be able to oxidize Mn^{2+} without hydrogen peroxide with decomposition of acids and concomitant production of peroxyl radicals (Maciel and Ribeiro, 2010; Hofrichter et al., 1999).

The interesting part about versatile peroxidase is that due to its unique molecular structure given by the presence of different oxidation-active sites (Falade et al., 2017; Ruiz-Dueñas et al., 2008), it's able to oxidize the substrate without redox mediators, unlike MnP and LiP (Ravichandran and Sridhar, 2016). Therefore, VP can have a great potential for future biotechnological applications (Busse et al., 2013).

The non-enzymatic reactions include aromatic ring cleavage, hydroxylation, demethoxylation, ether bond cleavage, side chain cleavage and phenol formation (Busse et al., 2013).

CONCLUSIONS

Lignin is the second most abundant biopolymer that represents 15-30% of lignocellulosic biomass.

There's an imperative need to find new and improved methods to valorise lignin, without affecting the other major components of lingo-cellulosic biomass.

The difficulty in degrading lignin is reflected by lignin complex and variable structure.

The most important enzymes involved in lignin depolymerisation are: laccase, lignin peroxidase and manganese peroxidase.

While lignin peroxidase attacks the non-phenolic structures of lignin, laccase and manganese peroxidase catalyse, without mediators, the oxidation of phenolic fragments of lignin.

These enzymes can work together if they are produced by the same microorganism. Therefore, an improvement on this subject could be the exploration and optimization of novel microbial sources that can produce these enzymes capable of depolymerizing lignin.

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RESEARCH CONCERNING THE INFLUENCE OF ALTERNATIVE METHODS FOR FIGHTING AGAINST WEEDS AND OF FOLIAR FERTILIZATION ON *Phylostachys pubescens* SPECIES DEVELOPMENT

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Abstract

Giant bamboo culture begins to gain increasingly more land in Romania, becoming an attractive crop for farmers, not only because of its multiple utilities, but also because of the ease with which it maintains culture immediately after setting up. Choosing the most effective schemes of fertilization and the practice of alternative methods of weeds control, represent two of the essential technological links which any farmer must take into account when proposing the establishment a culture of giant bamboo, according to some minimal inputs. It was found so that, taking 2 foliar treatments during the growing season of the crop, with total soluble and total assailable fertilizers for the crop, associated with mulching or setting up land cultivation of dwarf clover under „culture hidden" to combat weeds, have induced a bamboo plants accelerated growth and development, in the context of the use of technological practice gentle with the environment.

Key words*: foliar fertilizer, mulch, dwarf clover, eco-friendly, technological links.*

INTRODUCTION

The giant bamboo, *Phyllostachys eudilis*, has attracted worldwide attention as a versatile plant with multiple uses. Its uses varied from subsistence, commercial food (young shoots) to construction and furniture (Azziniand Salgado, 1981). It offers economical and ecological benefits to many people in the world.
Ecological plasticity, growth vigor and species diversity are characteristic of bamboo.
It is a plant found almost everywhere and is known for its rapid growth. A bamboo stem reaches the full height in about 60-90 days. In the three to five years, crops are already matured and can already be harvested, depending on intentional uses (Bezze et al., 2017).
Bamboo protects the environment and cleans the air we breathe. Bamboo strains release 35% more oxygen than tree stems. Some species of bamboo can hold up to 12 tons of carbon dioxide in the air per hectare (Lobovikov et al., 2005).
It can also reduce the intensity of light and protect humans from ultraviolet radiation (Benzhi et al., 2005).
Bamboo is a good plant for soil preservation. With its radicular system that explores a large volume of soil, bamboo can provide effective soil erosion control, support river dams and serve as a windbreaker against strong winds.
At the present stage, where more and more emphasis is placed on the practice of environmental friendly technology links, finding alternative weed control methods as well as finding the most effective fertilization schemes become two major objectives to be taken into account when we want to achieve the expected results in terms of minimum inputs.
Therefore, the use of mulch or perennial leguminous crops to eliminate plant competition with a wide range of weeds that invade crops a year is simple, at the fingertips of any bamboo grower.
At the same time, supplementing the necessity of nutrients by administering during the

fertilizer period completely soluble and totally assimilable by plants, fertilizers that can be applied both by plant leaf spraying on plants and by fertilization, is an easy and cheap method at the same time with maximum effectiveness due to the fact that the nutritional elements, found in ionic form (the preferred form of the plants), are rapidly and totally taken over by the nutrients, thus counteracting the possible deficiencies that may occur at a given moment (Dobrinoiu et al., 2014; Dobrinoiu et al., 2011).

In the present paper we intend to establish the impact of the two technological links on the growth and development of bamboo plants in order to protect the environment from aggressive interventions on the soil under the conditions of the traditional farming system.

MATERIALS AND METHODS

In order to achieve our objectives, namely to determine the impact of using alternative weed control methods and the use of foliar fertilizers on the biometric parameters specific to bamboo culture, we have devised a bifactorial type experience in the field according to the subdivision parcel method, the experimental factors taken in the study being the following:

Factor A - weed control method with 3 graduations:

a1 - mechanically mowed;

a2 - mulch layer;

a3 - clover in hidden culture.

Factor B - fertilization scheme with 3 graduations:

b1 - unfertilized;

b2 - fertilized with POLYFEED 14-14-28 + 2% MgO + ME, 10 kg / ha;

b3 - fertilized with POLYFEED 14-14-28 + 2% MgO + ME, 15 kg / ha.

The experience was set in 3 rehearsals, and following the combination of the two factors studied, 9 experimental variants (3 x 3) were obtained.

Interpretation of the experimental results was done by the variance analysis method, according to bifactorial experiments based on the subdivision parcel method.

In order to track the weed control level in the spring of 2017, the variants in which we proposed to combat the weeds by mechanically breeding were left in their natural state while, in the variant where the combat was carried out by the mulching of the soil, I placed a 15 cm thick mulch on the surface of the field, the mulch being chopped cereal straw at a length of 10 cm.

In the case of controlling the weeds by setting up the clover culture as a hidden culture, at the deprivation, we made the clover sowing using 5 kg seed/ha for this purpose.

Immediately we recorded the emergence of the weed species present in the crop, we performed the mapping operation, identifying both the total number of weeds present in each experimental variant and the weed species present in the crop, separately for each experimental variant.

Regarding the fertilization factor, foliar fertilizer administration was done by leaf sprays directly on the plant, in two distinct vegetation phenophases, namely the beginning of the strain stretch (May) and the intensive growth phase of the strains (August), using this for a fertilizer dose of 10 or 15 kg/ha of commercial product.

During the entire vegetation period of the bamboo culture, we carried out biometric observations and determinations, namely: the total number of stems present on the plant, the height of the stems, the branching degree of the stems, the diameter of the stems.

The experimental results obtained were centralized in synthetic tables and analyzed statistically, according to the method of setting the field experience.

RESULTS AND DISCUSSIONS

The competition of bamboo plants with weeds is one of the most important problems, especially in the first two years of setting up the plantation, which is why we need to take the most effective measures to counteract their growth and development, taking into account that the system root plants of bamboo plants did not have the time to explore a large volume of soil so as to cope with the over 800 species of weeds that naturally grow on the preluvosols in southern Romania.

Analyzing the number of weeds present in the experiment (Table 1), we find that the greatest number of weeds were found in experimental

variants where mechanical mowing was used to control their development, the differences from the average of the experience, taken as a witness, being statistically ensured from significant (a1b3) to very significant (a1b2 and a1b3).

Table 1. Influence of weed control method and fertilization scheme on weed number

EXPERIMENTAL VARIANT	NUMBER OF WEEDS/m^2	RELATIVE VALUES (%)	DIFFERENCE	SEMNIFICATION
a1b1	63	324.0	43.56	xxx
a1b2	37	190.3	17.56	xxx
a1b3	25	128.6	5.56	x
a2b1	17	87.4	-2.44	-
a2b2	11	56.5	-8.44	oo
a2b3	8	41,2	-11.44	ooo
a3b1	7	36.0	-12.44	ooo
a3b2	5	25.7	-14.44	ooo
a3b3	2	10.2	-17.44	ooo
AVERAGE (Controll)	19.44	100.0	Controll	Controll

$DL_{5\%} = 4.32$; $DL_{1\%} = 6.46$; $DL_{0,1\%} = 9.12$

The main weed species present in these experimental variants were: *Agropyron repens, Cynodon dactylon, Cirsium arvense, Sonchus arvensis, Sonchus asper, Convolvulus arvensis, Rumex acetosa*, predominantly perennial monocotyledonous species.

By practicing the soil mulching system and that of clover sowing in a hidden crop, we can see that the number of weeds has dropped drastically with statistical assurance from distinctly significant negative (a2b2) to very significant negative (a2b3, a3b2 and a3b3) , so that the combination of the two alternative ways of controlling weeds by supplementing the nutrients needed by the administration of foliar fertilizers becomes technologically worthwhile to account for and at the expense of any farmer (Table 1). The most representative weed species present in these experimental variants were *Sonchus arvensis, Sonchus asper, Convolvulus arvensis, Rumex acetosa* the perennial monocotyledonous weeds, virtually nonexistent. This aspect is particularly important in the context in which, as already known, perennial monocotyledonous weeds are considered to be a weed problem and their control can only be achieved by the use of total herbicides, which can not be achieved in bamboo plantations because, bamboo itself is a perennial graminee species.

Table 2. Influence of weed control method and fertilization scheme on the number of strains per plant (04.06.2017)

EXPERIMENTAL VARIANT	NUMBER OF STRAINS/plant	RELATIVE VALUES (%)	DIFFERENCE	SEMNIFICATION
a1b1	14	54.6	-11.6	ooo
a1b2	15	58.5	-10.6	ooo
a1b3	19	74.2	-6.6	ooo
a2b1	22	85.9	-3.6	oo
a2b2	24	93.7	-1.6	-
a2b3	28	109.3	2.4	x
a3b1	32	125.0	6.4	xxx
a3b2	36	140.6	10.4	xxx
a3b3	41	160.1	15.4	xxx
AVERAGE (Controll)	25.6	100.0	Controll	Controll

$DL_{5\%} = 2.12$; $DL_{1\%} = 3.42$; $DL_{0,1\%} = 5.9$

The total number of strains formed on a plant during the vegetation period varied within fairly wide limits, with statistical assurance from very significant negative for variants where weed control was achieved by mechanical mowing (a1b1, a1b2 and a1b3) (a3b1, a3b2 and a3b3), the highest number of strains being recorded in the case of variants where foliar fertilizations were applied with 10 and 15 kg respectively POLYFEED/ha (Table 2).

Table 3. Influence of weed control method and fertilization scheme on stems height (04.06.2017)

EXPERIMENTAL VARIANT	STEMS HEIGHT (cm)	RELATIVE VALUES (%)	DIFFERENCE (cm)	SEMNIFICATION
a1b1	83.5	63.6	-47.7	ooo
a1b2	96.5	73.5	-34.7	ooo
a1b3	98.3	74.9	-32.9	ooo
a2b1	116.2	88.5	-15.0	o
a2b2	124.8	95.1	-6.4	-
a2b3	147.4	112.0	16.2	x
a3b1	162.3	123.7	31.1	xxx
a3b2	168.7	128.6	37.5	xxx
a3b3	183.4	139.8	52.2	xxx
AVERAGE (Controll)	131.2	100.0	Controll	Controll

$DL_{5\%} = 14.78$; $DL_{1\%} = 19.23$; $DL_{0,1\%} = 24.52$

As a result of determinations related to the maximum height of the strains, it can be seen that minimal values of this biometric indicator were recorded in cases where weed control was done by mechanical mowing, (a1b1, a1b2 and a1b3). The rest of the experimental variants recorded a progressive increase of the value of this parameter, the maximum values being recorded for the alternatives used as an alternative weed control method, the establishment of the clover culture in the hidden culture (a3b1, a3b2 and a3b3), the experimental results obtained following the determinations being statistically very positive (xxx) (Table 3).

Table 4. Influence of weed control method and fertilization scheme on stems diameter (04.06.2017)

EXPERIMENTAL VARIANT	STEMS DIAMETER (mm)	RELATIVE VALUES (%)	DIFFERENCE (mm)	SEMNIFICATION
a1b1	3.0	34.1	-5.8	ooo
a1b2	5.0	56.8	-3.8	oo
a1b3	6.0	68.2	-2.8	oo
a2b1	7.0	79.5	-1.8	-
a2b2	9.0	102.2	0.2	-
a2b3	10.0	113.6	1.2	-
a3b1	11.2	127.2	2.4	x
a3b2	13.4	152.2	4.6	xxx
a3b3	15.1	171.6	6.3	xxx
AVERAGE (Controll)	8.8	100.0	Controll	Controll

$DL_{5\%} = 1.83$; $DL_{1\%} = 2.33$; $DL_{0,1\%} = 3.98$

The diameter of the strains increased, directly proportional to the increase in nutrient intake, and the maximum values of this dendrometric defecer were recorded in experimental variants where the foliar fertilization was supplemented by the symbiotic activity of clover plants (a3b2 and a3b3) significantly positive (xxx) (Table 4.).

Table 5. Influence of weed control method and fertilization scheme on the number of strains per plant (04.09.2017)

EXPERIMENTAL VARIANT	NUMBER OF STRAINS/plant	RELATIVE VALUES (%)	DIFFERENCE	SEMNIFICATION
a1b1	18	56.8	-13.7	ooo
a1b2	21	66.2	-10.7	ooo
a1b3	24	75.7	-7.7	ooo
a2b1	28	88.3	-3.7	oo
a2b2	32	100.9	0.3	-
a2b3	36	113.5	4.3	xx
a3b1	38	119.8	6.3	xxx
a3b2	43	135.6	11.3	xxx
a3b3	46	145.1	14.3	xxx
AVERAGE (Controll)	31.7	100.0	Controll	Controll

$DL_{5\%} = 2.63$; $DL_{1\%} = 3.12$; $DL_{0,1\%} = 5.52$

As plants progress into vegetation, there is a direct proportional increase in the value of the main dendrometric parameters taken into study. Thus, the number of strains formed on a plant varied from 18 strains/plant to 46 strains/plant, the largest number of strains being formed in the experimental variants where the elimination of bamboo weed plants competition was achieved by sowing the clover, the differences from the average of the experience (taken as a witness), being very significant positive (xxx) (Table 5).

Table 6. Influence of weed control method and fertilization scheme on stem height (04.09.2017)

EXPERIMENTAL VARIANT	STEMS HEIGHT (cm)	RELATIVE VALUES (%)	DIFFERENCE (cm)	SEMNIFICATION
a1b1	91.5	61.4	-57.4	ooo
a1b2	106.8	71.7	-42.1	ooo
a1b3	118.2	79.3	-30.7	ooo
a2b1	136.4	91.6	-12.5	oo
a2b2	144.3	96.9	-4.6	-
a2b3	158.7	106.6	9.8	-
a3b1	182.9	122.8	34.0	xxx
a3b2	198.2	133.1	49.3	xxx
a3b3	203.5	136.6	54.6	xxx
AVERAGE (Controll)	148.9	100.0	Controll	Controll

$DL_{5\%} = 12.46$; $DL_{1\%} = 18.32$; $DL_{0,1\%} = 24.22$

Combining foliar fertilization with clover sowing in hidden crops, as an alternative weed control method, has increased the growth rate of bamboo strains, increasing by a few tens of centimeters per month (Table 6.). Thus we notice that for these experimental variants, the intake of nutrients brought about by the practice of these two technological links resulted in very positive values (xxx) of this parameter. This must be taken into account when we aim to obtain a superior wood in both quantitative and qualitative terms, and also if we want to shorten the time of harvesting wood.

As regards the diameter of the strains (Table 7), it is observed that these increased with the advancement of the plants in the vegetation, the highest values being obtained under conditions of combining the weed control method by sowing clover in a hidden crop with the foliar administration of 15 kg of POLYFEED/ha.

Table 7. Influence of weed control method and fertilization scheme on strain diameter (04.09.2017)

EXPERIMENTAL VARIANT	STEMS DIAMETER (mm)	RELATIVE VALUES (%)	DIFFERENCE (mm)	SEMNIFICATION
a1b1	5.0	44.6	-6.2	ooo
a1b2	7.0	62.5	-4.2	oo
a1b3	8.0	71.4	-3.2	oo
a2b1	10.0	89.3	-1.2	-
a2b2	12.2	108.9	1.0	-
a2b3	13.3	118.7	2.1	x
a3b1	14.1	125.9	2.9	xx
a3b2	14.5	129.4	3.3	xx
a3b3	16.3	145.5	5.1	xxx
AVERAGE (Controll)	11.2	100.0	Controll	Controll

$DL_{5\%} = 1.92$; $DL_{1\%} = 2.82$; $DL_{0,1\%} = 4.46$

We can therefore conclude that, in order to obtain the greatest number of strains at the surface unit and higher values of the main dendrometric parameters, bamboo culture becomes imperative to choose the most efficient methods of stimulating the rhythm of growth and development of the plants, under conditions of practical alternative weed control, practical to be mild with the environment.

CONCLUSIONS

The issue of eliminating competition between bamboo plants and weed species is essential

especially during the first two years of setting up the plantation, therefore choosing effective methods to combat them is the key to successfor any farmer who wishes to set up such a plant plantation.

Combating perennial monocotyledonous weed species by using total herbicides is practically impossible, bamboo being itself a perennial monocotyledonous species.

Using weed control methods by using the mulch or clover culture as a hidden crop was very effective in weed control, so the number of weed caps decreased very significantly with plant growth, monocotyledonous species perennials almost disappearing.

The vegetative use of fertilizers, which are totally soluble and totally assimilable by plants, intensifies the growth and development of bamboo plants, the effect of which is reflected in the high growth rate in height and diameter of the stems.

Combining foliar fertilization with the alternative method of controlling weeds by setting up clover culture in hidden culture is two of the technological links that we must take into account when aiming to obtain bamboo of high quality wood but at the same time environment friendly technology links.

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RESEARCHES ON OBTAINING PRODUCTS WITH ADDED VALUE THROUGH SUPERIOR CAPITALIZING OF WHEY

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Abstract

The theme of the paper was chosen because we considered it important that people know not only the negative part about whey - it is a waste, but also the positive side - that it can be successfully used in the production of value-added products. The purpose of the paper was to obtain a probiotic product by superior capitalizing of whey. The objectives of the paper were: (1) Processing of whey in a slightly fermentable form for the cultivation of Saccharomyces cerevisiae and Lactobacillus bulgaricus strains; (2) Optimizing the composition of the whey culture medium in order to obtain the highest biomass productivity; (3) Obtaining probiotics based on Saccharomyces cerevisiae and Lactobacillus bulgaricus strains. Several media variants with different glucose concentrations (0%, 0.5%, 1%, 2%) were used for the biosynthesis of probiotics. In the case of yeasts, glucose supplementation of culture media increases the amount of wet biomass by 21% compared to glucose-free media, and 16% for lactobacilli. However, good results were also obtained on whey as such, without additional glucose, yielding 52 g/l yeast and 42 g/l lactobacilli.

Key words: *whey, probiotic, biomass.*

INTRODUCTION

Whey, a bio product obtained from cheese and sweet cheese was once considered a waste. The discovery of whey as a functional food with nutritional value has made it recognized as a co-product in cheese making (Banu et al., 2000).

Components of whey include α-lactalbumin, lactoferrin, β-lactoglobulin, glycoacropeptides, immunoglobulins, bovine serum albumin, lactoperoxidases, lactose and minerals (Geogescu et al., 2000; Pescuma et al., 2008).

Today, whey is considered a product that has antimicrobial activity, has an immunemodulatory role, improves muscle strength, and prevents cardiovascular disease and osteoporosis (Banu et al., 2000).

Advancing technology in ultrafiltration processes, microfilters, reverse osmosis, ion exchange have resulted in the discovery of new whey products (Costin et al., 2005).

Protein whey concentrate (80-95% protein), whey lactose, whey protein isolates, demineralised whey and hydrolysed whey are products currently available in the world market. Each whey product varies in amount of protein, carbohydrates, immunoglobulins, lactose, minerals and fats.

These variables are important factors in the selection of whey fractions for specific applications (Panayotov et al., 2015; Oprea et al., 2001).

Whey, a complex of milk-derived proteins has been recognized as having a large number of health benefits.

Why has the ability to behave like an antioxidant, antihypertensive, antitumor, antiviral, antibacterial agent (Pescuma et al., 2010).

The purpose of this work is to capitalize on whey and aims to:

1) Processing of whey in a easily fermentable form for the cultivation of *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* strains; 2) Optimizing the composition of the culture medium based on whey in order to obtain as high productivity in biomass (Ştefan et al., 2016); 3) Obtaining probiotics based on *S. cerevisiae* and *L. bulgaricus* strains (Champagne et al., 2002; Bîjnea et al., 2015).

MATERIALS AND METHODS

Raw materials

The main raw material is bovine whey.

The whey used in the experiments was purchased from a private manufacturer and was stored at 4^0C until processing.

Microorganisms

For the biosynthesis of probiotics, strains of *Saccharomyces cerevisiae* and *Lactobacillus bulgaricus* from the Microorganism Collection of the Faculty of Biotechnologies were used.

Culture media

For maintenance of yeast culture we used YPG medium and for lactobacilli we used MRS medium.

Yeast control medium = Medium S (g/v of whey)

Peptone	1%
Yeast extract	1%
Magnesium sulphate	0.2%

pH=4,8

Lactobacilli control medium = Medium L (g/v of whey)

Peptone	0.5%
Meat extract	0.3%
Sodium chloride	0.5
Magnesium sulphate	0.02%

pH=5,5

For the cultivation of microorganisms, several variants of culture media with the same components as control media were tested, to which glucose was added at different concentrations: 0.5%, 1%, 2%.

Processing of whey

1. Centrifugation. The purpose of centrifugation was to semipurification of whey by removing solid residues and grease (8000 rpm, 15 min, 3^0C).

2. Filtration was aimed at removing residual impurities after centrifugation. Filtration was performed under vacuum using a Buchner funnel and high quality filters.

3. Deproteinization aimed at removing residual protein left in whey after obtaining the cheese. Deproteinization was performed by treating the whey filtrate with a 20% trichloroacetic acid solution added at a concentration of 10%, based on the volume of processed whey. The precipitate was removed by vacuum filtration.

Obtaining the vegetative preparation

The solid media was inoculated with a cell suspension from a lyophilized preparation. The culture was statically grown, 24-48 hours at 30-32^0C, then stored at 4^0C.

Preparation of laboratory inoculum

From the maintenance culture, a microbial suspension is prepared in sterile distilled water. 2 ml of cell suspension is inoculated into 25 ml of liquid medium. The inoculum culture was grown in 100 ml Erlenmeyer flasks for 24 hours at 30-32^0C at 200 rpm for yeast and static for lactobacilli.

Obtaining probiotics

For the biosynthesis of probiotics, control media S and L and variants with different concentrations of glucose (0.5%, 1%, 2%) were used. The main objective of making different variants of culture media was to highlight the role of whey as the only carbon source for the cultivation of microorganisms.

Fermentations with yeast were carried out under the following conditions: 48 h, 30^0C, 200 rpm, pH = 4.8.

Fermentations with lactobacilli were carried out under the following conditions: 48 h, 35^0C, 20 rpm, pH = 5.5

Analytical control of the process of biomass production

1. Determination of wet biomass (WCW= Wet Cell Weight). In a centrifuge tube, an exactly measured volume of the sample (10 ml) was introduced and then centrifuged at 4000 rpm for 20 minutes. After the supernatant was removed, the biomass was weighed.

2. Determination of dry biomass (DCW= Dry Cell Weight). In a centrifuge tube, an exactly measured volume of the sample (10 ml) was introduced and then centrifuged at 4000 rpm for 20 minutes. After removing the supernatant, the biomass tube was dried step by step first at 60^0C for 4 hours, then at 105-110^0C, to constant weight.

Post-biosynthesis processing of fermentation media

After completion of the fermentation process, the first post-biosynthesis operation was the separation of biomass from the liquid medium by centrifugation at 4000 rpm for 20 minutes in the cold conditions. After centrifugation, wet biomass was collected and weighed, placed in the trays and dried in the oven at 105^0C. Finally, the total amount of dry biomass was weighed.

RESULTS AND DISCUSSIONS

Purity of cultures

Cultures of *S. cerevisiae* and *L. bulgaricus* used as inoculum in laboratory fermentations were examined microscopically and macroscopically, highlighting the characteristics mentioned in the literature for these species. The microscopic and macroscopic appearance of the cultures is shown in Figures 1-4.

Figure 1. Macroscopic appearance *S. cerevisiae*

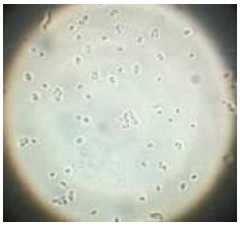

Figure 2. Microscopic exam *S. cerevisiae*

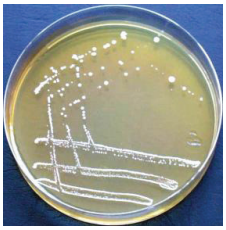

Figure 3. Macroscopic appearance *L. bulgaricus*

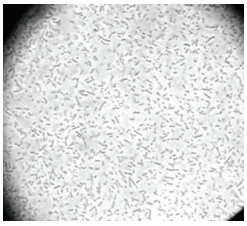

Figure 4. Microscopic exam *L. bulgaricus*

Processing of whey

From the data presented in the graph below (Figure 5), it can be concluded that whey deproteinization had a positive effect on microbial growth, as a higher amount of biomass was obtained in the trichloroacetic acid treated media (Table 1). This effect was more obvious in the case of yeasts.

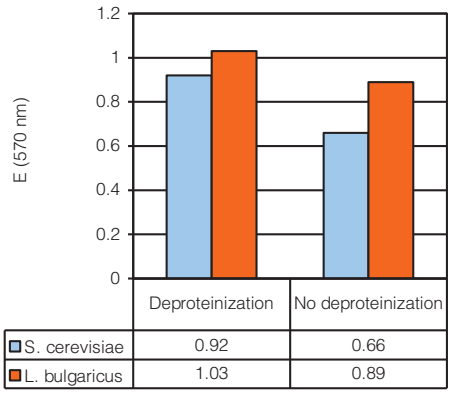

Figure 5. Influence of deproteinization treatment on microbial growth

Table 1. Influence of deproteinization treatment on the amount of biomass

Strain / Culture medium	*S. cerevisiae*		*L. bulgaricus*	
	Deproteinized medium	Undeproteinized medium	Deproteinized medium	Undeproteinized medium
WCW (g/100 ml)	5.23	3.86	4.2	3.51
DCW (g/100 ml)	1.02	0.77	0.85	0.72

From the examination of the data presented in table 1 and figure 5 it can be concluded that the whey deproteinization treatment is cost-effective since it results in much better results in the obtained biomass, by 33.7% in *S. cerevisiae* and 19% higher for *L. bulgaricus*.

Influence of glucose concentration on the accumulation of biomass

For probiotic biosynthesis, *control media S, control media* L and variants with different glucose concentrations were used (0.5% = test 1, 1% = test 2, 2% = test 3).

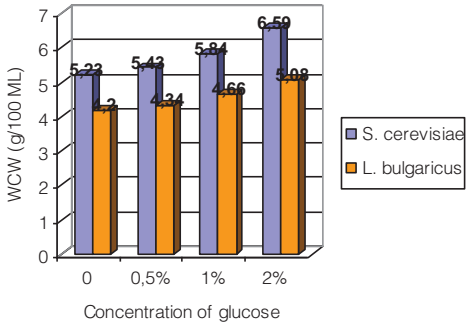

Figure 6. Influence of glucose concentration on the amount of biomass

Comparing the values of the wet biomass quantities obtained in the case of glucose-supplemented media to glucose-free media, the different glucose efficiency is clearly observed with the increase in its concentration (Figure 6). Thus, in the case of yeasts, the increase in the amount of wet biomass is with 26%, compared to the glucose-free media, and in the case of lactobacilli, the increase is with 21%.

However, it can be concluded that good results were obtained also on whey without glucose, 52 g/L of yeast and 42 g/L of lactobacilli, results that cannot be neglected and which, from the economic point of view does not justify additional costs for glucose use.

An idea to make the whole process worthwhile would be to use sources of carbohydrate that also result as residues from various industrial processes.

CONCLUSIONS

The following conclusions can be drawn from present researches:

- ✓ the deproteinization of whey is profitable as it results in much better results in the amount of biomass obtained, with over 33.7%, in case of *S. cerevisiae* and with approx. 19% higher for *L. bulgaricus* compared to unprocessed whey;
- ✓ by comparing the values of the wet biomass quantities obtained by supplementing the media with glucose vs the glucose-free media, the different glucose efficiency is clearly observed with the increase in its concentration. Thus, in the case of yeasts, the increase in the amount of wet biomass is 26%, and in the case of lactobacilli, 21%;
- ✓ good results were obtained also on whey without glucose, 52 g/L of yeast and 42 g/L of lactobacilli, results that cannot be neglected and which, from the economic point of view does not justify additional costs for glucose use.

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EFFECTS OF GAMMA RADIATION ON INULINASE PRODUCTION BY *Aspergillus terreus*

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Abstract

This work aims to study the effect of gamma radiation on the inulinase production by the fungus Aspergillus terreus. The fungus was screened for the ability to produce enzymes from other strains belonging to the Collection of Microorganisms of Industrial Importance of the National Institute of Chemical-Pharmaceutical Research and Development. Subsequently, the fungi were irradiated at 3 doses (500, 1000 and 1500 Gy), using the vegetative form of the fungi. A fermentation assay for enzyme production was made using the DNS method. The best enzyme activity was 770.7 U/L, obtained from the media with orange peels. The use of gamma radiation increased the production of enzymes compared to tests without radiation. Statistically, the best dose of radiation was 1000 Gy.

Key words*: Aspergillus sp., inulinase, radiation, orange peel.*

INTRODUCTION

Recently, inulinases have received much attention as they can be widely applied to hydrolyze inulin for the production of fuel ethanol, fructose, and fructo-oligosaccharides (Gao et al., 2009). The various oligosaccharides derived from inulin also find their application in the medical and dietary sector. High fructose syrup and fructooligosaccharides are two major industrial applications of inulinases. The industrial production of short-chain fructo-oligosaccharides and inulo-oligosaccharides is expanding rapidly due to the pharmaceutical importance of these compounds (Guimaraes et al., 2007; Maria Rosa Vela Sebastiao Fernandes and Bo Jiang, 2013). Inulinases are a group of hydrolases which target on the β-2,1 linkage of inulin and hydrolyze it into fructose and glucose. Inulinases can be divided into endo-inulinase and exo-inulinase. The endo-inulinase hydrolyzes the internal linkages in inulin to produce inulotriose, inulotetraose, and inulopentaose as the main products. The exo-inulinase hydrolyzes inulin into fructose and glucose, then, the formed fructose and glucose can be further fermented into ethanol by specific microorganisms (Li et al., 2013). Inulinases can be secreted by a variety of microbes including fungi, yeasts, and bacteria (Gavrailov and Ivanova, 2014; Ram Sarup Singh and Kanika Chauhan, 2016; Yun et al., 1997; Nirobol et al., 2012). Among them, *Aspergillus* and *Kluyveromyces* strains are generally preferred choices for commercial applications (Zhang et al., 2004). Recently, many studies have been conducted using inulinase from *Aspergillus* for enzymatic hydrolysis of inulin (Gill et al., 2006; Sirisansaneeyakul et al., 2006). Some efforts have been made to enhance enzyme activity of *Aspergillus* such as transgene expression (Zhang et al., 2004) and coculture with other species (Ge et al., 2009).

Artificial mutations are mutations that are experimentally induced using a wide range of mutagens. Mutagens are classified by nature in three categories: physical, chemical, and biological. Chemical or radiation-based mutagenesis (eg UV and gamma) are approaches that have the potential to generate gain of function mutations. In our study we have been working on obtaining mutants by ionizing radiation on the strain of *Aspergillus terreus*. Gamma radiation was use in order to increase inulinase productivity.

MATERIALS AND METHODS

The *Aspergillus terreus* microorganism is part of our Culture Collection of Microorganism of Industrial Importance, a newly isolated microorganism. The ionized radiation treatment was achieved using 3 different doses, 500, 1000 and 1500 Gy, conducted with the support of a team from IFIN-HH. The reagents (organic

solvents, analytical reagents and mineral salts) used for research were purchased from Merck and Sigma-Aldrich.

Obtaining mutants to increase bioproductivity of inulinases

After ionizing radiation treatment of the *Aspergillus terreus* strain ICCF 262, the fungus in liquid suspension was seeded on agarized media with 1% inulin content (1% inulin, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% agar). Isolated colonies were selected and passed to the inoculum phase and then to the bioprocess to observe the differences in enzymatic activity of the inulinases.

Culture media and cultivation conditions

Preinoculum phase. The 17 mutated *Aspergillus terreus* strains that were tested, were cultivated on solid agar medium, PDA (potato dextrose agar). They were incubated in optimal development conditions: 24-48 h / 28 - 29°C.

Inoculum phase. The preinoculum tubes were washed with 2 ml sterile inoculum medium and inoculated into 500 ml Erlenmayer flasks containing 100 ml of liquid medium.

The bioprocessing phase was performed according to the classical scheme, in optimal temperature conditions (28-29ºC), initial pH 6.5 and biosynthesis time (3 or up to 7 days), for the development of tested microorganisms, but also for the induction of inulinase production. The inoculum volume was 2% using 500 ml Erlenmayer vials with 100 ml of liquid medium (shaking 220 rpm). The main sources of carbon in the media were the different sources of inulin (laboratory grade inulin and orange peel (agro-food waste)) along with the other components indispensable to the development of microbial life: nitrogen sources (corn extract, peptone, ammonium salts, yeast extract) and secondary nutrients (potassium phosphate, magnesium sulphate, sodium chloride, citric acid).

The *Aspergillus terreus* strain ICCF 262 is grown on inoculum medium with the following composition: 1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, for 24 h and then grown for accumulation of inulinase, on biosynthesis medium with the following composition: 2% yeast extract, 0.3% NH_4NO_3, 0.4% $(NH_4)_2HPO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$. The fermentation conditions were 28-29°C, initial pH 6.5, agitation on a rotary shaker with 220 rpm and 2 cm agitator eccentricity for 7 days.

Isolation of microbial inulinase

After fermentation, the supernatant was separated by centrifugation at 8000 rpm, 4°C for 20 minutes, in a centrifuge (Hettich - Germany).

Measurement of enzyme activities

The inulinase activity was assessed as follows: 0.1ml of crude enzyme solution was mixed 0.9 ml of 2% inulin in acetic buffer 0.1M at pH 5.5. The sample was incubated at 50°C for 15 min. The reducing sugars were determined by the dinitrosalicylic acid (DNSA) method (Miller, 1959). One activity unit is defined as the amount of enzyme required to produce one micromole of reducing sugar per minute under assay conditions.

RESULTS AND DISCUSSIONS

To optimize the yield of inulinase production, we monitored both the evolution of cell growth by determining optical density, dry biomass, final pH value, and the enzymatic activity of extracellular inulinases.

Following the treatment with ionizing radiation, 17 mutant strains were tested to monitor the increase in inulinase bioproductivity (Figure 1, Figure 2 and Figure 3).

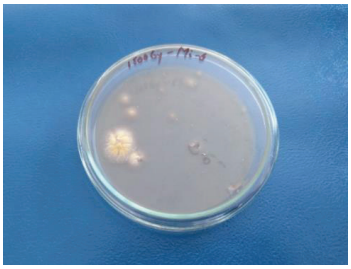

Figure 1. Mutants of *Aspergillus terreus* strain after gamma radiation treatment - 1500 Gy

It can be seen from Table 1 that five strains (6, 7, 8, 10 and 17) had the highest activity; these 5 strains were selected for bioproductivity testing in case of agro waste as C source (orange peel). The mutant strains G1, G2 and G5 (see Table 2) had the highest enzymatic activity.

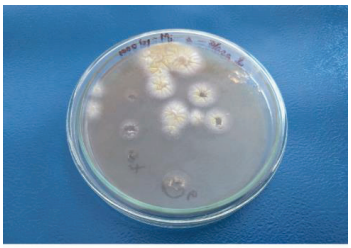

Figure 2. Mutants of *Aspergillus terreus* strain after gamma radiation treatment - 1000 Gy

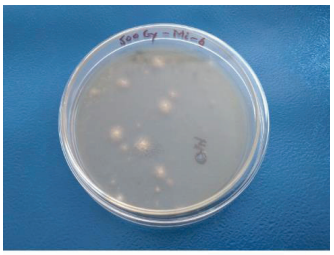

Figure 3. Mutants of *Aspergillus terreus* strain after gamma radiation treatment - 500 Gy

Table 1. Study to determine the bioproductivity of inulinases with pure inulin as C source (values after 72 h of bioproces)

Strain n (isolated mutants)	Final volume (mL)	pH	Enzymatic activity (U/ml)
1	45	3.51	0.21
2	47	3.36	0.19
3	47	4.47	0.22
4	36	3.92	0.19
5	56	2.92	0.06
6	**48**	**3.84**	**0.48**
7	**55**	**2.69**	**0.58**
8	**50**	**3.13**	**0.59**
9	42	3.24	0.24
10	**36**	**4.67**	**0.65**
11	38	3.32	0.05
12	47	3.23	0.19
13	43	3.39	0.21
14	37	4.0	0.25
15	54	3.50	0.23
16	36	3.25	0.14
17	**38**	**4.79**	**0.39**

Table 2. Study to determine the inulinases bioproductivity of the mutant strains with agro-food waste (orange peel) as C-source (values after 72 h of bioprocess)

Strain n°	pH	Enzymatic activity (U/ml)
G1	**2.8**	**0.64**
G2	**2.68**	**0.77**
G3	2.03	0.13
G4	1.93	0.49
G5	**2.39**	**0.55**

CONCLUSIONS

Research in the production and application of FOS and IOS is gaining momentum due to several health benefits and biofunctional properties of these compounds. Prebiotics are produced by crops such as chicory and Jerusalem artichoke. However, FOS can be synthesised *in vitro* from precursors such as sucrose using fructosyltransferase enzymes. Furthermore, IOS can also be produced from the enzymatic hydrolysis of inulin under controlled conditions. The main drawback of the production process is the low yields of FOS. It is therefore crucial to explore other methods such as molecular methods to improve the efficiency of the enzymes involved in the synthesis of FOS and IOS. More research on the efficacy and mode of action of prebiotics is critical to harness maximum benefits from the preparation and consumption of these oligosaccharides.

Aspergillus terreus strain was chosen to be the most important in terms of bioproductivity of inulinases on different nutrient substrates.
The optimization of the biosynthesis conditions of selected strains following screening of enzymatic activities was carried out by diversifying the carbon source using inulin and agro-alimentary by-products (orange peel). The highest inulinase activity was achieved with the mutant strain G2 (0.77 U/mL), grown on orange peel as a inulin source.
These values are comparable to the values reported in the literature for the *Aspergillus niger* strain (Rawat et al., 2015; Fawzi 2011; Cruz et al.,1998, Singh and Chauhan, 2016).
It is noted that the bioproductivity of the strains is improved with the use of substrate from agro-food waste.

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AN OVERVIEW ON MICROORGANISMS DERIVED BIO-MATERIALS

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Abstract

Petrochemical-based packaging materials represent a billion dollar industry that makes possible modern innovations in sustainable packaging design, but with severe footprint on the environment. Currently, polystyrene products take over 30% of worldwide landfill space which represents a real issue as sustainable disposal methods must be pursued, given the fact that polystyrene products are incredibly hard to recycle and biodegrade (approximately 500 years for styrofoam to organically degrade). The paper presents main biotechnological advances regarding mycelium based bio-materials used as sources of renewable protective packaging materials, structural bio-composites, thermal insulation materials, packaging materials, decorative objects etc. Biotechnological processes successfully explore the great potential of Basidiomycetes strains (E.g.: Pleurotus djamor, Pleurotus eryngii, Pleurotus ostreatus,Grifola frondosa, Ganoderma lucidum, Ganoderma oregonense, Lentinula edodes, Agrocybe aegerita, Coprinus comatus etc.) in obtaining mycelium-based novel bio-materials.

Key words*: fungi, basidiomycetes, composite materials, agricultural biomass.*

INTRODUCTION

Petrochemical-based packaging materials represent a billion dollar industry that makes possible modern innovations in sustainable packaging design. Even plastic packaging has obvious advantages, such as price/kg, manufacturing speed, physical-mechanical properties etc., their production involve high consumption of fossil fuels, energy and water, multiple complex manufacturing stages, each of them with severe footprint on the environment (Kremer, 2003). Disposal of such materials into the environment contributes to an array of environmental problems, affecting humans' health, soil and marine habitats degradation, ground water contamination, long-term pollution (Maachia et al., 2005). Currently, polystyrene products take over 30% of worldwide landfill space which represents a real issue as sustainable disposal methods must be pursued, given the fact that polystyrene products are incredibly hard to recycle and biodegrade (approximately 500 years for Styrofoam to organically degrade). Current researches focus on obtaining alternative packaging materials by development of environmentally safe and cost-efficient process for preparation of mycological materials for packaging as green alternative to highly polluting plastics. The production of 1 m^3 of polystyrene consumes 4,667 mega Joules (MJ) of energy and release into the atmosphere 462 kg of CO_2 while the production of 1m^3 of mycelium derived bio-material uses 652 MJ and releases 31 kg of CO_2.

Regarding the novelty of this research field, both scientific and technological future objectives are to be achieved with the sustained effort of multidisciplinary industries, which may include both SMEs and research organizations, representing the whole value chain, from producers and suppliers of agricultural and textile wastes, biotechnology processors to packaging producers.

CONTEXT

Robust, low-cost, non-toxic manufacturing processes represent major factors driving future markets and demand for bio-based materials in the European Union. According to EPA (United States Environmental Protection Agency) data provided by Ecovative, the pioneer of mycelium-based bio-materials, plastics - especially plastics like EPS (expanded

polystyrene) - take up 25 percent of US landfills by volume. The environmentally safe, fire resistant, VOC free, and 100% sustainable and compostable mycelium materials have a wide range of applicability: the packaging industry will be more competitive by replacing petroleum-derived polymers with sustainable and fully biodegradable/compostable materials.
Innovative biotechnological processing for transforming and improving exploitation of waste and by-products from agriculture, fruit and vegetable processing industry, plastic and textile industries, will reduce the environmental impact of wastes, will enhance the sustainable management of organic wastes as they can be used as feedstock for value-added bio-based materials. Sustainable packaging development addresses economic, environmental and social objectives, which are dynamically interconnected.
Bio-materials obtained by growth of fungi on industrial waste have generated tremendous interest worldwide due to the obvious benefits: the transformation of huge volumes of waste with low consumption of energy, water and chemicals, into valuable products with applications in the most diverse fields: packaging materials replacing polystyrene plastics (Heredia-Guerrero et al., 2016), constructions, architectural design, interior decorations, garments etc., economic, social and environmental benefits. However, the technology is still incipient due to the low reproducibility of the materials created, the final properties strongly depending on the variable composition of the waste, the type of strain used, the growth and post-treatment conditions, the design and the geometric shape of the enclosure which occurs in mycelium growth.
The market for plastic packaging (polystyrene, polyethylene terephthalate, polyethylene, polypropylene, polyvinyl chloride, polyamide, polyurethane and lactic acid) was estimated at $189.43 billion in 2015 and is expected to reach $262.68 billion 2021, at an RCAC (annual composite growth rate) of 5.71%. The annual consumption of rigid packaging is 30€ billion in the EU, while flexible packaging amounts to about 10€ billion. About 63% of all EU consumer goods are marketed in plastic packaging. The global market for biodegradable paper packaging materials are estimated to grow to an RCAC of nearly 11% between 2017 and 2021, bioplastic packaging materials dominating the global market, accounting for 54% of the market share in 2016 (Technavio report).

MICROBIAL STRAINS AND PROCESSES

Filamentous fungi are the most effective and competitive microorganisms used to produce mycelium-based materials due to their physiological, enzymatic and biochemical properties. They possess versatile and high secretory capabilities, good tolerance to high osmotic pressure conditions, exponential growth of biomass, large amounts of hydrolytic enzymes excreted at the top of their hyphae, allowing the penetration and degradation of nearly all solid substrates. The use of filamentous fungi properties to develop mycelia networks lead to the creation of safe and inert, light, strong and durable, fire - resistant and insulating materials (insulation value of R-3 or R-4) (Figure 1) that can successfully replace materials made of plastic (polystyrene) (Vilela et al., 2014).

(Source: www.smithsonianmag.com)

(Source: www.equipmentworld.com)
Figure 1. Mycelium based insulation materials

They can be used for a wide range of applications, including insulation, packing materials, building blocks, acoustic boards,

sandwich panels (Figure 2), decorative products (Figure 3) (Johansson et al., 2012).

Figure 2. Mycelium based
(Source: www.architectmagazine.com)

Figure 3. Mycelium based decorative products
(Source: www.wired.com)

Bio-composites based on fungal mycelium are considered to be one of the future generations of renewable materials due to their obvious advantages: rapid growth (approximately 10-14 days) on almost all types of waste transformed into valuable products, organic composition (cellulose, chitin, fats, carbohydrates, various nitrogen species, vitamins and minerals), low costs and broad availability (Vega et al., 2012). More than that, the production of packaging material from mycelia and waste uses 12% of the energy normally required in the manufacture of traditional plastic packaging and reduce carbon emissions by up to 90%. However, the technology is on baby footsteps due to the low reproducibility of the materials created by using biological organisms especially on wastes having variable composition. Also, the most challenging problem remains the final properties of the mycelium materials which strongly depends on the type of fungi and wastes used, the growing and post-treatment conditions, the design and geometrical shape of enclosure (Figure 4): the material could be frangible and brittle when is too much desiccated (Allan, 2017), fracturing occurring at the boundaries between areas of mycelium growth and undigested wastes, and chemical composition and final morphologies are highly different from substrate to substrate (Mazur, 2017; Travaglini et al., 2017).

Figure 4. Geometrical formulations of mycelium based structures
(Source: www.builderonline.com)

Fungal mycelium is mainly composed of natural polymers (chitin, cellulose, proteins, etc.) (Figure 5) which due to its unique structure and composition, correlated with enzymatic activity able to hydrolyze a wide variety of organic substrates, can be exploited with success in producing bio-materials (Vega et al., 2012).

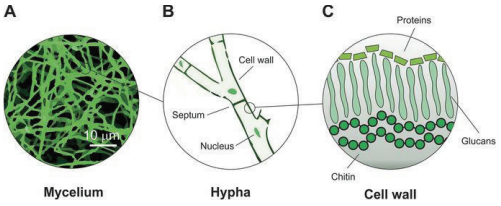

Figure 5. Representation of mycelium physiology
(Source: Haneef et al, 2017)

Patents US 2011/0306107 A1 and US8283153 B2 describe the methods to prepare mycelium from *Pleurotus djamor, Pleurotus eryngii, Pleurotus ostreatus, Pleurotus ostreatus var. columbines, Grifola frondosa, Ganoderma lucidum, Ganoderma oregonense, Lentinula edodes, Agrocybe aegerita or Coprinus*

comatus. Even though the mycelium prepared according to above mentioned show fast growth ability, the provided methods are suitable mainly for the development of biodegradable packaging from plastics and plastic foams. Muhammad Haneef described the use of white rot fungi (*Ganoderma lucidum* and *Pleurotus ostreatus*) for production of mycelium-based fibrous films, but pure amorphous cellulose and a mixture of cellulose and PDB are used as nutritive substrates to obtain a high growth of the mycelium materials and acceptable mechanical properties of the bio-films (Haneef et al., 2017). Patent US 2011/8001719 B2 provides the method of growing Basidiomycetes and Ascomycetes fungal fruiting bodies into a low density, chitinous material (Vega et al., 2012) that can replace balsa, bass, other woods, and also foam based plastics. Specific conditions and multiple stages are required to activate primordia maturation and induce fruiting of fungal primordium in the organism type. Patent US no. 5854056 discloses a process for the production of „phylum fungal pulp" but the method is optimized for *Neurospora crassa* as the filamentous fungus that can be used in the production of paper products and textiles. Patent US 2015/0342138 provides a method for producing dehydrated mycelium which can be re-hydrated and rapidly re-formed into many different shapes, such as bricks, blocks, pellets and the like elements wherein the adhesion of the elements is achieved through re-animation of a fungal organism which grows the elements together.

However, none of the patents describes the production in a cost-effective manner of an achievable fungal consortium able to degraded wastes with variable composition, so more versatile and higher yielding fungal strains are needed to up-scale the process. Also, the know-how about specific mechanism is very limited and precludes further optimization options including directed evolution approaches for selective fine tuning of processes.

MAIN CHALENGES

Despite all efforts, the technologies for making mycelium based materials are still in incipient phases due to low reproducibility of the materials created by the use of biological organisms on waste with variable composition (Papagianni, 2004). Also, the most difficult problems remain the final properties of materials obtained by fungal mycelium, which strongly depend on the type of used fungi and waste, growth conditions, post-treatment processes, the design and geometric shape of the final enclosures where microbial growth takes places (Figure 6), which can cause several mechanical changes in the final products: chemical composition and final morphologies differ greatly with strain inoculation on various substrates.

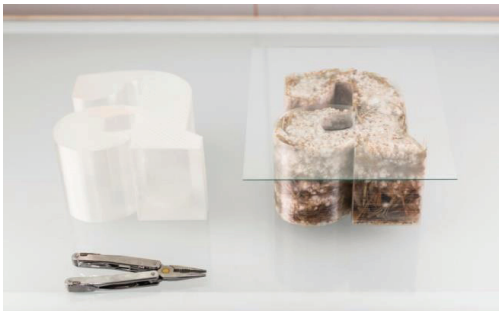

Figure 6. 3D-printed mold for mushrooms growing (Source: www.make.land)

To improve the process, complex experimental determinations are needed, more versatile and with higher yields. Also, the know-how about the specific mechanism is very limited and hinders additional optimization options, including targeted approaches for regulating the fine selectivity of fungal mycelium growth on various organic wastes.

Patents US 2008/0145577 A1, US 2012/0315687 A1 and US 2016/0002589 A1, describe the use of agricultural waste as nutritive substrate (cotton seed hulls, coconut coir), minerals (horticultural perlite, vermiculite, diatomaceous earth), industrial waste (sawdust, waste cellulose pulp from paper mill or recycled paper, foam based products and polymers) for obtaining of mycelium-based materials. However, until now, the most challenging, not yet solved problem of waste substrate is the high heterogeneity of the mixture components with different structures which makes difficult the uniform growth of the mycelium mass, and leads to varying quality of obtained packaging

materials. Another challenge is the bio-contamination with unwanted fungi and/or bacteria which could hamper the further bio-processing.

APPLICATION FIELDS AND KEY PLAYERS

The key players for bio-plastic packaging materials include Amcor Ltd., Crown Holdings Inc., Bemis Company Inc., Basf SE, Huhtamaki Oyj, Mondi, Sealed Air Corp., Sonoco Products, Saint-Gobain etc. The global production capacity of bio-plastics will increase to about 6.1 million tons in 2021, with packaging accounting for nearly 40% (1.6 million tons) of the total bio-plastics market. Trends show an increase in demand for bio-plastics in the automotive and transport sector (14%, 0.6 million tone) and the construction sector (13%, 0.5 million tons). Even if the benefits of mycelium-based materials are obvious, such as 25% reduction of waste landfills, biodegradability, there are very few companies producing such bio-composites. Ecovative Design, USA, is the leading bio-composites company engaged in research and development of ecological packaging materials (EcoCradle) (Penelope, 2012) by transforming and casting agricultural waste into the required packaging forms using mycelium. Mycological materials are used to package products made by various companies such as Ikea, Dell, Surf Organic Boards and Danielle Trofe Design to develop sustainable surfboards, lampshades and plant pots (Figure 7).

Figure 7. Ecovative Design's proprietary EcoCradle® mycelium-based materials
(Source: www.ecovative.com)

Architectural elements (garden decorations, floral foam materials), furniture and other building materials are developed by architect David Benjamin (New York, USA). Various research institutes and universities are involved in the study of mycelium-based materials: Karlsruhe Institute of Technology, ETH Zurich, University of Leeds, UK, Het Nieuwe Instituut, Netherlands, in collaboration with Vitra Design Museum, makes architectural elements. TuDelft University, Netherlands, is involved in a project aimed at the development of mycelium composite materials with different physical properties ranging from elastic to rigid, hydrophilic/hydrophobic and porous/ compact.

The markets for bio-materials include many applicative industrial sectors such as:

- Constructions sector: insulating panels, sandwich panels, bricks, acoustic tiles (Johansson et al., 2012), office furniture, furniture packaging;
- Electrical and electronic applications: packaging materials for LCD flat screens, electronic devices, telephones, IT & C components;
- Packing materials for sports, leisure and design, consumer goods;
- Outdoor and garden products: decorative materials, biodegradable flower pots (Figure 8);
- Automotive and transport sector: panels and door bars, lightweight automotive panels, machine parts packaging.

Figure 8. Mycelium based flower pots
(Source: www.danielletrofe.com)

CONCLUSIONS

This novel field proposes obtaining of bio-material that harvest the great potential of higher fungi which pose high bio-efficiency and are able to break down a wide variety of nutritive substrates, rapid hyphae grow (netting) and versatility towards cultivation

parameters changes on a wide range of substrates. The potential for waste to be converted into biodegradable packaging will dominate research efforts in recent years due to their high availability, low environmental footprint, a wide range of achievable products (insulation, building blocks, acoustic boards, sandwich panels, decorative products, architectural design, decorations interior, packaging materials etc.) (Johansson et al., 2012).

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TOMATO BY-PRODUCTS AS A SOURCE OF NATURAL ANTIOXIDANTS FOR PHARMACEUTICAL AND FOOD INDUSTRIES – A MINI-REVIEW

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Abstract

Antioxidants are substances that are able to prevent or inhibit oxidation processes in human body as well as in food products. The entire tissue of fruits and vegetables are rich in bioactive compounds and in most cases the waste by-products can present similar or even higher contents of antioxidant compounds. The tomato processing industry generates large quantities of tomato peel residues, usually creating environmental problems. The tomato by-products mainly constituted by tomato skins and seeds represent one of the richest sources of lycopene, a carotenoid with a noncyclic, not branched structure which has demonstrated antioxidant properties and an important role in the prevention of chronic diseases. Tomatoes skin can, in fact, contain up to 5 times more lycopene than the pulp. So, although these by-products of tomato industry represent a major disposal problem for the food industry, they are also a promising source of compounds which may be used in the food, pharmaceutical and cosmetic industries because of their antioxidant or nutritional properties.

Key words*: lycopene, peel tomatoes, properties, applications, antioxidant.*

INTRODUCTION

Lycopene is an important carotenoid in tomatoes, responsible for the red color of tomatoes. Tomatoes and tomato products are the major sources of lycopene compounds which can represent more than 85% of all the carotenoids present in the fruits (Abreu W. et al., 2011). Lycopene is an important biological compound and has received great interest in the past decade because of its important role in preventing chronic diseases, such as atherosclerosis, skin cancer and prostate cancer (Cruz B.R.M. et al., 2013). It is an antioxidant that displays higher efficiency than vitamin E and other kinds of carotenoids. Tomato skins can be a viable source of lycopene, as the per unit mass of tomato skins contain about five times more lycopene than the whole tomato pulp (Muhammad W. et al., 2017). George et al. (2004) studied 12 genotypes of tomatoes, and found that the free polyphenolic content (expressed as mg catechin/100 g fresh weight) in pulps ranged from 9.2 to 27.0 mg/100 g, compared to 10.4 to 40.0 mg/100 g in skin, and also that for each genotype, the polyphenolic content in skin was higher than in pulp. A similar observation has been made by Toor and Savage (2005), who reported that the total polyphenolic content (expressed as mg gallic acid equivalents/100 g) of skin and seeds of tomatoes were, respectively, 29.1 and 22.0, compared to 12.7 mg/100 g in the pulp. However, when tomatoes are processed into products like ketchup, sauces or juice, 3-7% of their weight become waste. Tomato waste, since it contains a significant amount of skin and seeds, is a potential source of natural antioxidants (Savatovic M.S. et al., 2010). Considering that more than one third of the tomatoes delivered to processing plants end as processing wastes, mainly constituted by seeds and skins, the recovery of this carotenoid could represent an alternative for the valorization of the by-products of the tomato industry. Commercial processing of tomato produces a large amount of waste at various stages and constitutes the major part of the waste that comes from the pulper. Because tomato skins were found to give the highest yield of lycopene, it has been reported that tomato processing residues, including skins, are ideal for extracting large amounts of lycopene (Sandei L. et al., 2006). Industrial production of

lycopene from tomatoes appears to be in high demand by food companies for the development of functional foods (Kaur D. et al., 2004).

Discarded tomato skins from the production of tomato juice have been found to be the best source for lycopene extraction. Enrichment of tomato paste with tomato peel is an interesting option for increasing lycopene and β-carotene intakes (Mortensen A., 2006). The lycopene content in tomatoes typically ranges from 70 to 130 mg/kg and depends on the variety, geographic location, cultivation technique, climatic conditions and ripeness of the tomato.

The lycopene content increases as the fruit ripens. Tomato sauce and ketchup contain lycopene at concentrations of 33 to 68 mg per 100 g, while raw tomatoes contain lycopene at concentrations of 3.1 mg per 100 g.

The lycopene concentration in the non-blanched tomato peels was 62.92 mg/100 g, whereas it was 134 mg/100 g in the blanched tomato peels (Rao A.V. et al., 2004). It is one of the pigments widely used by the food industry as a food additive due to its strong colour and non-toxicity. It has many health benefits and is in increasing demand as a red colorant and antioxidant agent (Krinsky et al., 2005; Chauhan K. et al., 2011).

LYCOPENE CHEMISTRY

Structurally, it is a tetraterpene assembled from eight isoprene units, composed entirely of carbon and hydrogen. Lycopene, a C40 polyisoprenoid compound containing 13 double bonds, is the most abundant carotenoid, accounting for approximately 80–90% of the total pigment contents in ripe tomatoes. With its 11 conjugated and two non-conjugated double bonds, it was found to be a more efficient antioxidant (singlet oxygen quencher) than β-carotene, α-carotene, and α-tocopherol (Chun Yi et al., 2009; Liana M.A et al., 2009). The acyclic structure of lycopene makes it more soluble in organic solvents, such as chloroform, hexane, benzene, methylene chloride, acetone and petroleum ether (Shi J., 2000). Structure and physical properties of lycopene are shown in Figure 1 and Table 1 (Pratik M.K. et al., 2007; Raey M.A. et al., 2013).

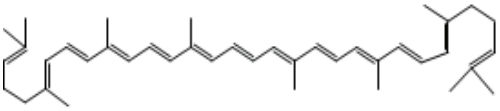

Figure 1: Structure of lycopene

Table 1: Physical properties of lycopene

Molecular formula	$C_{40}H_{56}$
Molecular weight	536.85 Da
Melting point	172-175^{0}C
Crystal form	Long red needles separate from a mixture of carbon disulfide and ethanol.
Powder form	Dark reddish brown.
Solubility	Soluble in chloroform, hexane, benzene, carbon disulfide, acetone, petroleum ether and oil; Insoluble in water, ethanol and methanol.
Stability	Sensitive to light, oxygen, high temperature, acids, catalysts and metal ions.

The main concerns in using lycopene extracted from tomatoes and waste tomatoes are solubility and stability. Carotenoids are susceptible to degradation due to high temperatures, oxidation and UV light, which further limit their application in the food industry.

EXTRACTION METHODS

Nowadays, there is an increasing trend towards utilization of food processing by-products as a source of functional components. Many studies have been carried out on the extraction of lycopene from by-products, especially tomato waste.

Several different methods have been used to extract lycopene, such as supercritical fluid extraction (SCFE) with CO_2 and solvent extraction (Rozzi N.L., 2002; Vagi E. et al., 2007). Solvents for extracting carotenoids, include ethyl acetate (100%) or different mixtures of solvents such as ethanol/hexane (1:1), acetone/ethanol/hexane (1:1:2), ethyl acetate/hexane (1:1) or acetone/hexane (1:1), ethyl acetate and ethyl lactate being non-toxic solvents. Recent studies describe a lycopene extraction process based on supercritical CO_2, which avoids using harmful solvents. Over 60% of the lycopene in tomato waste was extracted

with this process. This type of extraction is extremely efficient with non-polar carotenoids (lycopene) with a total of carotenoids recovery of 96% (Topal U. et al., 2006; Akbari et al., 2014). Successful extraction technology for lycopene recovery from tomato pomace (seeds and skin) can significantly improve the economic aspects of tomato industry besides making available one of the most potent antioxidants for formulating health supplements for human beings (Naviglio D. et al., 2008).

ANALYTICAL METHODS

Various analytical methods have been developed to measure and analysis of lycopene. These include ultraviolet-visible (UV-VIS) spectrophotometry, liquid chromatography (LC), thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). UV-VIS spectrophotometry is more convenient, faster and less expensive than HPLC analysis and large numbers of samples can be processed in a relatively short time. However, UV-VIS spectrophotometry cannot detect very small quantities of lycopene (less than 1 μg), whereas HPLC can detect quantities as small as 1 ng. Although HPLC analysis allows accurate quantification of pigments and separation of isomers, it is laborious and a high level of skill is required to produce consistent results (Knoblich M. et al., 2005).

THE MAJOR HEALTH BENEFITS OF LYCOPENE INCLUDE:

• Antioxidant activity

Lycopene is one of the most potent antioxidants. Its singlet-oxygen-quenching ability is twice that of β-carotene and ten times higher than that of α-tocopherol. As an antioxidant, it traps reactive oxygen species, increasing the overall antioxidant potential and reducing the oxidative damage to lipids (lipoproteins, membrane lipids), proteins (important enzymes) and DNA (genetic material), thereby lowering oxidative stress. This reduced oxidative stress leads to reduced risk of cancer and cardiovascular heart disease. As lycopene levels in the blood increase, the levels of oxidized lipoproteins, proteins and DNA compounds decrease (Rao A.V. et al., 2002; Erdman J.W. et al., 2009).

• Reducing prostate cancer

Studies show that taking high doses of lycopene can slow the progression of prostate cancer. The estimated intake of lycopene from various tomato products was inversely proportional to the risk of prostate cancer, a result not observed for any other carotenoid. Consuming ten or more servings of tomato products per week reduced the risk by almost 35%. The protective effects were highest for more advanced or aggressive prostate cancer (Basu A. et al., 2007; Zhang J. et al., 2007).

• Inhibiting cancer cells

Lycopene has a protective effect against stomach, colon, lung and skin cancers. Free radicals in the body can damage DNA and proteins in the cells and tissues, resulting in inflammation which may lead to cancer. Hence, the antioxidant properties of lycopene in eliminating free radicals may reduce the risk of cancer (Giovannucci E. et al., 2002). Research in breast, lung and endometrial cancers has shown that lycopene is even more effective than the other bright vegetable carotenoids α- and β-carotene in delaying the cell cycle progression from one growth phase to the next, thus inhibiting growth of tumor cells. Lycopene also plays a role in modulating intercellular communication by regulating irregular pathways that may be associated with cancer. Multiple studies have investigated whether intake of tomatoes or tomato-based products helps prevent digestive tract cancers, including oral, pharyngeal, oesophageal, gastric, colon, and rectal cancer. People with a higher intake of lycopene have been shown to have a reduced risk of developing cervical and breast cancer (Keleman L.E. et al., 2006; Rao A.V., 2006).

• Reducing blindness

Age-related macular degeneration (ARMD) is the most common form of blindness in elderly people in the Western world. Lycopene is the only micro-nutrient whose serum level is shown to be inversely related to the risk of ARMD. Lycopene also helps reduce the incidence of cancers and cardiovascular diseases which play a role in eye health (Mendelova A. et al., 2013).

• Reducing atherosclerosis and heart disease

Lycopene may be helpful in people with high cholesterol, atherosclerosis or coronary heart

disease, possibly due to its antioxidant properties. Lycopene prevents oxidation of low density lipoprotein (LDL) cholesterol and reduces the risk of arteries becoming thickened and blocked (Rao A.V. et al., 2000). Most published studies in this area used tomato juice as a treatment (Kritchevsky S.B., 1999; Arab L. et al., 2000; Ito Y. et al., 2006).

• Reducing osteoporosis

Epidemiological data indicates, lycopene prevents osteoporosis in post-menopausal women. This is a new and exciting finding and could stimulate serious dietary considerations for all people seeking to protect against this disease (Rao L.G. et al., 2007).

• Preventing skin damage

Lycopene can reduce inflammation and help to protect the skin from damage resulting from UV sun exposure. It is a common ingredient in anti-aging creams and lotions but because it degrades easily, containers must be properly sealed between uses (Stahl W. et al., 2006).

FUNCTIONAL USES OF LYCOPENE IN FOOD

Because of lycopene benefits, there is a growing interest in using lycopene as a value-added or functional ingredient in food products. Lycopene extract from peel tomatoes can be used as a nutritional supplement in several food categories such as baked goods, breakfast cereals, dairy products including frozen dairy desserts, dairy product analogues, spreads, bottled water, carbonated beverages, fruit and vegetable juices, soybean beverages, candy, soups, salad dressings, and other foods and beverages. Lycopene is a natural food colouring, thus eliminating the adverse effects of artificial food colorants. It provides colour shades ranging from yellow to red.

There are initiatives by food scientists to recycle lycopene-rich by-products as food ingredients. Fortifying dry fermented sausage with lycopene can be achieved by adding dried tomato peel to the meat mixture during sausage production. Extrusion processing allows barley-tomato pomace blends to be formulated into snacks.

Research shows that heat processing converts lycopene in tomatoes to a form the body can absorb more easily. One study showed that lycopene is absorbed 2.5 times better from tomato paste than from fresh tomatoes (D'Evoli et al., 2013).

CONCLUSIONS

By-products derived from food processing are considered highly advantageous for their development as nutraceuticals and food ingredients, additives, functional fruits, and are also used in the pharmaceutical industry due to the various bioactive components and colour pigments present in them.

Tomato peels, usually eliminated during tomato processing as by-product, are a valuable source of carotenoids. Wastes from processing tomato products contain the carotenoid rich skins and the seeds, and are available in large quantities. Extracting lycopene from tomato processing wastes is economical and these by-products could become a cheap source or lycopene, carotenoids and/or natural oils for formulating new nutraceuticals, pharmaceuticals, and cosmetic products. Lycopene is particularly important because it has a dual influence on production and quality as a natural color and nutrient for the food and pharmaceutical industries. Color also serves as a measure of total quality for tomato and tomato products. Lycopene can play an important role in human health and provide protection against a broad range of epithelial cancers.

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RESEARCHES CONCERNING THE LEVEL OF FERMENTABLE SUGARS FROM FEED MATERIALS IN RELATION WITH CELLULASE HYDROLYSIS BY CARBOHYDRASE ENZYME

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Abstract

The objective of this experimental study was to evaluate the enzymatic hydrolysis process in order to obtain fermentable sugars from feed materials (maize, field peas, field beans), usually used in animal nutrition. For breaking down the carbohydrates from cell materials raw, it was used a carbohydrase enzyme preparation (IUB No. 3.2.1.6) with activity of 50 FBG/g. The enzyme was tested at concentration of 0.05-0.5% (w/v). For measuring accumulation of reducing sugars, it was used 3,5 dinitrosalicylic acid (DNS) assay. Results from experimental samples supplemented with carbohydrase enzyme were compared with control sample without enzyme addition. The activity of carbohydrase enzyme was evaluated using a carboxymethyl cellulose medium (4.19 UDNS/ml). For the experimental data, the total amount of available sugars was significantly higher (P<0.0001) for peas (19.60±0.105), followed by maize (17.17±0.105) and beans field (10.50±0.055) at a concentration of 0.5% enzyme. The results suggest that hydrolytic potential of enzyme preparation to produce fermentable sugars was superior on field peas, knowed as source of protein in animal nutrition and can be used as feed additive to improve nutrient availability.

Key words: *enzymatic hydrolysis, carbohydrase enzyme, reducing sugars.*

INTRODUCTION

Lignocellulose is the major component of biomass, involves by three polymers: cellulose (35-50%), hemicelluloses (20-35%) and lignin (10-25%), for typical feedstuffs which are arranged in a complex way (Dumitru and Jurcoane, 2017; Ganna, 2012).

Lignocellulose is a high source of cheap car-bohydrates. Over the past decades, it was used as raw material for the production of a range of high value products, such as bioethanol, organic acids, enzymes and biodegradable plastics (Ravindran and Jaiswal, 2016).

The cellulose (fiber) is a major component of plant cell wall, composed of glucose molecules linked together by β-1,4 glycosidic bonds, which the pig's digestive tract is unable to unlink them and hence is degraded by microbial fermentation in the hindgut (Diguță et al., 2007, Banino, 2012).

The cellulose has been identified as the principal polysaccharide in plants with a rigid structure very difficult to decompose (Oyetunji, 2009). The basic unit of cellulose is glucose. It is the most common organic group in nature, and is one of the main energy sources for plants and animals. Cellulose's hydrolysis determines her degradation of cellulose to glucose or other simple sugars, in order to use for the production of energy, in our study, in animal nutrition (Oyetunji, 2009).

Dietary carbohydrates constitute a major fraction feed, which can be divided according to glycosidic linkages into sugars (mono and disaccharides), oligosaccharides and two broad classes of polysaccharides; starch and non-starch polysaccharides (NSP) Therefore, nutrition's swine are made up primarily of grains, along with protein supplements and other vitamins and minerals. Cereal grains make up 50% to 85% of the ingredients, which in turn provide much of the energy to the

animal (Velayudhan et al., 2015). Maize grain is among the leading cereal used in swine nutrition with a greater energy density than other cereal grains. For example, small grains, such as barley, wheat, oats, rye, and triticale form other practical ingredients in swine feeding programs.

Measurement the concentration of reducing sugars (RSs) from raw materials, can provide valuable information about the analyzed sample, for understanding the amount of sugar from feedstuffs and the activities of some enzymes which are responsible for the hydrolysis of polysaccharides (Negrulescu et al., 2012).

In this study, three types of raw materials (maize - *Zea mays* L., field peas - *Pisum sativum* L. and field beans - *Vicia faba*), used in animal nutrition, were supposed to enzymatic hydrolysis process. Investigating the efficiency of glycoside bonds, with a positive effect over polysaccharides, it was determined the level of fermentable sugars yield from different source of feed materials.

MATERIALS AND METHODS

Substrate

The raw materials (maize, field peas and field beans) used in this study, were supposed to determine the concentration of reducing sugars. The samples were provided through the National Research Development Institute for Biology and Animal Nutrition (IBNA - Baloteşti, Romania). The samples were milled to 1-3 mm, to increase the accessibility of hydrolytic enzyme to the substrate.

Enzymatic hydrolysis process of raw materials

The enzymatic treatment was performed in one step process, by using a carbohydrase enzyme preparation produced by submerged fermentation of an *Aspergillus aculeatus* microorganism with activity of 50 FBG/g (Fungal β-glucanase activity/per gram). The enzyme preparation contains endo-1,3(4)-β-glucanase, hemicellulase and polygalacturonase activities. It was used different concentrations of enzyme (0.05-0.5% w/v). The hydrolysis incubation took place at 55°C, pH=5-5.5, on a rotary shaker as 150 rpm, for 16 h. During hydrolysis process, the samples were taken and centrifuged at 5500 rpm for 20 min., to remove remaining raw materials. The experiment was performed in triplicate for each substrate and the results are presented as mean values. For all trial, were prepared a control sample (without enzyme additions) (Dumitru and Jurcoane, 2017).

Determining the reducing sugars by DNS method

It was used 3,5-dinitrosalicylic acid (DNS) (Miller, 1959) as colorimetric method, for assessing the RS extracted from various raw materials (corn, field peas, field beans), by using an carbohydrase enzyme preparation (EC/IUB 3.4.21) (Dumitru et al., 2017; Jurcoane et al., 2006).

DNS assay allows measuring the concentration of total RSs obtained as a result of enzymatic hydrolysis of polysaccharides contained in lignocellulose materials. The concentration of RSs in a sample was estimated by diluting 1 mL of the hydrolysis's liquid to 25 mL with deionized water. From diluted sample (1:25 v/v), 0.5 ml was added on 3 mL DNS reagent, supplemented with 0.5 ml buffer citrate (0.05 M, pH=4.8) and then the test tube was inserted at 100°C, for 10 min. The tubes were inserted into an ice water bath, in order to stop the reaction and to stabilize the color. The absorbance of the sample is directly proportional to the amount of RSs and was acquired at wavelength 640 nm using a Biomate 3 spectrophotometer UV-Vis.

Procedure of glucose assay

RSs can be investigated by DNS method employing glucose (0.1%) as the standard calibration curve, described by Petterson and Porath (Iordăchescu and Dumitru, 1980). DNS reacts with free carbonyl group of RSs under alkaline condition, forming 3-amino-5-nitrosalicylic acid, an aromatic compound with maximum absorption at 640 nm.

Assessing the enzymatic activity

CMCase activity was assayed follow by Petterson's and Porath's method, at 50°C, for 10 min., using 1% (w/v) CMCase (carboxymethylcellulose, medium viscosity) as substrate in the enzyme activity measurements.

One unit (U) of CMCase activity was defined as the amount of enzyme, which forms with the DNS reagent, the same optical density, similar to a milligram of glucose per minute under the assayed condition used.
The analytical data were compared using variance analysis (ANOVA) with STATVIEW for Windows (SAS, version 6.0). The results were expressed as mean values ± standard deviation, the differences being considered statistically significant for P <0.05.

RESULTS AND DISCUSSIONS

All the raw material samples were hydrolyzed. The enzymatic was performed by using a carbohydrase enzyme in different concentration (0-0.5%).
For the experimental data, the total amount of available sugars was significantly higher (P<0.0001) for peas (19.60±0.105), followed by maize (17.17±0.105) and beans field (10.50±0.055) at an addition of 0.5% carbohydrase enzyme (Figure 1).The best results were obtained for field peas (19.60).

The results from ANOVA showed statistically significant differences (P<0.05), between all substrates used. Without addition of enzyme product, beans field present 4.64 RSs, compared with maize, respectively peas field, where the RSs yield are approximately similar as value.
According to Figure 1, the RSs in control maize (without enzyme addition) is lower, compared with the sample where, at an addition of 0.5% carbohydrase enzyme, the level of RSs was approximately 10 times higher.

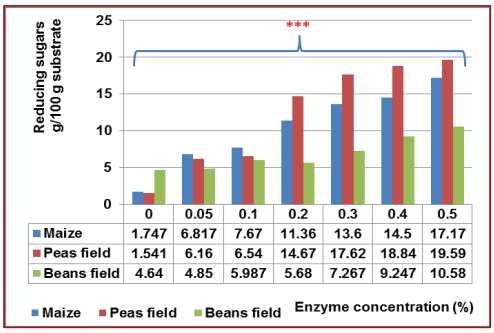
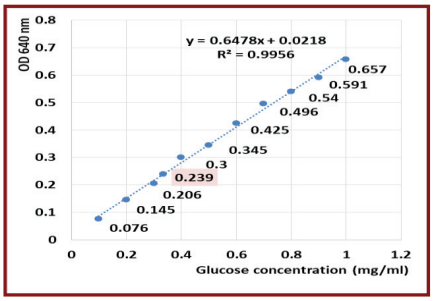


Figure 1. Reducing sugars yields for raw materials



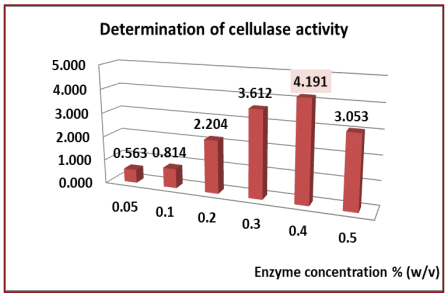


Figure 2. The enzymatic activity of carbohydrase enzyme

Figure 1 shows the variation of reducing-sugars concentration of hydrolysis liquid during the enzymatic hydrolysis. For all samples, the hydrolysis liquid did not contain sugars at the beginning, a lesser beans field, where the RSs fold registered 4.64.
The RSs increased due to the degradation of cellulose and hemicellulose at addition with enzyme. For example, the majority of feed ingredients used in monogastric animal nutrition, have in their composition carbohydrates constitute, quantitatively as the most important energy source (Banino, 2012). The small and large intestines of pigs, are both carbohydrate digestion sites and the chemical composition of carbohydrates determines if they are degraded by enzymes or microbes.
In our study, the carbohydrase enzyme preparation, at 0.4% (w/v), presents an celullase activity of 4.19 [UDNS/ml], compared with 0.5% addition, when the level of CMCase activity is lower 3.053 [UDNS/ml) (Figure 2).
The results suggest that hydrolytic potential of enzyme preparation to produce fermentable sugars was superior on field peas, followed by maize and beans field at 0.5% enzyme addition.

CONCLUSIONS

Hydrolytic potential of enzyme preparation to produce fermentable sugars was superior on field peas, knowed as source of protein in animal nutrition and can be used as feed additive to improve nutrient availability.

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CANINE AMNIOTIC MEMBRANE DERIVED MESENCHYMAL STEM CELLS- POTENTIAL SOURCES FOR REGENERATIVE MEDICINE

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Abstract

Canine mesenchymal stem cells (MSCs) can be defined with self renew potential and specific differentiation capacity. Amiotic membrane represent an important source of MSCs, which can be harvested by minimally invasive methods. The aim of our study was to evaluate the growth characteristics of canine amniotic membrane derived mesenchymal stem cells. The placenta samples were collected after cesarean section from healthy mixed breed dogs. MSCs isolation was performed using enzymatic method. Isolated cells were cultured in propagation medium: Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (Sigma-Aldrich). The medium was changed after 4 days. The cell doubling number, cell proliferation capacity, cell doubling time, daily duplication rate and clonogenic efficacy were evaluated. Our study demonstrate the self renew potential of canine amniotic membrane derived mesenchymal stem cells, and can represent a potential source of stem cells for canine regenerative medicine.

Key words*: canine, stem cells, amniotic membrane, proliferation, cells growth.*

INTRODUCTION

Mesenchymal stem cells (MSCs), are multi-potent cells with unique immunoregulatory properties and self-renewal capacity (Ullah et al., 2015; Pall et al.; 2015; de Bakker E. et al., 2013). MSCs can be obtained from mesodermal tissues, endodermal tissues and ectoderm-derived tissues (Phinney et al., 2007; de Bakker E. et al., 2013).

According to the International Society for Cellular Therapy, MSCs are defined by their plastic adherence, expression of some specific markers and *in vitro* differentiation potential (Dominici et al., 2016).

For regenerative therapy in veterinary medicine, mesenchymal stromal cells (MSC) have been traditionally isolated from bone marrow or adipose tissue.

Neonatal tissues, normally are discarded after birth from all species (Saulnier et al., 2016; Seo et al., 2009).

These cells have been described as primitive cells with proliferative and immunosuppressive potential (Saulnier et al., 2016; Maymó et al., 2018).

The aim of our study was to evaluate the growth characteristics of canine amniotic membrane derived mesenchymal stem cells.

MATERIALS AND METHODS

Samples (n=3) were collected during cesarean-section deliveries from healthy mixed breed dogs. The samples were harvested after owner's agreement. The amniotic membrane was separated from chorionic membrane mechanically.

The samples were minced and treated with collagenase type I (Sigma-Aldrich) at 37℃ for 1 h. After centrifugation the cell pellet was resuspended in basal culture medium DMEM-F12 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Invitrogen, USA) 1% AA (antibiotic-antimycotic, Sigma-Aldrich).

The cells were incubated at 37°C in humidified atmosphere with 5% CO_2. After 4 days, non-adherent cells were removed and the medium was replaced.

The passages was performed at a confluence of 80-90%. The clonal capacity and population-doubling times (DTs) have been calculated.

The clonal capacity of canine amniotic tissue derived cells was assessed using CFU-F assay. $5x10^5$cells/well was cultured in basal medium, after 10 days the cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO) in 10% methanol for 20 minutes. Colony-

forming efficiency was calculated as percentage of the ratio of number of colonies counted to number of cells initially seeded. In order to evaluate the growth curves the cells were plated at a density of $4x10^3$ cells (six-well plate).
After every 72 h of culture, cells from one well were counted. proliferation rate was determined as previously reported (Lange-Consiglio et al., 2012; Corradetti et al., 2014).
The amniotic tissue derived cells (p1-p5) were plated at a density of $4x10^3$ cells/cm^2. Every 4 days the cells were trypsinized, counted and reseeded at the initial density ($4x10^3$ cells/cm^2).
Doubling time were calculated for each passage using the formula: doubling time (DT)=culture time/number of cell generations. Cell generations =log (Nc/No)/log2 (Nc, number of cells at confluence; No, the number of seeded cells).
For evaluation of cell phenotype, cell suspensions were incubated for 20 minutes at 4°C with CD34-FITC, CD45-FITC, CD90-FITC CD44-PE. Samples were analyzed with a FACSCanto II and Diva software (BD Biosciences).

RESULTS AND DISCUSSIONS

Collection of placental tissue during cesarean-section deliveries from canines did not have any adverse effect on the donors. Epitelial layer of amniotic tissue were separated and treated enzymatically. The cells suspension was cultured in standard propagation media. After 48h single cells with round, flatted, stellate, spindle shape morphology was observed. After 72 h the non adherent cells were removed.
Aderent fibroblast-like cells formed round clusters and after 14-15 days of culture, they reached 80 to 90 % confluency.
After several passages the degree of heterogeneity decreased, cells proliferated uniformly maintaining a homogeneous fibroblast-like morphology.
Total number of cells and cells viability was measured in each passages (Figure 1). In P1 the cells viability was 84.00±1.73 After the first passages the cells viability was 94.66±3.05.
The cell viability remained at approximately constant level, but after P3 cell viability decreased slightly.
The total number of cells also showed an increased tendency (Figure 1, 2).

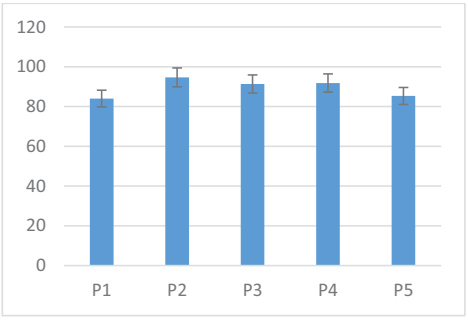

Figure 1. Canine amniotic tissue derived cells viability P1-P5

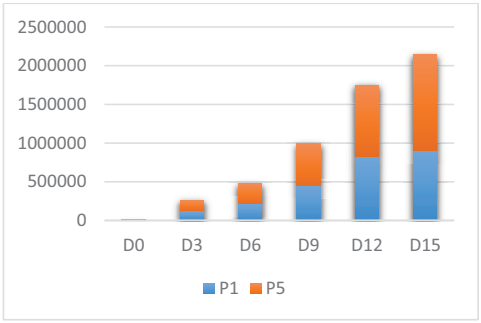

Figure 2. Total number of cells and cell viability P1-P5

The population doubling time (DT) at P1 was 1.26±0.11, 1.73±0.20 at P2 whereas at P5 the DT was 3.03±0.20 (Figure 3).

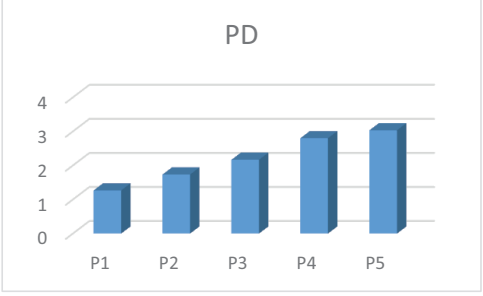

Figure 3. Doubling times at different passages (P1-P5)

The clonogenic potential of MSCs was assessed by CFU-F assay. Isolated cells displayed colony-forming ability; the frequency of colony forming cells was 42.88%.
The isolated cells expressed high levels of CD44 and CD90, and were negative for CD34, CD45 (Figure 4).

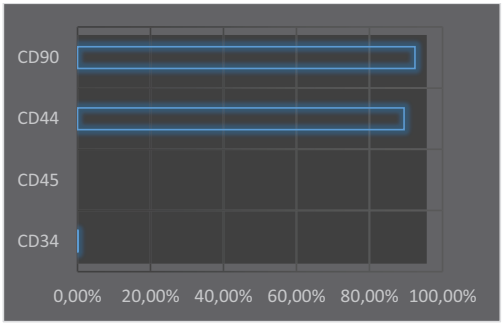

Figure 4. Flow cytometric analysis of canine amniotic tissue derived mesenchymal stem cells

The aim of our study was to identify a valuble sources of stem cells for regenerative medicine. Mesenchymal stem cells (MSCs) were first isolated from bone marrow (Noth et al., 2008). Recent study has identified alternative sources of MSCs, including umbilical cord blood (Martin-Rendon et al., 2008; Reed et al., 2008) adipose tissue (Eirin et al., 2012; Elashry et al., 2017), ovary (Trindade et al., 2017), placenta (Fukuchi, 2004; Fernandes et al., 2012; Yu et al., 2013), palatal tissue (Pall et al., 2017), dental pulp (Huang et al., 2009), synovial membrane (Hermida-Gomez et al., 2011), peripheral blood (Tondreau et al., 2005), periodontal ligament (Park et al., 2011), endometrium (Schwab et al., 2008), umbilical cord (Sarugaser et al., 2005), Wharton Jelly (Davies et al., 2017). The principal task of regenerative medicine is to identify a safe and valuable source of stem cells that can be used for treatment of different diseases (Si JW et al., 2015; Maymó et al., 2018). Stem cells derived from fetal tissues are an attractive source of cells for regenerative medicine (Noth et al., 2008; Parveen, 2018). Two types of stem cells can be isolated from the amniotic membrane: the stromal cells and the epithelial amniotic cells (Maymó et al., 2018). The presence of mesenchymal stem cells was described for the first time in human by Kaviani et al., 2001. Our study showed that mesenchymal stem cells could be successfully isolated form canine amniotic tissue.

CONCLUSIONS

Our results indicate a novel sources of stem cells with clonogenic and proliferative potential which can serve as a model system of mesenchymal stem cells for veterinary regenerative medicine.

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MISCELLANEOUS

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CHARACTERIZATION OF SOME BIOACTIVE COMPOUNDS RELEASED BY *Inonotus obliquus* IN SUBMERGED CULTURE

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Abstract

Inonotus obliquus (chaga) is a birch phytoparasitic mushroom with an irregular shape that represents dark-colored mycelial agglomeration. It has a high pharmaceutical value because of its rich content in bioactive compounds, polysaccharides, phenols, or triterpene (betulin, for example). The purpose of this study was to determine the composition of the fermented medium in different active compounds, while also identifying the antioxidant potential expressed by the fermented medium of I. obliquus mycelium. In a general analysis of the obtained results, the potential of the I. obliquus mycelium to release in the fermented medium compounds with antioxidant potential is primarily due to the medium composition. Particular attention was determined by the presence of other compounds, such as ascorbic acid, which was identified in a reduced number of samples. The results led to the conclusion that fermentation medium with I. obliquus mycelium had the potential to be cultivated via a technological process to obtain valuable compounds.

Key words*: carbon source, chaga mushroom, phenolic compounds, DPPH.*

INTRODUCTION

Inonotus obliquus (chaga mushroom) grows predominantly on birch trees, as the bark is up to 22% rich in betulin (Todd, 2016). Betulin is deficiently absorbed into the human body, even when administered intravenously (Müllauer, 2011). The fermentative process is not frequently used given the lack of optimization (Singh, 2016). The mushroom composition includes the following: complex polysaccharides (which boost immunity in the cardiovascular system and serves as a liver detoxifier) (Robinson, 2018), polyphenols, vitamins (Sanchez, 2017), minerals, and other antioxidant compounds (Vasile, 2017). Betulin is absorbed from bark and biotransformed into betulinic acid (Liu, 2011). This is a functional compound that has antitumor action. It is assumable that such antitumor effects represent a mixture between the action of betulin and the bioactive compounds that exert antioxidant effects, which can reduce the oxidative stress at the cellular level (Krol, 2015).

The purpose of the study was to characterize the bioactive compounds and antioxidant potential expressed by a medium fermented following inoculation with *I. obliquus* mycelium.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from Sigma Aldrich GmbH (Sternheim, Germany). All other unlabeled chemicals and reagents were of analytical grade.

Mycelium cultivation and fermentation conditions

I. obliquus mycelium was obtained and authenticated by Mihaela Ene, Bucharest, Romania. The corresponding number for the specimen was 17011723IF. The mycelia were revitalized on a medium of malt extract agar. The inoculum was prepared by cultivating the mushroom on a laboratory rotary shaker at 150 rpm, for a minimum of 5 days, at 23ºC, in 250 mL Erlenmeyer flasks with 100 mL of the culture medium containing the following (g%) (Vamanu, 2014): Sample 1 (glucose 1, fructose 4, malt 1, yeast extract 4, starch 1, and lactose 4); Sample 2 (glucose 1, fructose 4, malt 1, yeast extract 4, starch 4, and lactose 1); Sample

3 (glucose 1, fructose 4, malt 4, yeast extract 1, starch 1, and lactose 4); Sample 4 (glucose 1, fructose 4, malt 4, yeast extract 1, starch 4, and lactose 1); Sample 5 (glucose 4, fructose 1, malt 1, yeast extract 4, starch 1, and lactose 4); Sample 6 (glucose 4, fructose 1, malt 1, yeast extract 4, starch 4, and lactose 1); Sample 7 (glucose 4, fructose 1, malt 4, yeast extract 1, starch 1, and lactose 4); and Sample 8 (glucose 4, fructose 1, malt 4, yeast extract 1, starch 4, and lactose 1).

Bioactive compound quantification

Ascorbic acid was determined using Quantofix® test strips. Exopolysaccharides were determined by precipitation with cold absolute ethanol (Sun, 2015). The total soluble phenolic and flavonoid contents were determined using spectrophotometry, according to previously described methods (Vamanu, 2018).

Antioxidant activity quantification

The antioxidant activity of the fermentation broth was determined using spectrophotometry to assess DPPH scavenging activity (Vamanu, 2013) and lipid peroxidation inhibition (Vamanu, 2014).

Statistical analysis

All parameters for antioxidant activity were assessed in triplicate, and the results were expressed as the mean ± standard deviation (SD) of three observations. The mean values and SD were calculated with the Excel program from Microsoft Office 2016 (Microsoft Corporation, Redmond, WA, USA).

RESULTS AND DISCUSSIONS

The overall contents of polyphenols showed some significant variations between the eight medium formulas being tested. The maximum polyphenol levels were reached in Sample 1, whereas the minimum amount was reached in Sample 3. The difference between Samples 7 and 8 was 38.77% higher, on average.

The accumulation of flavonoids was inversely proportional to the accumulation of phenolic compounds, which is correlated with the distinct antioxidant profile (Figure 1). Thus, in the samples that had high phenol content, the flavonoids did not exceeded a maximum value of 10 µg/mL quercitin equivalent. The overall share of flavonoids was 15.5%, on average. In Sample 5, for instance, the overall flavonoid content was 75.04% higher.

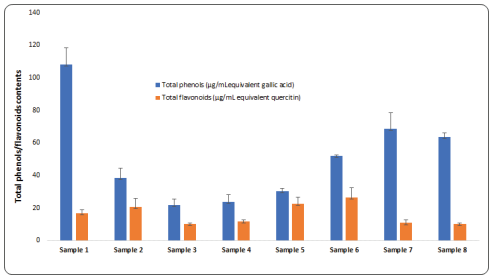

Figure 1. Overall contents of phenols and flavonoids in the fermented medium

This study represents the first assessment of the presence of these bioactive compounds in medium fermented by mycelium *I. obliquus*.

The value of the antioxidant potential (Figure 2) of the medium was determined in relation to the contents of the main bioactive compounds (Figure 1).

The differences noted between the antioxidant activity (specifically, the anti-radical activity) was duly interpreted based upon several distinct phenolic profiles, as determined by the fermented medium formula.

The high accumulation of flavonoids induced antioxidant activity (which was duly expressed under the form of lipid peroxidation inhibition); this effect was inversely proportional to the anti-radical activity (for instance, Sample 4).

However, the lipid peroxidation inhibition did not drop below 50%, which illustrates how valorization of the fermented medium holds great potential for reducing oxidative stress.

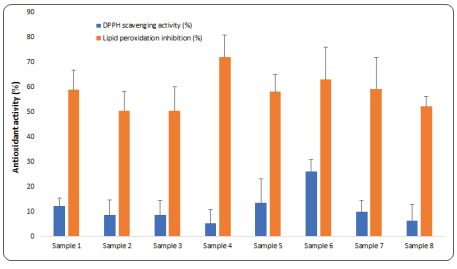

Figure 2. Inhibiting the DPPH radical and lipids peroxidation in the fermented medium

Thus, the value of index R^2 between the overall phenolic contents and anti-radical activity was

-0.499 (Figure 3). Conversely, the flavonoid content showed a very good correlation (R^2 = 0.6169; Figure 4) with the DPPH radical-inhibiting activity. This behavior was duly expressed by an inversely proportional correlation between the overall accumulation of phenols and that of flavonoids, as this was the main method of inducing antioxidant effects.
The correlation between the two methods was high, which showed direct dependency between the composition of the medium and the antioxidant response.
The correspondence between the two activities has proven that the biological value is not directly dependent on the presence of one single biological component.
This is why the polysaccharide ratio is significantly influential; it maintains part of the phenolic ratio, while the response of the samples shall consider this component too. Upon conducting a preliminary analysis, one has noticed that a reduction in the overall phenol content matches this particular fermentative behavior, which was translated by a high level of antioxidant protection (Figure 2).

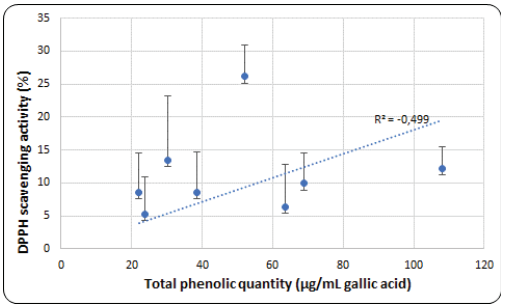

Figure 3. Correlation between scavenging activity and total phenolic quantity

Upon a general review of the results obtained in this study, the fermentative capacity of mycelium *I. obliquus*, in terms of its ability to release antioxidant compounds into the fermented medium, is primarily due to the formula of the medium.
The presence of other compounds, such as ascorbic acid, was also identified in a low number of samples.
This profile was interpreted as an accumulation within the mycelium, which triggered the lowering of the biological response (Moldoveanu, 2015) in the fermented medium.

CONCLUSIONS

The results have led to the conclusion that the medium fermented by the mycelium of *I. obliquus* holds potential as a technical procedure that can facilitate the fast and reproducible production of high-value biological compounds.
Future studies shall examine the division of the phenolic ratio, for instance, in view of identifying the phenolic profile and assessing the metabolic capacity of selecting an optimal substrate for the tested species.

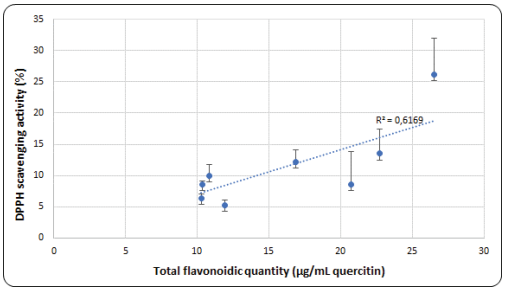

Figure 4. Correlation between scavenging activity and total flavonoid quantity

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EVALUATION OF NUTRITIONAL, PHYSICAL, TEXTURAL AND SENSORIAL PROPERTIES OF GLUTEN FREE COOKIES SUPPLEMENTED WITH DRIED *Rosa damascena* Mill. PETALS

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Abstract

In the present study, the effects of dried and ground Rosa damascena Mill. petals (Rosa flour-RF) for improving the nutritional and some characteristics of gluten-free cookies (GFC) were investigated. The RF was used to replace 0-2.5-5-7.5-10% of gluten free flour (GFF) formulations. RF supplementation increased lightness and yellowness but decreased redness whereas a slight increase was observed at both moistures and spread ratio. RF addition lead to a significant reduction in the hardness of GFC while different levels of RF did not shown such an effect. RF provided darker samples and cookies became more brittle. RF addition led to an increase in total dietary fibre, total phenolics, and antioxidant activity of GFC. GFC containing RF were more appreciated than GFC with no added RF regarding taste and aroma. At the same time, a higher purchasing decision was found for cookies with added RF. Results showed that acceptable GFC couldbe produced by using RF up to 7.5% level to enhance the nutritional value of GFC that could be important for the gluten-free industry.

Key words*: antioxidant activity, celiac disease, dietary fibre, gluten-free, Rosa damascene.*

INTRODUCTION

Rosa damascena, regarding the economic and therapeutic point of view, has an essential place among medicinal and aromatic plants. There are about 15,000 tons of rose flower production per year in the world. It is commonly grown mainly in Turkey and Bulgaria (Anonymous, 2017).

Rosa genus consists of more than 200 species and 18,000 cultivars, among them *Rosa damascena* Mill*., Rosa gallica* L*. and Rosa centifolia* L*.,* have found industrial scale application for their flavouring and fragrance properties (Mahboubi, 2016). The Isparta Rose, *Rosa damascena* Mill*.,* stands in *Rosaceae* family, *Rosa* genus (Gül et al., 2015). *Rosa damascena* Mill*.* is usually grown in Isparta, Burdur and Denizli provinces of Turkey and mainly used for the production of rose oil (Gül, 2000).

Rosa damascena is a valuable raw material for cosmetic perfume, and drug industry (Şentürk and Doğan, 2017). Today, it is also used for the production of various food products such as: dessert (Gül et al., 2010), bread, delight, jam, syrup and vinegar.

Rosaceae family contains various components such as terpenes, glycosides, flavonoids and anthocyanins. Thus it possesses a wide range of pharmacological activities including antioxidant, antimicrobial, analgesic, anticancer, anti-inflammatory, antimutagenic, antidiabetic and antidepressant properties (Boskabady et al., 2011; Mahboubi, 2016). Memory enhancing effects of *Rosa damascena* due to the antioxidant effects were also reported by Mohammadpour et al. (2015).

Rosa damascena Mill*.* petals and leavens show significant antimicrobial and antioxidant effects (Şengül et al., 2017). Among flavonoid skaempferol and quercetin, in petals of *Rosa damascena* Mill. were found by Jaimand et al. (2010). Antimicrobial, anti-inflammatory, antioxidant, anticancer, protective neuronal, cardiac, gastrointestinal and hepatic effects of *Rosa damascena* Mill*.* in 21 *in vivo* and 30 *in vitro* animal studies were reviewed by Nayebia et al. (2017).

Dried flowers, petals, of *Rosaceae* family can solve problems with the digestive system (Boskabady et al., 2011). The dried buds and petals of the rose are used as laxative agent and flavouringin foods (Mahboubi, 2016).

Celiac disease which can be defined as an autoimmune disorder that is seen genetically predisposed individuals after eating foods including gluten and/or other environmental factors (Green et al., 2015).

Syndromes of this disease such as diarrhea, malabsorption, growth issues etc. can be prevented only by being tightly bound to gluten-free diet during lifelong.

There has been an increase in the number of patients suffering from the celiac.

Therefore the demand for gluten-free products increases day by day. Unfortunately, both the low quality and the nutritional deficiencies are significant problems due to the starch-based composition of these products (Hayıt and Gül, 2017a).

There are extensive research to produce gluten-free products at least partially resembles the gluten-containing counterparts regarding nutritive, sensory and technological quality (Pellegrini and Agostoni, 2015).

All age groups have extensively consumed cookies since for a long time for their convenience of ready to eat, long shelf life and rich nutrient composition (Acun and Gül, 2014; Gül et al., 2016; Gül et al., 2017). At the same time cookies are also preferred and consumed in large quantities by celiac patients.

Valitutti et al. (2017) were investigated the pattern of cereal-based products consumption among people with celiac disease. Celiac disease patients' median three-day intake of biscuits and crackers was found higher compared with a control population (65.8 g vs. 22.7 g and 44.7 g vs. 10.6 g, respectively), by these researchers.

There ismore researches regarding the improving of nutritional content and technological quality of gluten-free cookies (Hadnađev et al., 2013; Giuberti et al., 2018; Molinari et al., 2018; Porcel et al., 2017), and other gluten-free bakery products (Özer and Dizlek, 2016; Hayıt and Gül, 2017b; Hayıt and Gül, 2017c).

To the best of our knowledge, we found no study to use dried and ground *Rosa damascena* Mill. petals (RF) in the formulations of gluten-free cookies. In this context, the effect of increasing levels (0-2.5-5-7.5-10%) of RF on chemical, physical, textural, nutritional and sensorial properties of GFC were investigated.

MATERIALS AND METHODS

Materials

Rosa flour, as dried and ground form of *Rosa damascena* Mill. petals, was obtained from Kurucum Gıda (Isparta Turkey). Potato and corn starch from Tat Construction Industry and Trade Incorporation (İzmir, Turkey), corn and rice flour from Hüsnü Özmen Food Industry Incorporation (İzmir, Turkey), sodium bicarbonate from Şişecam Chemicals Group Soda Industry (Mersin, Turkey) were purchased. Corn syrup (42%) was kindly supplied from Sunar Corn Integrated Plant Inc. (Adana, Turkey). Hydrogenated vegetable oil, fine granulated sugar and salt were purchased from local markets.

Chemical analysis

Moisture, ash, protein, total lipid, total dietary fibre content of RF, corn starch, potato starch, corn flour, rice flour and cookie samples were determined by using AACC Method 44-01.01, AACC Method 08-01.01, AACC Method 46-12.01, AACC Method 30-25.01 and AACC Method 32-05.01 (AACC, 2001), respectively. pH was determined according to Sertakan, 2006. For the measurement of water activity (Woody, 2003), the cookies were crushed into small granules, and approximately 2 g of sample were placed in plastic dishes designed for use with an a W-meter (Novasina, LabTouch-aw, Lachen, Switzerland). Total phenolic content (TPC) and antiradical activity of RF and cookies were detected by using methods of Singleton and Rossi, (1965) and α-diphenyl-β-picrylhydrazyl DPPH method of Dorman et al. (2003).

Experimental design

RF was used to replace 0-2.5-5-7.5-10% of gluten free flour (GFF) formulations. GFF included 7% corn starch, 8% corn flour, 40% rice flour and 45% potato starch. Response Surface Methodology analysed this mixture and range of ingredients in our previous study (not pressed yet).

GFC preparation

Cookies were prepared according to AACC method 10-50.05 (AACC, 2000) with slight modifications. 225 g GFF mixture (14%

moisture basis), 64 g hydrogenated vegetable oil (cream shortening), 130.0 g fine granulated sugar, 2.1 g salt, 2.5 g sodium bicarbonate ($NaHCO_3$), 33 g high fructose corn syrup (42%), and 16.0 g distilled water was used for preparation of cookies. No added RF (0%) GFC sample was taken as control. Preparation of dough was made in a mixer (Hobart, N-50) according to the procedure given in the method 10-50.05 (AACC, 2000). The dough was then gently scraped from the bowl and sheeted by rolling pin to 5 mm thickness. Then it was shaped circular with a diameter of 60 mm with a circular scone cutter. Baking was performed in a convection oven (Fimak FSET4, Turkey) at $205^0C \pm 2^0C$ for 10 min. Cookies were cooled at room temperature and packed in high-density polyethene bags with hermetic cover until further analysis.

Geometrical analysis

The physical characteristics of cookies were analysed for width (W), thickness (T), and spread ratio (W/T) values by digital calliper in mm. The cookies were laid edge to edge and were measured for diameter and measured after rotating them through 90^0. The spread ratio was obtained by finding the ratio between the average width and thickness of the cookies. Six cookie samples were taken in each experiment.

Color analysis

Color parameters of RF and GFC were determined using colourimeter (Minolta CR-310, Minolta Co Ltd., Tokyo, Japan). Average L (lightness), a (redness) and b (yellowness) values were recorded after five readings for each measurement.

Texture analysis

The maximum force „hardness" and the distance at the point of break „fracturability" values of GFC were measured by using a texture analyser (TA-XT PLUS, Stable Micro Systems, Surrey, UK) equipped with a 3-Point Bending Rig (HDP/3PB). 5 kg load cell was used for calculation of these texture parameters. The pre-test speed of 1.0 mm/s, the test speed of 3.0 mm/s, the post-test speed of 10 mm/s, a distance of 5.0 mm and data acquisition rate, 500 pps. All textural tests were performed in triplicate.

Sensory analysis

Cookies were evaluated by ten untrained judges from food engineering department according to Gül et al. (2013) after 4 hours of baking. Hedonic scale from 1 to 5 point; 'dislike extremely' to 'like extremely' respectively, was used. Each panellist evaluated five samples of the cookies for the following sensory attributes, colour, surface structure, texture (hardness and brittleness), flavour, odour, mouthfeel, and overall acceptance. Along with that purchasing intent of cookies were evaluated according to Gül and Şen (2017) on a five-point scale (5: definitely would buy, 1: definitely would not buy).

Statistical analysis

All the analysis was done in triplicate. Analysis of variance (ANOVA) was conducted by using the software, statistical package for social science (SPSS 16.0) procedures. Duncan's multiple range test with significance defined at $P < 0.01$ was used to determine significant difference among the various samples.

RESULTS AND DISCUSSIONS

Chemical composition of RF, and gluten-free flour ingredients

Moisture, ash, protein, total dietary fibre and total lipid content of RF and GFF ingredients are given in Table 1.

Table 1. Chemical composition of soft wheat flour, potato starch, corn starch, corn flour and rice flour[1]

Material	Moisture (%)	Ash (% d.b)	Protein (% d.b)	Total lipid (% d.b)	TDF (%)
RF	5.75±0.01^a	4.51±0.05^a	2.6±0.02^b	4.8±0.08^b	61.40±1.15^a
Potato starch	8.30±0.03^c	0.79±0.09^c	0.19±0.91^c	0.45±0.01^c	0.69±0.32^d
Corn starch	8.34±0.02^c	0.69±0.11^c	0.25±0.81^c	0.82±0.02^d	0.47±0.29^d
Corn flour	9.10±0.21^b	1.12±0.03^b	7.14±0.61^a	5.15±1.00^a	9.16±1.09^b
Rice flour	9.29±0.22^b	0.47±0.01^d	7.7±0.52^a	1.25±0.19^c	4.88±1.07^c

[1]Mean values in the same column with different letters are significantly different ($p<0.01$), RF: Rosa flour, TDF: Total dietary Fibre.

Ash and total dietary fibre content of RF were observed significantly higher than other GFF ingredients while its moisture found lower than others. In terms of lipid content, RF ranked second after corn flour with 4.8%. These results suggest that RF is a rich source of dietary fibres, minerals and other nutrients. Since it is a good source of dietary fibre, so it can provide

many beneficial effects on health such as; lower risk for developing coronary heart disease, hypertension, stroke, obesity diabetes and certain gastrointestinal diseases (Anderson et al., 2009).
The vegetable material must contain more than 50% dietary fibre to identify it as antioxidant dietary fibre (Saura-Calixto, 1998). According to this statement,RF could be interesting for the potential use as afood ingredient to improve the fibre profile of food products.

Total phenolic and antioxidant capacity of RF

TPC and DPPH antiradical activity of RF were calculated as 414.2 g/kg GAE (Gallic acid equivalent) and 73.15 IC_{50}, µg/ml. In the present study antiradical activity of RF was found higher when compared with the results of Sakač et al. (2015), who determined DPPH scavenging activity of light buckwheat flour which is used in gluten-free formulations as IC_{50} 1.61 mg/ml.

Chemical composition of cookies

The addition of RF to GFF has slightly affected the moisture, aw and ash content of GFC (Table 2). These values were an expected result because of the higher moisture and ash content of RF (Table 1) than the amount of other GFF components.

Table 2. Chemical composition of GFC supplemented with RF[1]

RF (%)	Moisture (%)	Aw	Ash (%)	pH	Protein (%)
0	5.11±0.13^{b}	0.36±0.00^{c}	2.05±0.03^{c}	7.33±0.12^{a}	4.80±0.11^{a}
2.5	5.83±0.28^{ab}	0.42±0.01^{b}	2.03±0.03^{c}	7.11±0.03^{b}	4.24±0.25^{b}
5	6.10±0.10^{a}	0.41±0.00^{b}	2.21±0.01^{b}	7.00±0.05^{bc}	4.26±0.25^{b}
7.5	6.14±0.70^{a}	0.42±0.00^{b}	2.22±0.01^{b}	6.91±0.03^{c}	4.02±0.03^{bc}
10	6.42±0.23^{a}	0.44±0.00^{a}	2.56±0.01^{a}	6.88±0.08^{c}	3.70±0.10^{c}

[1]Mean values in the same column with different letters are significantly different (p<0.01), RF: Rosa flour, Aw: Water activity

The pH and protein ranged from 7.33 to 6.88 and 4.80 to 3.70, respectively. Both of them decreased with increase in RF concentration. The decrease in the protein content of GFC was due to the decrease at the concentration of corn and rice flour which had high protein content in the formulations. A similar dilution effect on protein content was previously reported on cookies substituted with an unripe plantain flour (Garcia-Solis et al., 2018).

Total dietary fibre content of cookies

The dietary fibre content of the RF supplemented gluten-free cookies are given in Table 3.

Table 3. Total dietary fibre, TPC and antioxidant activity of GFF supplemented with RF[1]

Addition level of RF (%)	Total dietary fibre (%)	TPC (g/kg GAE)	Antiradical activity DPPH IC_{50} mg/ml
0	5.73±0.02^{d}	0.92±0.67^{d}	ND
2.5	5.23±0.20^{d}	61.40±1.52^{c}	ND
5	6.76±0.02^{c}	105.09±1.18^{b}	7.92±0.05^{a}
7.5	8.23±0.07^{b}	125.97±1.07^{b}	6.95±0.08^{b}
10	10.21±0.09^{a}	169.61±2.15^{a}	5.81±0.02^{c}

[1]Mean values in the same column with different letters are significantly different (p<0.01), RF: Rosa flour, ND: Not detected, TPC: Total Phenolic content, GAE: Gallic acid equivalent

Total dietary fibre content of the GFC was found to be significantly different (P<0.01) after the addition level of RF more than 2.5%. This significant increase can be associated with the higher content of dietary fibres in RF. An increase in dietary fibre content of GFC by fibre addition was also described by Garcia-Solis et al. (2018). On the other side, authors Sakač et al. (2015) found lower total dietary fibre (2.94 g/100 g) in gluten-free cookies containing 30 % light buckwheat flour.

Total phenolic content and antioxidant capacities of cookies

TPC and antioxidant capacities of cookies enriched with RF are presented in Table 3. It is obvious that increasing RF levels resulted in remarkable increases in the TPC and antioxidant activity of GFC. TPC content of GFF was increased from 0.92 g/kg GAE to 169.61 g/kg GAE by the addition of RF at 10% level. It is noteworthy that, while control GFC with no added RF and GFC with 2.5% added RF, did not shown any antiradical activity, whereas the antioxidant activity of 5%, 7.5% and 10% RF containing cookies were increased gradually due to their higher total phenolic content. Similar results were found in gluten-free cookies supplemented with light buckwheat flour (Sakač et al., 2015) and sugar beet molasses (Filipcev et al., 2016).

Geometrical properties of cookies

Geometrical properties of cookies are presented in Table 4.

Table 4. Geometrical properties of GFC supplemented with RF[1]

RF (%)	Width (W, mm)	Thickness (T, mm)	Spread ratio (W/T)
0	70.75±0.80^{a}	9.31±0.55^{b}	7.60±0.38^{a}
2.5	64.41±0.26^{b}	11.76±0.69^{a}	5.47±0.33^{b}
5	63.61±0.17^{bc}	11.40±0.91^{a}	5.58±0.43^{b}
7.5	62.62±0.25^{c}	10.87±0.47^{ab}	5.76±0.26^{b}
10	61.32±0.44^{d}	10.81±0.25^{ab}	5.67±0.16^{b}

[1]Mean values in the same column with different letters are significantly different (p<0.01), RF: Rosa flour.

A significant difference (p<0.01) was occurred between geometrical properties of control and RF supplemented GFC. Control cookies with no RF had the highest width and spread ratio whereas had the lowest thickness. There was a progressive decrease observed in the width values of RF added GFC. Hydrophilic nature of the RF may be led to decrease in width values of cookies. After the addition of RF, the content of free water in the empty spaces of the gluten network could be limited which indicates high compactness of this network (Nawrocka et al., 2016) which led to obtaining more compact cookies.

Color values of cookies

Surface color with texture and flavour of cookies are the main features considering the preference of consumers. Surface colour values of cookies are shown in Table 5. The addition of RF affected significantly (P<0.01) the L, a, and b values of GFC.

Table 5. Color and textural properties of GFC supplemented with RF[1]

RF (%)	L	a	b	H (g)	F (mm)
0	60.3±0.17^{a}	6.0±0.09^{d}	20.6±0.01^{a}	8228.1±92.65^{a}	40.59±0.02^{c}
2.5	48.6±0.16^{b}	8.1±0.06^{c}	9.9±0.69^{b}	4646.2±194.61^{b}	41.49±0.24^{b}
5	42.1±0.27^{c}	10.2±0.11^{b}	6.5±0.05^{c}	4229.7±126.23^{c}	42.91±0.05^{a}
7.5	37.6±0.88^{d}	10.9±0.31^{a}	4.2±0.06^{d}	4135.6±91.76^{d}	42.69±0.25^{a}
10	34.2±0.78^{e}	11.4±0.32^{a}	3.7±0.04^{d}	4083.5±63.05^{e}	42.82±0.36^{a}

[1]Mean values in the same column with different letters are significantly different (p<0.01), RF: Rosa flour, H: Hardness, F: Fracturability

Control cookies with no added RF were found lighter than RF added GFC (Figure 1). Substitution of RF caused a gradual decrease in both lightness and yellowness values and a increase in redness. Lower L and b values observed for RF cookies are possibly related to the specific darker colour of RF (Table 1). At the same time, the formation of colour widely known as browning, is the result of Maillard reaction and caramelisation (Purlis, 2010).

Figure 1. Photographs of the cookies, from left to right: control, and Gluten-free cookies (GFC) with 2.5-5-7.5-10% RF added

Textural properties of cookies

The texture values, hardness and fracturability of GFC are presented in Table 5. There was a significant difference detected between the hardness values of control and RF added cookies. RF supplementation was led to a sharp decrease in the hardness of GFC. This should result from the higher moisture and total lipid content of RF (Table 1). Although there was a gradual decrease in hardness values of GFC in increasing levels of RF, it is not as noticeable as the drop was seen between control and RF containing cookies. Supplementation of RF was causedby a slight increase in the fracturability values of RF cookies as compared with control. Increasing concentrations of RF, except 2.5% level, did not affect significantly the fracturability of GFC.

Sensory properties of cookies

Sensory evaluation scores for GFC added with four different levels of RF are shown in Figure 2. There was a tendency towards the higher scores for 10% RF containing cookies, whereas their surface structure was scored lowest. Brittleness, mouthfeel, flavour and aroma points were increased, more prominently with cookies made with RF when compared with control cookie. Among all cookies, 7.5% RF added GFC were scored higher regarding odour, aroma and mouth feel.

General acceptability and purchasing intent of cookies were shown in Figure 3. These values were scored slightly higher at 2.5 and 5% enrichment levels. They became more dominant at the 7.5 addition level of RF. However, further increase of RF level to 10% led to decrease in the overall acceptability and in purchase intent of GFC. The lowest acceptance level and purchasing intent were reported for cookies made with 2.5% RF and control.

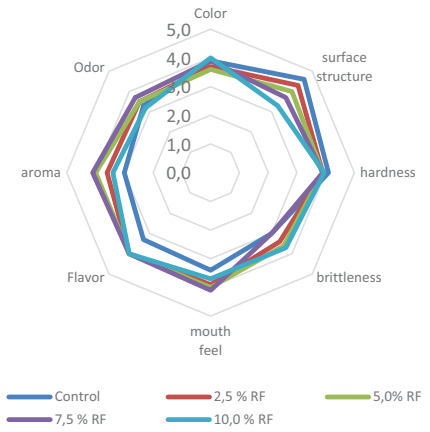

Figure 2. Sensory attributes of cookies supplemented with Rosa flour (RF)

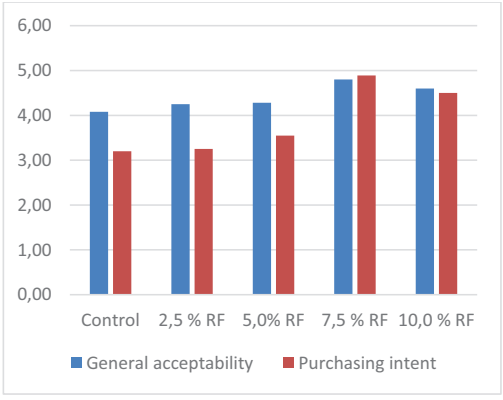

Figure 3. General acceptability and purchasing intent of cookies, RF: Rosa flour

CONCLUSIONS

The addition of RF in to GFC at an increasing rates lead to a slight increase on moisture, aw and ash content while pH and protein content of GFC were affected adversely. It is obvious that increasing RF levels resulted in remarkable increases in the total dietary fibre, total phenolic content and antioxidant activity of GFC. Sharp decrease on the spread ratio and hardness were observed when the RF was added to GFC formulations. However its increasing concentrations did not lead to a significant differences both on spread ratio and fracturability. Addition of RF made GFC darker and redder than control samples. Odour, aroma and mouth feel of GFC supplemented with 7.5% RF was scored higher. At the same time its overall acceptability and purchasing intent were found higher than other cookie samples.

When all of these constituents are evaluated together, they may have various beneficial effects on human health. However, gluten-free products have lower dietary fibre, and phenolic compounds due to the based on starch or other compounds have limited nutritional content. The overall results showed that the gluten-free cookies with acceptable quality and improved nutrition could be prepared from RF up to 7.5% level. As a result, it can be suggested that the petals of *Rosa damascena* Mill. can be used to enhance nutritional and sensorial profiles of gluten-free cookies.

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BARRIERS IN ONLINE MARKETING, FEAR IN CONSUMERS' MENTALITY

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Abstract

Technology has transformed the mentality in retail business is done with leading players shifting to mobile devices specific platforms. Through about 19 million internet connections which were in first semester of 2016 in Romania, users made a traffic of almost 2,7 million terabytes. More and more people discover how easily can be to use internet, to find any information on a smartphone, a tablet or a laptop or desktop. A year before, in 2015, about 48% of consumers used the internet but only 17% also for buying some products and services online, comparative with an average of 63% European consumers. First and second barriers in mentality to buy online are online payment security and lack of transaction people to people. The aim of this work was to update the information regarding internet access and usage by the Romanian consumers.

Key words*: internet, online consumers, business, barriers.*

INTRODUCTION

Online marketing is a package of powerful tools and methodologies used for promoting products and services through the internet. Online marketing includes a wider range of marketing elements than traditional business marketing due to the extra channels and marketing mechanisms available on the internet. Online marketing, named also internet marketing or web marketing or digital marketing or search engine marketing (SEM), takes business development to a much higher level than traditional marketing. In online marketing, to reach targeted customers basic depends on internet connections in any place of the world. (Condei R. et al., 2014.). More and more people discover how easily can be to use the internet, to find any information on a smartphone, a tablet or a laptop or desktop. In the recent years, through about 19 million internet connections which were in the first semester of 2016 in Romania, about 48% of consumers used the internet but only 17% also for buying some products and services online, comparative with an average of 63% European consumers (Vasilache A., 2017). For this reason, the aim of this work was to update the information regarding internet access and usage by the Romanian consumers.

WHAT CUSTOMER IS TARGETED IN ONLINE MARKETING?

At first side, all the customers can be targeted in online marketing. All over the world. (Pavel I.A., 2015). But not all customers have a credit or debit card, can write what is demand in special spaces on the site or fulfill the banking card data to send an order. The language barrier remains the last reason for those customers who want to buy abroad.

DISADVANTAGES OF ONLINE MARKETING

Generally speaking, the main limitation of online marketing is the lack of tangibility, which means that consumers are unable to try on, or try out items they might wish to purchase. So, on balance, generous return policies are the main way to circumvent such buyer apprehension. But many consumers seem to be fear when have to pay online some

products or services. (Gupta A., Arora N., 2017).

It is also a problem of trust, such I have no trust in online payments, what it happens with banking card data, what if I lose more money that appears on screen, what if the product does not fit to me or to person I want to make a gift, how much time I spend to finally I recover the money or receive another product instead, what if the product is stolen on the way to me and so on (Popa M.E., Popa A., 2012).

So, first and second barriers in mentality to buy online are lack of transaction people to people and customer's fear face-to-face with online payment security.

Last year, NIS from Romania published a study on four years regarding the population access at the informational technology and communications (table 1).

ROMANIA: BARRIERS AND POSSIBILITIES IN ACCESSING ONLINE PRODUCTS

After the National Institute of Statistics, in 2016, two of three households in Romania have access to the home internet, also 65% of them are located in the urban area (Figure 1).

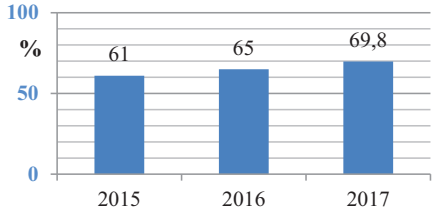

Figure 1. Romanian households with access to the internet between 2015-2017(http://www.insse.ro)

Table 1. Total households with access to the internet - % repartition after the head of the family domain of occupation

Year	Domain of occupation for the head of the family				
	Employee	Employer	Unemployed	Retired	Another inactive person (including pupil, student)
2014	58.8	9.9	2.4	25.2	3.7
2015	58.8	10.4	1.6	25.8	3.4
2016	58.8	9.4	1.7	27.1	3.0
2017	56.0	11.0	1.9	27.3	3.8

(*http://www.insse.ro/cms/sites/default/files/field/publicatii/accesul_populatiei_la_tehnologia_informatiei_si_comunicatiilor_romania_2017.pdf)*

According to the same source, reasons about not having the internet access at home in the same areal of time are described below (table 2). About the home internet type of broadband, first place is taken by fixed broadband connection, followed by the mobile ones and at a long distance the narrowband connections (Figure 2).

Table 2. Reasons for not having internet access at home

Year	Reasons which have not internet access at home						
	Access to the internet elsewhere	Not considered useful, interesting	Equipment is too expensive	Lack of skill	Fear about security and confidentiality of data on the internet	No connection at the broadband in home area	Another reason
2014	9.2	32.8	35.8	41.8	0.6	0.7	11.7
2015	7.9	42.8	30.4	43.3	1.1	1.2	9.8
2016	5.6	42.4	26.6	44.2	1.0	1.0	10.3
2017	6.2	40.6	22.4	50.3	1.9	1.1	14.4

(*http://www.insse.ro/cms/sites/default/files/field/publicatii/accesul_populatiei_la_tehnologia_informatiei_si_comunicatiilor_romania_2017.pdf)*

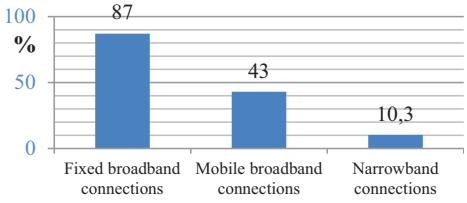

Figure 2. Types of internet connection on households (*https://www.comunicatii.gov.ro/)*

Also, we have a growing percent regarding the internet users.
More than 70% of persons between 16 and 74 years used the internet in 2017, 70% of persons between 16 and 74 years used the internet in 2016 (about 10.6 million users), 1.2% increase from 2015.
In this trend, unfortunately, we have a large percent of person who does not prefer to use mobile payments (Figure 3).

Internet Users in Select Countries Who Prefer vs. Don't Prefer to Use Mobile Payments, Aug 2017
% of respondents

	Prefer		Don't prefer
China	64%	Bulgaria	71%
Mongolia	63%	Greece	68%
Brazil	46%	Hungary	65%
Kenya	46%	Romania	64%
Chile	44%	Finland	61%
Colombia	41%	France	57%
Russia	41%	Germany	57%
Ukraine	39%	Luxembourg	56%
Cambodia	38%	US	54%
Saudi Arabia	36%	Ireland	53%
Myanmar	36%	Australia	53%
Sweden	35%	UK	52%
India	33%	Canada	52%
Mexico	33%	Cambodia	51%
Israel	33%	Myanmar	49%

Note: top 2 box "prefer mobile payment" and bottom 2 box "no mobile payment"
Source: Kantar TNS, "Connected Life," Oct 18, 2017

Figure 3. Internet users in August 2017 who prefer or do not prefer to use mobile payments (*www.emarketer.com)*

CONCLUSIONS

Barriers to online marketing, fear of consumer's mentality are represented below:

- lack of a household internet connection (applied to the person who has not a job and possibilities to make online shopping being at the job);
- all the customers can be targeted in online marketing. But not all customers have a credit or debit card;
- unknowing to write what is demand in special spaces on the site or fulfill the banking card data to send an order;
- untrust of measurements appeared at different sites,customer's fear face-to-face with online payment security;
- lack of transaction people to people;
- no trust in online payments and all the what if questions;
- more money that appears on screen, what if the product does not fit me or to person I want to make a gift, how much time I spend to finally I recover the money or receive another product;
- the language barrier remains the last reason for those customers who want to buy abroad.

Online marketing is an essential part of running a successful business in today's digital world.
Aside from advertising online, your online reputation is very important, even if you do not conduct business over the internet - before a new customer decides to patronize your business chances are they will check online reviews, so building a reputation for quality and customer service is very important.
Online marketing can deliver benefits such as:

- Growth in potential;
- Reduced expenses;
- Elegant communications;
- Better control;
- Improved customer service;
- Competitive advantage.

Unhappy customers are more likely to leave online reviews than satisfied ones, so having a strong reputation and plenty of positive online reviews are vital to business success in today's digital world.

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THE USE OF MOBILE APPLICATIONS IN THE FIELD OF BIOTECHNOLOGIES

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Abstract

The number of mobile users today is greater than the number of desktop users. Mobile applications have the advantage of utilizing features of a mobile device like camera, contact list, GPS, phone calls, accelerometer, compass, and so on. Such device features, when used within an app, can make the user experience interactive and fun.
The aim of the paper is to describe the making of the first electronic Romanian - language dictionary with biotech terms, ***being the first mobile application of its kind*** *and to highlighting the importance of using mobile applications in biotechnology both educational and industrial field.*
Mobile applications like dictionaries are useful for both researchers and students in various domains for a facile access to the necessary information directly from mobile phone so they can work or study wherever they are.

Key words: *biotechnology, education, e-learning, internet, mobile, mobile application.*

INTRODUCTION

Spectacular discoveries in the fields of biology, biochemistry, microbiology, genetics, enzymology, and the need to apply this knowledge in practice have led to the emergence of a new science called generic Biotechnology.
According to the European Federation of Biotechnology: *The integrated use of biochemistry, microbiology and engineering sciences in order to achieve technological application of the capabilities of microorganisms, cultured tissue, cells* is the definition of Biotechnology (The European Federation of Biotechnology-EFB, 1981).
In the field of biotechnological education, in recent years, there has been notable progress in terms of teaching and learning techniques. Using the Internet and modern technology in education have resulted in changes of substance (Toma, 2013; Toma, Pomohaci, 2005).
Computer and electronic (digital)/ multimedia materials are used as support in teaching, learning, assessment, or as a means of communication (Toma et al., 2016; Mărgărit et al., 2016)
The number of mobile users today is greater than the number of desktop users.
Mobile applications have the advantage of utilizing features of a mobile device like camera, contact list, GPS, phone calls, accelerometer, compass, and so on. Such device features, when used within an app, can make the user experience interactive.
Mobile applications like dictionaries are useful for both researchers and students in various domains for a facile access to the necessary information directly from mobile phone so they can work or study wherever they are (Toma, 2016).

MATERIALS AND METHODS

The huge part of the mobile devices' software uses the Android system.
Android is a mobile operating system based on a modified version of Linux (for hardware, process and memory management) and Java libraries (for telephony, connectivity, graphics, user interface programming).
As mobile applications are the ones that bring competitive advantage, the benefit of Android is the unified approach to application development. In other words, an application developed under the Android API will be able to run on multiple mobile devices where the operating system is installed.
The Linux kernel (with some modifications) contains drivers for various hardware (screen, camera, keyboard, Wi-Fi antenna, flash

memory, audio devices), responsible for process, memory, peripherals (audio / video GPS, WiFi), input / output devices, network and power consumption.
The application level contains both the products with which the mobile device is delivered (Browser, computer, Camera, Contacts, Clock, FM Radio, Launcher, Music player, Phone, S Note, s planner, Video Player, Voice Recorder) and the products installed from the Play Store or those developed by programmers (Figure 1).

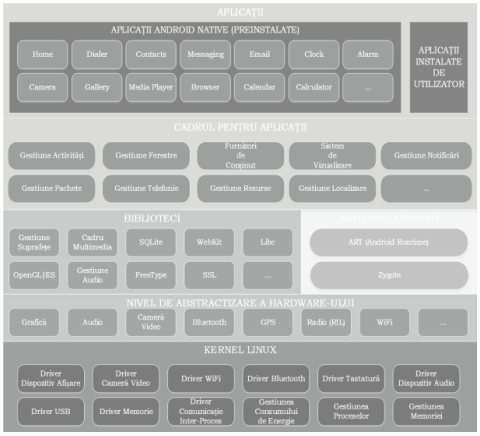

Figure 1. Android structure

The application presented is a hybrid application. Hybrid mobile applications are applications that are typically developed using WEB technologies (JavaScript, HTML, CSS).
The software is an electronic Romanian - language dictionary with biotech terms, being the first mobile application of its kind. The application is designed to help biotechnology workers ease their work if they need a dictionary with terms used in biotechnology. One can enter a word into the application, and he is given the definition of that word.
The Android mobile software described in this paper is built with the Unity engine with C # and Java programming elements.
Unity is a powerful engine and an extremely user-friendly interactive application development environment. It has the advantage of being very easy to use, both by the people who do not have the solid knowledge of programming as well as experienced ones.
The engine uses three programming languages: C #, Boo and Unity JavaScript and can be used to develop applications for most operating systems, even mobile ones (Figure 2).

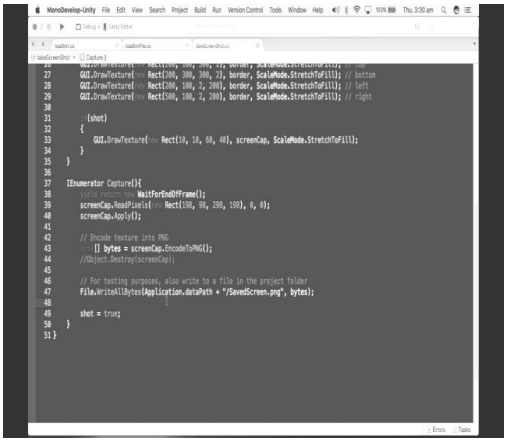

Figure 2. Unity screenshot

Unity can publish the output in Windows, OS X, and through the Web Player plug-in. Web Player is a browser plug-in that works with all known browsers and offers the same performance as the stand-alone desktop application.

RESULTS AND DISCUSSIONS

The definitions have been translated from an online English Dictionary. The dictionary contains about 300 words with their definitions. The action was repeated for each word, that is, a definition in part (Figure 3).

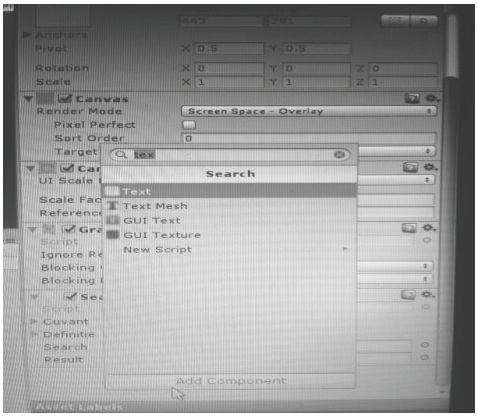

Figure 3. Unity screenshot

The next step was to create the Search button. After this step was created the Text field where

the definitions will appear after the Search action.
The following stage is the scene layout of the actions (Button, Text field, Field Search). The Scene page is shown in the following image (Figure 4):

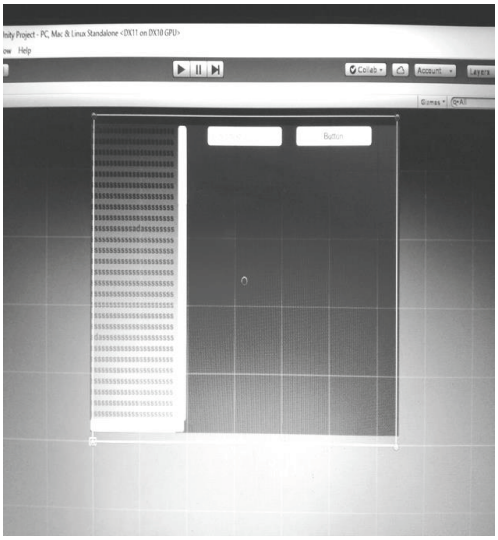

Figure 4. Scene screenshot (Unity screenshot)

When the app is started on the device a page with the name of the app is launched (Figure 5).

Figure 5. Open screen of application

The application is designed to help students ease their work if they need a dictionary with terms used in biotechnology. One can enter the word into the application, and the definition of that word is given as the result. Some examples of such definitions are given in the pictures below (Figures 6, 7).

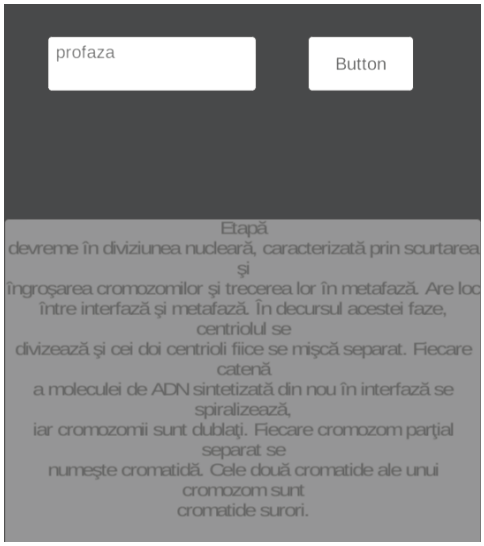

Figure 6. Screenshot of application

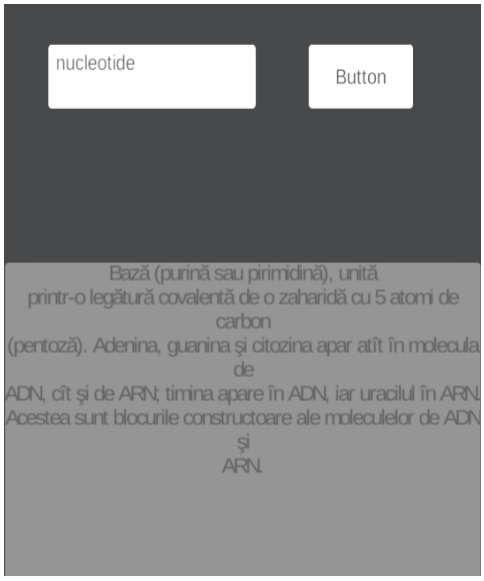

Figure 7. Screenshot of application

The font of the text is Arrial and its size is 18. The keyboard used is the Querty that Android uses (Figure 8).

Figure 8. Screenshot of application

The final result is a mobile application for Android smartphones (APK) of a dictionary of biotechnology terms, with about 300 terms.

CONCLUSIONS

Creating such a dictionary-based application is beneficial to biotechnology researchers as it facilitates access to the necessary information.
At the same time, the application is also useful for biotechnology students, or related fields, providing them with a modern, perfectly portable educational tool to quickly access information of interest as computer and electronic (digital)/ multimedia materials are used as support in teaching, learning, assessment, or as a means of information.
The application presented is a hybrid application. Hybrid mobile applications are applications that are typically developed using WEB technologies (JavaScript, HTML, CSS). Nowadays, hybrid applications are starting to grow stronger because many development environments are becoming more stable and provide access to more and more hardware features. Developing hybrid mobile applications can bring the following **benefits** to those who create them:
- Low development time for a wide range of operating systems;
- Faster learning of development technologies, being in principle WEB technologists;
- The app has a higher visibility on the platform where it is launched because it is distributed through the application markets used by most mobile users;
- Free development tools.
Hybrid applications also have **negative** points as follows:
- Dependence on tool developers, which may delay the launch of an application running on a new version of an operating system or delays in repairing technical issues that may occur especially with new versions of mobile operating systems;
- Lower performance in some places;
- Low reputation among loyal users of a particular mobile platform;
- Loss of time to fix the problems found by not running on all platforms as well.

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STUDIES ABOUT THE FISH FARMING DEVELOPMENT IN AQUAPONIC SYSTEMS: A REVIEW

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Abstract

Recirculating aquaculture systems have experienced a remarkable growth over the last decade all over the world. Aquaponics is a soilless agriculture system that synergistically combines aquaculture and hydroponics. The paper presents different types of aquaponic system designs that can sustain many fresh water species of fish like Carp and Tilapia. Aquaponics has great potential to become a sustainable technology for nitrogen-rich waste remediation with simultaneous year-round production of high quality fish and vegetables while conserving the water. The common carp (Cyprinus carpio) was the first species developed to have an organic production standard in 1994 in Austria. Later on organic aquaculture standards have been created for other species of fish and sea food. This review provides some necessary background to worldwide conventional small scale aquaponic systems.

Key words*: ecological aquaculture, aquaponics, recirculating aquaculture systems.*

INTRODUCTION

„*Ecological aquaculture is an integral part of our common planetary wisdom and cultural heritage, and is an essential part of our future evolution as a sophisticated species living in peace with the Earth's complex ecosystems."* (Costa Pierce, 2015).

The importance of aquaculture can be evaluated from an economic, social and environmental point of view, but especially from the multiple uses of the final product which can directly be offered for human consumption or can be distributed as raw material towards different industries. According to Food and Agriculture Organization, fish products own more than 14% of the animal protein products consumed globally. Moreover, we must admit that the development of the fish products industry can be the answer to a major problem that our fast growing population is facing. Demand and offer dynamics is characterizing the fish products market. Modified fishery resources, economy's climate and environmental conditions directly influence the market. Modernized aquaculture systems can be the solution to all these fluctuations. Aquaculture represents the fastest growing industry in the animal protein foods sector. Recording an annual growth rate bigger than 10%, fisheries can easily represent a quarter of the global fish production.

1. THE HISTORY AND CONTRIBUTION OF AQUAPONICS

Early civilizations from Asia and South America have applied the concept of using fish waste to fertilize plants millennia ago. Studies conducted in the late 1970's by the New Alchemy Institute and by the North American and European academic institutions have helped aquaponics to evolve into the modern food production. The success of aquaculture and hydroponics integration before 1980's was poor due to technological limitations. Later in the 1980's and 1990's, the system design has evolved. Recirculating aquaculture system was the design that permitted water recycling and also optimal nutrient buildup for plants to grow. Biological filtration and optimal fish to plant ratio were the key concepts that allowed this integrated system to work. A study led by the North Carolina State University demonstrated that the integrated hydroponic and aquaculture system can be suitable for growing plants and raising fish in arid and water poor areas. Water consumption in the integrated system was just 5 percent of that used in a regular pond for growing fish. Although the system is in use since the 1980's,

there are few researchers and practitioners that follow this new method of food production (Somerville et al., 2014).

James Rakocy is known as the father of aquaponics. Through his research at the University of Virgin Islands he developed vital ratios and calculations in order to maximize fish and vegetable production while maintaining a balanced ecosystem. During his research, interests for malnutrition, world hunger, wastewater management, ornamental fish, were brought together and combined into aquaponics. His main idea focused on diminishing the need for daily water exchange with new water due to buildup of nitrate ions in RAS (recirculating aquaculture systems). Aquaponic systems usually exchange less than 1% of the system's daily water compared to a 5-10% water exchange in RAS. This problem was solved by using plants to remove nitrates (Rakocy et al., 2006).

Subhendu Datta talks about Organic aquaculture as representing a new approach in fisheries development. As defined by the USDA's National Organic Standards Board (NOSB) „Organic agriculture is an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. It is based on minimal use of off-farm inputs and on management practices that restore, maintain and enhance ecological harmony." (Datta, 2006).

This description of organic agriculture can help us to better understand organic aquaculture. The term of organic is a very sensitive one. Organic practices cannot ensure that final products are absolutely free of residues. A purpose would be to minimize and reduce as much as possible air, water and soil pollution. The main goal of an organic food production process is to optimize the health and productivity of soil life, plants, animals and people (Datta, 2006).

Aquaculture represents the production of plants and aquatic animals under controlled conditions. There are multiple types of aquaculture systems that can combine one or more species of fish and other aquatic animals with different plants. Environmental conditions can influence conditions and species selection. Water salinity, temperature, oxygen level are probably the most important when concluding an aquaculture system. The major aquaculture systems are pond culture, cage culture, raceways, integrated and recirculating. Every system has different characteristics that can influence the production of organic foods or not.

Are recirculating aquaculture systems revolutionary? Aquaponics represents a system that combines recirculating aquaculture with hydroponics. This could be answer to the organic aquaculture because it is gaining increased attention as a bio integrated food production system.

2. TYPES OF AQUAPONIC SYSTEMS

There are three types of most commonly used aquaponic systems, classified based on types of grow bed, namely nutrient film technique (NFT), floating-raft (deep water culture) and media-filled (flood and drain) (Engle, 2015).

2.1. Media bed technique

For small - scale aquaponics, the most popular designs are media-filled bed units (Figure 1).

Media-filled type is the simplest aquaponic system that does not require separate biofilters because it contains media (e.g., pumice stones or clay beads) in the grow bed for nitrification (Zou et al., 2016). A siphon is used to fill and drain the water in order to supply oxygen by direct contact between plant roots and the air (Bernstein, 2011). Strongly recommended for most developing regions, these designs are suitable for beginners because of their simplicity, have a relatively low initial cost and are efficient with the space. The medium is used, in media bed units, to support the roots of the plants and also the same medium has the function of a filter. Both biological and mechanical, these double functions being the main reason why media bed units are considered the simplest. Anyway, this technique of media bed might become, at a larger-scale, relatively expensive and unwieldy. If fish stocking densities exceed the bed's carrying capacity, media may become clogged and separate filtration might be needed due to this. In media beds with larger surface area exposed to the sun the water evaporation is higher. There are some very heavy media (Somerville et al., 2014).

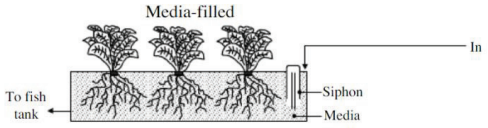

Figure 1. Media bed aquaponic system

2.2. Nutrient film technique

A hydroponic method using horizontal pipes each with a shallow stream of nutrient rich aquaponic water flowing through it is the NFT (Figure 2). The plants are able to use the thin film of nutrient-rich water from the top of the pipes where they are placed, within holes.

Popular methods for commercial operations, both the NFT and DWC are, when scaled up, more viable financially than media bed units.

Due to the fact that the water is completely shielded from the sun, this technique has a very low evaporation but it might not be appropriate in some locations with inadequate access to suppliers and it is far more expensive and complicated than media beds.

In urban applications, especially when some aspects such as weight limitations or vertical space are taken into consideration, this technique is proved to be most useful. NFT type provides high oxygen to the plant roots that facilitates high yield of vegetables (Somerville et al., 2014).

However, NFT is only suitable for small vegetable species because the grow bed cannot support high quantity of roots due to potential blockage of recirculating flow (Chérif et al., 1997; Engle, 2015).

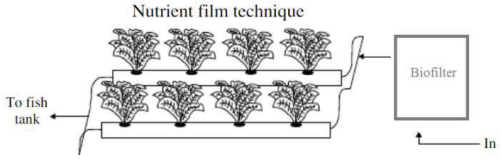

Figure 2. NFT aquaponic system design

The aquaponic systems have several common and essential components which include a fish tank, a biofilter, hydroponic containers and a mechanical filter.

Energy is used by all systems to circulate the water through the plumbing and pipes as aerating the water.

For every unit a crucial component is represented by the fish tanks and so, up to 20 percent of the entire cost of an aquaponic unit can be formed by the fish tanks cost.

In order to survive and thrive, the fish require certain conditions and consequently, the fish tank should be chosen really wisely. Several important aspects to consider are the shape, color and material (Somerville et al., 2014).

2.3. Deep water culture technique

Another method is DWC which requests suspending plants in some polystyrene sheets, while having their roots descending into the water (Figure 3).

For large commercial aquaponics that grow one specific crop (typically basil, lettuce or salad leaves), this method is more suitable for mechanization being also the most common.

Where access to materials is limited, this technique might not be suitable for some locations, being also, on a small-scale, more complicated than media beds (Somerville et al., 2014).

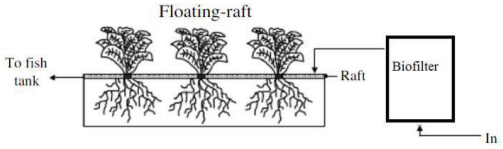

Figure 3. DWC aquaponics system

In 2006, Wilson A. Lennard and Brian V. Leonard conducted a comparison between these three subsystems. Murray Cod (*Maccullochella peelii peelii)*, Green Oak lettuce, and *Lactuca sativa* were used to test for differences.

The Murray cod recorded identical feed conversion ratio and biomass gains in all the 3 subsystems. Lettuce yields also gained biomass in the following relation MFBS > DWC > NFT. Phosphate removal recorded no significant difference in the compared subsystems while nitrate removal was significantly less efficient in the NFT. In conclusion NFT is the least effective subsystem used in this test when referring to biomass gain and nutrients removal probably due to low levels of root contact with water (Wilson et al., 2006).

In 2017, another comparison was made by A.A. Forchino, H. Lourguioui, D. Brigolin, R. Pastres. The objective was to evaluate the environmental impact. The test compared two different aquaponics techniques Raft System and Media Filled Beds System using the Life Cycle Assessment. Rainbow trout (*Oncorhynchus mykiss*) and lettuce (*Lactuca sativa*) were considered as cultivated species in both systems. Functional unit (FU) was set for 1kg of produced lettuce and the trout was defined as a co-product. The ratio of production resulted in an allocation of 91.6 % for lettuce and 8.4 % for trout. Both

systems used the same fish tank and the same water input. Emissions of nitrates and phosphorus were not taken into account because they have a neutral impact on the environment as previously underlined by Foteinis and Chatzisymeon (2016). Period of the test was set to 1 year and life cycle production of the lettuce was set to 21 days. Abiotic Depletion (AD), Global Warming Potential (GWP), Acidification (AC), Eutrophication (EU) and Cumulative Energy Demand (CED) were compared for the 2 subsystems. Environmental impacts studied by A.A Forchino can be observed (Figure 4). It is conclusive that Media Filled Bed System has a higher impact on the environment. In both techniques energy consumption played a key role, electricity representing the highest energetic cost. In conclusion, energy could be saved by optimizing the water flow, growing the number beds served by each pump and lowering the number of water pumps. (Forchino et al., 2017).

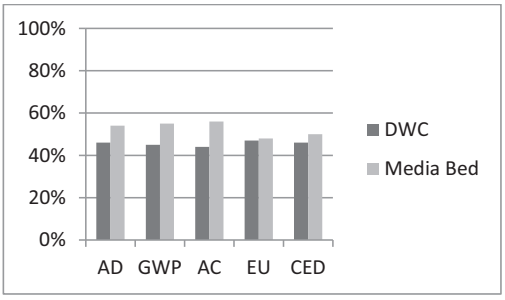

Figure 4. Comparison between DWC system versus Media-Filled Beds system using CML-IA and the Cumulative Energy Demand methods

3. WATER QUALITY IN AQUAPONIC SYSTEMS

Water is the most important subject to understand. It is the life-blood of the recirculating system and the medium through which the plants get the necessary nutrients and the fish receive the oxygen. There are discussed the following key water quality parameters: temperature, dissolved oxygen (DO), total nitrogen, pH and alkalinity of the water. The effects understanding of each parameter is decisive due to the fact that every parameter has an impact on all three organisms in the unit - plants, fish and bacteria. Keeping the high quality of the water in the aquaponic system is crucial. Even though some water parameters seem difficult to understand there is simple test kit that allows the user to easily interact and control the water (Somerville et al., 2014).

As a form of integrated agriculture, aquaponics combines the techniques of aquaculture and hydroponics. The culture water is evacuated from the fish tank which contains the fish metabolic waste, passing first through a mechanical filter which retains solid waste and after that through a biofilter which oxidizes ammonia to nitrate, all of it in a continuously recirculating unit. Travelling then through plant grow beds, where the plants assimilate the nutrients, the purified water returns finally to the fish tank (Figure 5). Bacteria can convert fish waste into accessible nutrients for plants with the help of the habitat offered by the biofilter. Dissolved in the water, these nutrients can be then absorbed by the plants. Cleaning the water and preventing the water from becoming toxic due to the harmful forms of nitrogen (ammonia and nitrite), the process of nutrient removal allows the plants, fish and bacteria to prosper symbiotically. If the system is properly balanced, all the organisms work together to create a healthy environment for each other (Somerville et al., 2014).

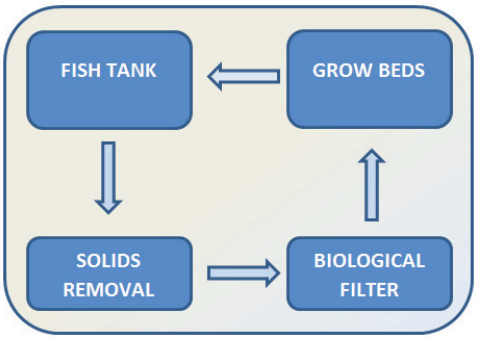

Figure 5. Aquaponic system water cycle

3.1. Biofiltration

The conversion of ammonia and nitrite into nitrate by living bacteria is commonly known as biofiltration. Due to the fact that the fish waste is dissolved directly in the water and the size of these particles is too small to be mechanically removed, most fish waste is not filterable using a mechanical filter and so, aquaponic systems use microscopic bacteria in order to process this microscopic waste. Because ammonia and nitrite are toxic even at low concentrations but the plants need the nitrates to grow, biofiltration is

essential in aquaponics. Besides, the very fine solids not captured by the clarifier will be broken down by the dynamic movement of water within the biofilter and that will prevent the further waste build up on the plant roots in DWC and NFT. Still, following the design of the system developed at the University of the Virgin Islands, some large aquaponic facilities do not use a separate biofilter because they mostly rely on the units' wet surfaces, on the plant roots and direct plant uptake to have the ammonia processed. Thanks to the fact that the grow beds themselves are perfect biofilters, the separate biofiltration is unnecessary in the media bed technique. In terms of aquaponics, mineralization refers to the way that bacteria processes and metabolizes the solid wastes into nutrients for plants. Although processing these wastes differs from biofiltration and requires a separate consideration, solid wastes that are trapped by the mechanical filter contain nutrients, too. More nutrients will be added back to the plants by retaining the solids within the overall system because there are subjected to some mineralization any wastes which remains on the mechanical filters, within the biofilters or in the grow beds. If the wastes are left in place for longer, more mineralization will be allowed and a longer residence time of the waste in the filters is leading to more nutrients being retained in the system and more mineralization too. Nevertheless, if not properly managed and mineralized, this same solid waste will consume oxygen, block water flow and lead to anoxic conditions, these leading to production and denitrification of the dangerous hydrogen sulfide gas (Somerville et al., 2014).

3.2. Mechanical filtration

Mechanical filtration is arguably the most important aspect of the design. Mechanical filtration is the separation and removal of solid and suspended fish waste from fish tanks. It is essential to remove these wastes for the health of the system, because harmful gases are released by anaerobic bacteria if solid waste is left to decompose inside the fish tanks. Moreover, the wastes can clog systems and disrupt water flow, causing anoxic conditions to the plant roots. There are several types of mechanical filters. The simplest method is a screen or filter located between the fish tank and the grow bed. This screen catches solid wastes, and needs to be rinsed often. Similarly, water leaving the fish tank can pass through a small container of particulate material, separate from the media bed; this container is easier to rinse periodically. These methods are valid for some small-scale aquaponic units, but are insufficient in larger systems with more fish where the amount of solid waste is relevant. There are many types of mechanical filters, including sedimentation tanks, radial-flow clarifiers, sand or bead filters and baffle filters; each of them can be used according to the amount of solid wastes that needs to be removed. A dedicated vessel that uses the properties of water to separate particles is called a clarifier. The water that is flowing faster is able to carry more particles than the water that is moving slower, usually, and consequently, the clarifiers are constructed in such a way as to slow down or to speed up the water so that to remove the particles concentrate on the bottom (Somerville et al., 2014).

3.3. Nitrification process

A crucial element of the whole nitrogen cycle seen in nature, the nitrification process is the most important biological process in aquaponics that transforms NH_4^+ to NO_3^- in the presence of oxygen (Hu et al., 2015).

The essential chemical element for all life forms is the chemical element Nitrogen (N) which is present in all amino acids that compose all proteins, these being essential for many key biological processes for animals such as enzyme regulation, cell signaling and the building of structures (Somerville et al., 2014).

Nitrogen is an essential element for all living organisms because it is the component of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), amino acids, protein and other cell components (Pratt and Cornely, 2014).

The major source of nitrogen input in aquaponic systems is fish feed, which is excreted by the fish in the form of ammonia nitrogen (90%) (M.B. Timmons et al., 2002).

The waste produced by fish is mostly made of ammonia (NH_3) that is metabolized by a specific group of bacteria, named nitrifying bacteria. These bacteria are very important for aquaponics because they are able to transform first the ammonia into nitrite compounds (NO_2^-) and after that into nitrate compounds (NO_3^-). Even if for the plants are useful both ammonia and nitrates to

help them in the growing processes, the nitrates can be easier assimilated by their roots. The process of nitrification by bacteria, which is natural and takes place in soil, similarly happens in the water, too. The fish excreta that are released in the culture tanks are the animal wastes for aquaponics. Changing the fish waste ammonia into the easily assimilated nitrate, so useful for plants, is made by the same nitrifying bacteria which live on land and will also naturally be found in the water or on every wet surface (Somerville et al., 2014).

In aquaponic systems, nitrification ensures nutrients for the plants and releases ammonia and nitrite that are toxic. In the nitrification process there are involved two main groups of nitrifying bacteria: the ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB). First, AOB bacteria change ammonia (NH_3) into nitrite (NO_2^-). Next, NOB bacteria transform nitrite (NO_2^-) into nitrate (NO_3^-). After the ammonia is oxidized (oxygen added to) by the AOB, it is created the nitrite (NO_2^-) and further the NOB oxidize the nitrite (NO_2^-) into nitrate (NO_3^-) (Figure 6) (Ebeling, Timmons and Bisogni, 2006).

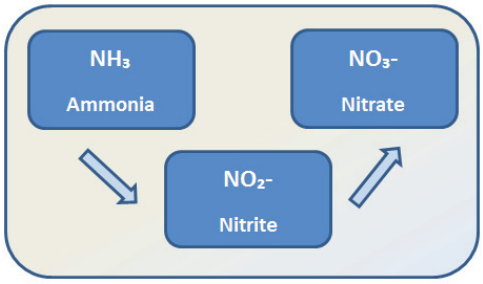

Figure 6. The nitrification process

In aquaponics the most common AOB is the genus *Nitrosomonas* and the most common NOB is the genus *Nitrobacter*. Ammonia oxidizing archaea (AOA) is responsible for oxidizing ammonia under extremely low NH_4^+ concentrations (about 2 µg N/L) due to their physiological diversity, leading to toleration and adaptation to extreme nutrient limitations (Martens-Habbena et al., 2009). Thus, nitrification by AOA does not significantly occur in aquaponic systems (Hu et al., 2015).

As a conclusion, the ecosystem from the aquaponic unit relies totally on the bacteria. Ammonia concentrations in the water tank will kill the fish if there are no bacteria. In order to manage the ammonia level and to keep it close to zero it is vital to maintain a healthy bacterial colony all the time (Somerville et al., 2014).

3.4. Water pH and temperature

An important impact on all aspects of aquaponics, mainly on the plants and bacteria, has the water pH. This controls the access of the plants to micronutrients and macronutrients. pH is a main factor that controls fish metabolism, microbial activities and affects the availability of nitrogen to plants (Kuhn, et al., 2010).

All nutrients are readily available at a pH of 6.0 - 6.5 but if this range is exceeded, the nutrients become hardly accessible for plants. A pH of 7.5 can actually lead to iron, phosphorus and manganese nutrient deficiencies. Below a pH of 6 the nitrifying bacteria experience difficulties and its capacity to transform ammonia into nitrate is reduced. Due to this, the biofiltration can be reduced and, as a result, the bacteria reduce the level of the conversion of ammonia to nitrate and so, the levels of ammonia can begin to increase, this leading to an unbalance of the system which is stressful for the other organisms. pH can be periodically adjusted by using potassium hydroxide (KOH) and calcium hydroxide (Rakocy, Shultz, Bailey, Thoman, 2004). Having their own specific tolerance ranges for pH as well, the most used fish in aquaponics have a pH tolerance range of 6.0-8.5. Due to the fact that the pH affects the ammonia toxicity to fish, a higher pH leads to higher level of toxicity. In summary, the ideal water for aquaponics has an optimum pH range of 6-7 and it is slightly acidic. The pH range of 6-7 keeps the functioning of the bacteria at a higher capacity and, at the same time, allows the plants to absorb all the essential nutrients. A pH higher than 8 or lower than 5, requires an immediate attention because such values can jeopardize the entire ecosystem (Somerville et al., 2014).

An important parameter for bacteria mainly, and for aquaponics in general, is the temperature of the water. For bacteria growth and productivity 17-34°C is the ideal range of temperature. The bacteria productivity will decrease if the water temperature drops below 17°C. The productivity can be reduced by 50 percent or even more for temperatures below 10°C because these low

temperatures have important effects on unit management (Somerville et al., 2014).

3.5. Dissolved oxygen

The essential element needed for all three organisms involved in aquaponics: plants, fish and nitrifying bacteria is the oxygen. The amount of molecular oxygen from the water is described by the DO level, which is measured in milligrams per liter. This parameter of the water quality has on aquaponics the fastest and strongest effect. In order to maintain high levels of productivity of the nitrifying bacteria, it is permanently needed an adequate level of dissolved oxygen in the water. Nitrification is an oxidative reaction where oxygen is used as a reagent and thus the reaction stops without oxygen. The most favorable levels of DO are 5-6 mg/liter to avoid the stress to fish and plants (S. Bernstein, 2011).

If DO concentrations drop below 2.0mg/liter, the nitrification will decrease. DO concentration of above 1.7 mg/L was recommended in biofilters to maintain the activity of nitrifiers (Ruiz et al., 2003). Furthermore, in the absence of sufficient DO concentrations another type of bacteria can develop and will transform the valuable nitrates back into unusable molecular nitrogen, through denitrification, which is an anaerobic process (Somerville et al., 2014).

3.6. Total nitrogen

The fourth crucial parameter for the water quality is the nitrogen and, as part of all proteins, it is needed for all life. Usually labelled as crude protein and measured as a percentage the nitrogen is entered, originally, in aquaponic systems from the fish feed. The fish use for their growth some of this protein. The main form of this waste is ammonia (NH_3) which is released by fish through the gills and as urine. They release also solid waste, some of it being converted into ammonia by microbial activity. At certain concentrations, nitrogenous wastes are poisonous for fish but ammonia and nitrite are about 100 times more poisonous than nitrate. Even if the nitrogen compounds are nutritious for plants and they really are the basic components of plant fertilizers, they are toxic for fish. The plants are able to use all three forms of nitrogen (NH_3, NO_2^- and NO_3^-). Nitrate being by far the most accessible one. Ammonia and nitrite levels should be close to zero in a fully functioning aquaponic unit, or at most 0.25-1.0 mg/liter. Almost all the ammonia and nitrite should be converted by the bacteria from the biofilter into nitrate (Somerville et al., 2014).

3.7. Ultraviolet light

Being photosensitive organisms, ultraviolet (UV) light from the sun is a threat for the nitrifying bacteria, especially in the case of the initial formation of the bacteria colonies, when a new aquaponics system is set up. The UV light does not pose any major problem after the bacteria have colonized a surface (3-5 days). Covering the filtration components and the fish tank with UV protective materials and making sure that the water in the hydroponic component is not exposed to the sun, at least until the bacteria colonies are fully formed, is a very simple way to remove this threat. Nitrifying bacteria are able to grow on large surface area materials being sheltered by UV protective material and having appropriate water conditions (Somerville et al., 2014).

4. TYPES OF FISH SPECIES SUITABLE IN AQUAPONIC SYSTEMS

There are more fish species that have recorded excellent growth rates in aquaponics units. Such fish species, which are suitable for aquaponics farming, include: common carp, tilapia, silver carp, grass carp, barramundi, jade perch, trout, salmon, largemouth bass and catfish. Worldwide available, some of these species grow especially well in aquaponics units and they are discussed more detailed in the following sections. It is crucial to appreciate the importance of the availability of healthy fish from reputable local providers when planning an aquaponic facility (Somerville et al., 2014). Some species and some production systems may prove quite difficult to adapt to a traditional „organic" system (Boehmer et al., 2005).

4.1. Tilapia

Blue tilapia (*Oreochromis aureus*)

Nile tilapia (*Oreochromis niloticus*)

One of the most popular freshwater species which grow in aquaculture systems worldwide are the tilapias, originary from East Africa. Resistant to many parasites and pathogens and handling well the stress, they do best in warm temperatures and are able to tolerate a wide range

of water quality conditions. In spite of the fact that tilapias can briefly tolerate extremes temperatures of the water like 14°C and 36°C they do not feed or grow below 17°C, and they even die below 12°C. Because the ideal range for tilapias, in order to ensure good rates of the growth is between 27-30°C, in temperate climates tilapias might be suitable for winter seasons only if the water is heated. For cool climates, an alternative method is to grow multiple species all year long: during the warmest season tilapia and during the winter changing to trout or carp. If they have ideal conditions, in about 6 months tilapias can grow to maturity (500 g) from fingerling size (50 g).
As omnivores, tilapias feed on both plant and animal based feed and they are candidates for many alternative feeds. *Moringa olifera*, duckweed, *Azolla* spp. and other high-protein plants have been used to feed tilapias, but they must be very carefully fed in order to ensure a nutritionally complete feed. The tilapias eat other fish and this should be taken into consideration when breeding and they should be separated by size because if not, they eat their own young. Tilapias which are less than 15 cm eat smaller fish, but when they are larger than 15 cm, they are generally too slow and, due to this fact, they are not a problem anymore. In small-scale and medium-scale aquaponic systems can be easily bred tilapias (Somerville et al., 2014) (Turek Rahoveanu et al., 2018).

4.2. Carp

Common carp (*Cyprinus carpio*)
Silver carp (*Hypophthalmichthys molitrix*)
Grass carp (*Ctenopharyngodon idella*)
The most cultured fish species globally at the present time, carps are originary of Eastern Europe and Asia. The same like tilapias, the carps are tolerant to poor water quality and relatively low DO levels, having a much larger tolerance range for water temperature, nevertheless. Their ability to survive at temperatures as low as 4°C and as high as 34°C, makes carps, in both temperate and tropical regions, an ideal selection for aquaponics. The best growth rates are obtained when temperatures are between 25°C and 30°C and they can grow in less than a year (10 months) from fingerling to harvest size (500-600 g) in proper conditions. When temperatures are lower than 12°C, the growth rates decrease dramatically. Even if they are smaller than females, the male carps are still able to grow in the wild up to 40 kg and 1-1.2 m in length. They are bottom feeding omnivores, in the wild, eating a large range of foods. They prefer feeding on invertebrates such as insect larvae, water insects, mollusks, zooplankton and worms. There are some herbivorous carp species which also eat the stalks, leaves and seeds of aquatic and terrestrial plants, and decaying vegetation, too. It is easy to train the cultured carps to eat floating pellet feed (Somerville et al., 2014).

4.3. Ornamental fish

Produced mainly for the ornamental fish industry rather than for food fish industry, Gold or Koi carps have also a high tolerance to a variety of water conditions being, therefore, very good candidates for aquaponic systems. Being sold to aquarium stores and individuals for considerably more money than fish sold as food, a popular choice for vegetarian aquaponic growers are Gold or Koi carps and other ornamental fish. Choosing a carp species to be cultured in aquaponics should be done, beyond the climatic characteristics and fish management issues, after a cost–benefit analysis which takes into account the advantages of culturing a fish that is bonier and usually has lower market prices than other species and consequently, less profit (Somerville et al., 2014).

4.4. Catfish

Channel catfish (*Ictalurus punctatus*)
African catfish (*Clarias gariepinus*)
An extremely hardy group of fish that tolerate very well wide swings in DO, temperature and pH are the catfish. The fact that they are also resistant to many parasites and diseases makes them ideal for aquaculture. Catfish are able to be easily stocked at densities up to 150kg/m3, which is a very high value. These stocking densities require comprehensive mechanical filtration and solids removal, topics that are beyond those discussed in this material. One of many species in the *Clariidae* family is the African catfish. Air breathers, these species are ideal for aquaculture and aquaponics due to the fact that there would not be any fish mortalities in case of a sudden and dramatic drop in DO. For beginners and also for aquaponists who want to grow fish in areas where the electricity supply is not reliable, the

easiest species are catfish. If there is an adequate mechanical filtration, given the high tolerance to low DO levels and high ammonia levels, they can be stocked at higher densities. Regarding the management of the waste, it must be noted that a factor that facilitates greater mineralization is that suspended solid waste produced by catfish is less voluminous and more dissolved than catfish, as tilapias prefer a temperature of 26°C and grow best in warm water, but, the growth stops below 20-22°C in the case of African catfish. The catfish physiology is different from other fish because, even if the can tolerate well high levels of ammonia, as stated by the recent literature, their appetite might be reduced due to an internal regulatory control trigged by high levels of nitrate in their blood for nitrate concentrations higher than 100 mg/liter. Being benthic fish, the catfish occupy only the bottom portion of the tank and raising them at high densities can be affected by the fact that they do not spread out through the water. The catfish can hurt each other with their spines if the tanks are overcrowded and so, one option, when raise catfish, is to allow the fish to spread out along the bottom using a tank with greater horizontal space than vertical space. As an alternative, catfish are raised by many farmers together with another species of fish that utilize the upper portion of the tank, commonly bluegill perch, sunfish or tilapia. The catfish are also able to be trained to eat floating pellets (Somerville et al., 2014).

4.5. Trout

Rainbow trout (*Oncorhynchus mykiss*)

Cold water fish, belonging to the salmon family, trout are carnivorous fish that need colder water than the species previously mentioned. Their preferred temperature range is 10–18°C and 15°C as an optimum temperature. That's why trout are considered, especially in winter, ideal for aquaponics in Nordic or temperate climate regions. When the temperature increases above 21°C, the growth rates decrease significantly. Trout might not be able to utilize DO properly, even if available, above this temperature. Compared with tilapia and carp, trout need a higher protein diet that means adding larger quantities of nitrogen in the overall nutrient pool per unit of fish feed. While maintaining a balanced aquaponic unit, this occurrence allows for more cultivable areas of leafy vegetables. Even though generally trout have a very high tolerance to salinity, and many varieties are able to survive in freshwater, brackish water and marine environments, yet these species need better quality of the water than tilapia or carp, especially regarding the ammonia and DO. A successful aquaculture of trout requires a frequent water quality monitoring and also air and water pumps backup systems (Somerville et al., 2014).

4.6. Prawn

Giant river prawn (*Macrobrachium rosenbergii*)

"Prawn", as term, refers to a various group of stalk-eyed freshwater decapod crustaceans with slender legs, long antenna and long, narrow, muscular abdomens that are found on the bottom of most estuaries and coastlines, as well as in freshwater systems. Their most species are omnivores and they can live from one to seven years. Commonly referred to saltwater and freshwater species, the names of shrimps and prawns are, especially in the culinary sense, often confused. Due to the fact that prawns ingest uneaten fish food, fish waste and whatever organic stuff they find in the water or on the bottom, they can be a very clever addition to any aquaponic system, helping to clean and supporting system health and accelerating organic material decomposition. Because prawns cannot be grown in densities high enough to produce adequate wastes for the plants, it is better to grow prawns and mid-water fish at the same time, in an aquaponic system. Being very territorial, the prawns need a substantial allocation of lateral space as horizontal surface area determines the number of individuals that can be raised. However, surface area and quantity could be increased by stacking layers of netting. Even if the number of individuals that can be stocked is low, there have been tested some polyculture systems with tilapia which had different degrees of success. The needs of most prawns are similar. These including warm temperatures (24-31°C), good water quality and hard water, still, these conditions need to be adjusted considering the grown species. Taking into consideration that prawns have a four-month growing cycle in ideal conditions, it would mean, theoretically, that three crops grow annually. Post-larvae prawns must be bought from a hatchery. Considering the fact that the larval

cycle of prawns is fairly complex and requires special feed and carefully monitored quality of the water, breeding prawns is recommended just for experts, even if they are easier possible on a smaller scale.

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