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ANTIOXIDANT AND PESTICIDE POTENTIAL OF SAGE HYDROSOLS

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Abstract

The aim of this study was to evaluate the properties of sage (Salvia officinalis L.*) hydrosols in terms of the antioxidant activity and inhibition of acetylcholinesterase, in order to be used in novel formulas of biopesticides for preventing aphid infestation of crops. Sage hydrosols were obtained as by-products of steam and reflux distillation, respectively, after essential oil extraction from dried aerial parts of the plant. The hydrosols were analyzed for terpenes and polyphenols composition by GC/MS and HPLC. The antioxidant activity of sage hydrosols was evaluated using Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical inhibition assay and cupric reducing antioxidant capacity assay (CUPRAC). Their pesticide potential was assessed using an experimental model in vitro and colorimetric measurement of acetylcholinesterase inhibition kinetics. The results showed that sage hydrosols contained significant amounts of phenolic acids and flavonoids, exhibiting a positive correlation with the antioxidant activity values. At the same time, sage hydrosols were responsible for acetylcholinesterase inhibition in a dose-dependent manner. In conclusion, sage hydrosols are natural effective products recommended for pest management solutions.*

Key words*: acetylcholinesterase, aphids, sage hydrosol, polyphenols, terpenes.*

INTRODUCTION

Hydrosols, also known as hydrolates or floral waters, are by-products of the essential oils extraction process through aromatic plants distillation. In the recent years, they were valorized as natural ingredients in aromatherapy, skin care products and preparation of cakes and drinks (Paolini et al., 2008; Aazza et al., 2011). Hydrosols consist of water-soluble plant compounds (polyphenols) and the aqueous fraction of essential oils, which gives them a pleasant odor. Therefore, the rose and orange hydrosols are the most used in food industry, in the Mediterranean area.

Several studies were focused on the chemical composition analysis of hydrosols resulted from aromatic plants present in different geographical areas, such as Morrocan thyme (*Thymus vulgaris*) (Aazza et al., 2011), Polish lavender (*Lavandula angustifolia*) (Prusinowska et al., 2015), Algerian *Calendula arvensis* (Belabbes et al., 2017) or Mexican oregano (*Poliomintha longiflora*) (Cid-Perez et al., 2019).

Moreover, the evaluation of their antioxidant, antibacterial and antifungal properties indicated their potential to act as natural pesticides, as such, or in combination with surfactants (Tornuk et al., 2011; Hay et al., 2015; Georgiev et al., 2019). It was reported that the redox and neuro-toxic/protective processes could be controlled by bioactive phytochemicals, such as terpenes, phenols, alkaloids, present in hydrosols or essential oils (Degirmenci & Erkurt, 2020). Some compounds, such as rotenone, an isoflavone extracted from *Derris elliptica* and azadirahtin, a secondary metabolite from neem oil, were identified and could act as plant biofungicides and bioinsecticides due to their toxicity and repellent activity (Spochacz, 2018; Hernandez-Carlos, 2019).

All these studies showed that, unlike synthetic pesticides, the hydrosol - and essential oil - based biopesticides had rapid biodegradation,

low toxicity, low risk of developing pest resistance and low cost (Rizvi, 2019).

In this context, the present study aimed to evaluate the chemical composition of Romanian sage hydrosols obtained by steam and reflux distillation and their antioxidant and pesticide activity, in order to be used in eco-agrosystems pest control.

MATERIALS AND METHODS

Extraction of sage hydrosols

The hydrosols were obtained from flowering aerial parts of sage (*Salvia officinalis* L.) by hydrodistillation using a Clevenger-type extraction apparatus (J.P. Selecta). The plant material was air-dried, powdered and an aliquot (25 g) was moistened in ultrapure water. Two extraction methods were applied, one based on steam distillation and the other on reflux using ultrapure water (750 mL), at 100°C, for 2 h. At the end of the incubation step, the essential oil was separated from the aqueous fraction, representing the sage hydrosol, based on the density difference. The steam distilled sage hydrosol (SSH) and the reflux sage hydrosol (RSH) were filtered and stored in the dark, at 4°C, until analysis.

Gas-chromatography/mass-spectrometry analysis

Sage hydrosols were analyzed by gas-chromatography coupled to mass-spectrometry (GC/MS) using a Focus GS-type equipment coupled to a mass spectrometer DSQ II (Thermo Electron Corporation, USA). The separation was performed on Macrogol 20,000 R capillary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1 mL/min. The mass spectrometer was operated at 70 eV with a scan interval of 0.5 s and scan range between 40-1000 m/z. The identification of the main constituents was performed by comparing the spectra with NIST mass spectral database.

HPLC analysis

Polyphenolic compounds from sage hydrosols were investigated by HPLC analysis. A reverse phase column C18 Zorbax Eclipse XDB (150 x 4.6 i.d. mm) was mounted on an Agilent 1200 HPLC system consisting of a quaternary pump, thermostated autosampler and diode array detector (Agilent, Germany). A sample (10 µl) was injected and then eluted using mobile phase A consisting of 2 mM sodium acetate, pH 3 and mobile phase B, acetonitrile, using the following gradient: 2-20% B, 0-30 min; 20-30% B, 30-40 min; 30% B, 40-50 min; 30-2% B, 50-60 min (Craciunescu et al., 2012). The identification of compounds was performed by comparison to the retention times (RT) of phenolic acids (gallic acid, caffeic acid, chlorogenic acid, ferulic acid) and flavonoids (rutin, luteolin, quercetin 3-O-glucoside (isoquercetin), kaempferol 3-O-glucoside (astragalin), luteolin-7-O-glucoside (cynaroside), quercetin, apigenin, kaempferol) standards (Sigma), at 280 and 370 nm. Quantification of the identified compounds was performed by peak area integration using standard curves built on the range of standard concentrations between 10-500 µg/mL.

Determination of total phenolic and flavonoid content

Total phenolic content (TPC) was determined by Folin-Ciocalteu method, as previously described (Moldovan et al., 2011). Briefly, the sample was mixed with Folin-Ciocalteu reagent (1:5, v/v) and incubated in the dark, for 5 min. Then, 2 mL of 12% sodium carbonate was added and the mixture was incubated at the room temperature, for 30 min. The solutions optical density (OD) was measured at 765 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The standard curve was built using different concentrations of caffeic acid in the range of 0-500 µg/mL. The results were expressed as caffeic acid equivalents (CAE) per 100 g of sample in dry weight (d.w.).

Total flavonoids content (TFC) was determined by aluminum chloride method, as previously described (Gaspar et al., 2014). Briefly, the sample was mixed with methanol (1:3, v/v), 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water. After incubation at the room temperature, for 30 min, the OD of the mixtures was read at 415 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The standard curve was built using different concentrations of quercetin in the range of 0-150 µg/mL. The results were expressed as quercetin equivalents (QE) per 100 g of sample in d.w.

Determination of free radicals scavenging activity

The Trolox equivalent antioxidant capacity (TEAC) assay was based on sample's ability to scavenge cationic free radicals of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and it was performed according to a previous protocol with minor modifications (Hay et al., 2015). Briefly, the stock solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1, v/v) and incubation at the room temperature, in the dark, for 16 h. Then, the solution was diluted to obtain an OD of 0.70 ± 0.02 at a wavelength of 734 nm. Different concentrations of sample were mixed with ABTS solution (1:10, v/v) and incubated at the room temperature, in the dark, for 10 min. The OD was read at 734 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The standard curve was built using an analog of vitamin E, Trolox, on the range of concentrations 0-250 µM. The results were expressed as Trolox equivalents (TE) per 100 mg of sample in d.w.

Determination of free radicals inhibition

The assay was based on sample's capacity to inhibit the formation of free radicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and it was performed according to Sugahara et al. (2015) protocol with slight modifications. Briefly, 150 µL of sample at different concentrations (10-500 µg/mL) were mixed with 0.9 mL of 0.1 M Tris-HCl buffer, pH 7.4 and 1.35 mL of 0.25 mM DPPH solution. The sample was replaced by an equal volume of buffer to prepare the blank. The mixtures were incubated at the room temperature, in the dark, for 30 min and then, the OD was read at 517 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The results were calculated using the following formula:

$$\%\ DPPH\ inhibition = (1 - OD\ sample)/OD\ blank \times 100$$

A known synthetic antioxidant agent, butylated hydroxytoluene (BHT) and a natural agent, ascorbic acid were similarly processed to serve as controls. The sample concentration (µg/mL) that inhibited 50% free radicals represented the IC50 value.

Determination of the antioxidant capacity

The assay was based on cupric ion reducing antioxidant capacity (CUPRAC) of the sample and it was performed according to an adapted protocol (Georgiev et al., 2019). Briefly, 1 mL of 10 mM $CuCl_2$ solution was mixed with 1 mL 7.5 mM neocuproine and 1 mL of 1 M ammonium acetate buffer, pH 7. The mixture was vortexed and incubated at the room temperature, for 10 min, to develop the complex. Then, 100 µL of sample and 1 mL of distilled water were added and incubation continued at the room temperature, for 1 h. The OD was read at 450 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The sample was replaced by an equal volume of distilled water to prepare the blank. The standard curve was built using Trolox in the range of concentrations 0.1-1.0 mM. The results were expressed as TE per 100 mg of sample in d.w.

Determination of acetylcholinesterase inhibition *in vitro*

The colorimetric assay was based on thiocoline reaction with Ellman's reagent and it was performed using a 96-well microplate adapted protocol (Mathew & Subramanian, 2014). Briefly, in the wells of a microplate, 100 µL of 3 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reagent was mixed with 20 µL of acetylcholinesterase (AChE) (0.125 U/mL) in 50 mM Tris buffer, pH 8 containing 0.1% BSA and 20 µL sample of different concentrations. The plate was incubated at 25ºC, for 15 min and the OD was read at 412 nm (OD control) using a SpectroStar nano microplate reader (BMG Labtech, Germany). Then, 20 µL of 7.5 mM acetylthiocholine iodide were added as enzymatic substrate and the kinetics of the hydrolysis reaction was recorded as OD at 412 nm, every 3 min, for 30 min (OD sample). A blank was similarly processed after sample replacement with an equal volume of buffer (OD blank). The results were calculated using the following formula:

$$\%\ AChE\ inhibition = \frac{(OD\ sample - OD\ control)}{(OD\ blank - OD\ control)} \times 100$$

The sample concentration (µg/mL) that inhibited 50% AChE activity represented the IC50 value.

Statistical analysis

The experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) (n = 3). Statistical differences were calculated by two-tailed, two-sample equal variance Student *t*-test and they were considered significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

In this study, sage hydrosols were obtained as brown solutions with pleasant odor by steam (SSH) and reflux (RSH) hydrodistillation of the aromatic plant after separation of the essential oil. The yield of SSH extraction was 11.92 ± 1.09% (w/w), while the yield of RSH extraction was 18.11 ± 1.22% (w/w).

Chemical composition of sage hydrosols

Chemical analysis of RSH by GC/MS showed the presence of 15 volatile compounds (Figure 1), while in SSH they were detected only in traces. The identified constituents, their RT and abundance (%) are presented in Table 1.

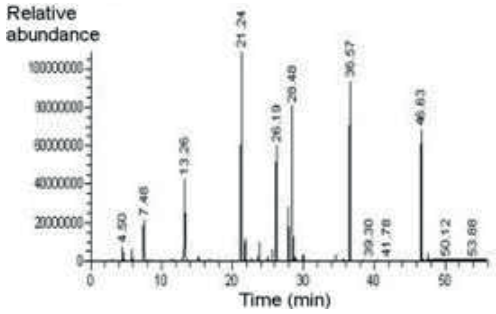

Figure 1. GC/MS chromatogram of sage hydrosol

Table 1. Percentage composition of volatile compounds determined by GC/MS in sage hydrosol

No.	Major compound	RT	% of total peak area
1.	α-Pinene	4.50	1.67
2.	Camphene	5.78	1.58
3.	β-Pinene	7.48	7.44
4.	1,8-cineole	13.26	12.69
5.	Trans-β-ocimene	15.14	0.57
6.	α-Thujone	21.23	17.06
7.	β-Thujone	21.80	2.50
8.	Camphor	23.73	1.57
9.	Bornyl acetate	25.63	0.94
10.	α-Caryophyllene	26.19	14.59
11.	β-Caryophyllene	27.90	5.87
12.	Borneol	28.48	9.53
13.	Cadinene	29.96	0.72
14.	Viridifloral	36.57	11.81
15.	Epimanool	46.63	11.46

RSH consisted mainly of monoterpenes and their oxygenated derivatives. The main components were β-pinene (7.44%), 1,8-cineole (eucalyptol) (12.69%), α-thujone (17.06%), α-caryophyllene (14.59%), borneol (9.53%), viridifloral (11.81%) and epimanool (11.46%). A similar composition was reported for the essential oil extracted from Romanian sage, but components abundance was different, the oil being rich in α-thujone (34.63%), β-thujone (13.10%) and camphor (16.02%) (Popescu et al., 2018). GC-MS analysis of the essential oil extracted from Italian *S. officinalis* seeds showed that the main constituents were 1,8-cineole (6.67%), α-thujone (14.77%) and camphor (13.08%), which indicated a similar composition to that of sage flowering aerial parts (Taarit et al., 2014). The presence of these water-soluble volatile compounds in sage hydrosols indicated their potential to be used as valuable, quality products, instead of by-products and wastes (Baydar et al., 2013).

Polyphenolic composition of sage hydrosols

HPLC chromatograms of SSH and RSH are presented in Figure 2.

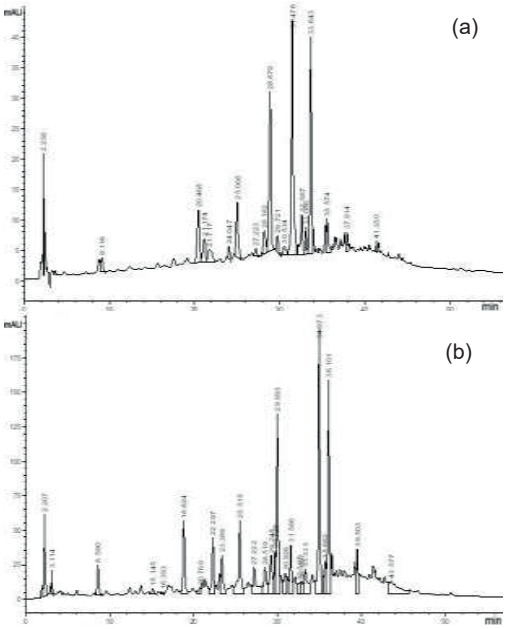

Figure 2. HPLC chromatograms of sage hydrosols obtained by steam (SSH) (a) and reflux (RSH) (b) hydrodistillation

SSH had 8 major peaks at 2.23, 20.46, 25.00, 28.87, 31.47, 32.58, 33.64 and 35.61 min. RSH had 10 major peaks at 2.20, 8.59, 18.82, 22.29, 23.38, 25.51, 29.99, 31.58, 34.97 and 36.10 min. The identified and quantified polyphenolic compounds based on the RT and integrated areas, respectively, are presented in Table 2. The results showed that SSH had higher quantities of phenolic acids, such as caffeic and ferulic acid, and flavonoids, like rutin and the glycosylated derivatives of quercetin, luteolin

and kaempferol, compared to those in RSH (Table 2). Free flavonoids of quercetin, luteolin and kaempferol were not detected, as against their glycosylated forms.

Table 2. HPLC analysis of phenolic acids and flavonoids in sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation

No.	Compound	SSH (mg/g d.w.)	RSH (mg/g d.w.)
1.	Gallic acid	ND	ND
2.	Chlorogenic acid	ND	1.05
3.	Caffeic acid	7.08	1.84
4.	Ferulic acid	10.70	5.63
5.	Rutin	56.62	10.72
6.	Isoquercetin	27.75	4.13
7.	Luteolin-O-glucoside	29.53	5.28
8.	Kaempferol-O-glucoside	17.71	8.63
9.	Quercetin	ND	ND
10.	Luteolin	ND	ND
11.	Apigenin	ND	ND
12.	Kaempferol	ND	ND

The results of TPC and TFC determination are presented in Table 3.

Table 3. Total polyphenolic (TPC) and flavonoid (TFC) content and antioxidant activity of sage hydrosols

Sample	TPC (mg CAE/ 100 g d.w.)	TFC (mg QE/ 100 g d.w.)	TEAC (mM TE/ 100 mg d.w.)	CUPRAC (mM TE/ 100 mg d.w.)
SSH	32.55 ± 1.71	5.93 ± 0.03	245.17 ± 11.68	241.04 ± 13.83
RSH	29.89 ± 2.20	3.08 ± 0.05*	208.75 ± 6.94*	210.02 ± 9.54*

*p<0.05, compared to SSH value

Data showed similar content of polyphenols in both sage hydrosols (~30 mg CAE/100 g d.w.), but flavonoids content was double in SSH obtained by steam distillation (6 mg QE/100 g d.w.), compared to RSH (3 mg QE/100 g d.w.).

Antioxidant activity of sage hydrosols

Three methods based on different scavenging mechanisms were used to assess the antioxidant activity of sage hydrosols, i.e. TEAC assay based on hydrogen atom transfer (HAT) mode, CUPRAC assay based on single electron transfer (SET) mode and DPPH assay with mixed mode of action. The results of the antioxidant activity of sage hydrosols determined by TEAC and CUPRAC assays are presented in Table 3. The capacity to scavenge ABTS radicals was significantly ($p<0.05$) higher for SSH (245 mM TE/100 mg d.w.) than for RSH (208 mM TE/100 mg d.w.). Similar, the capacity to reduce Cu (II) ions was significantly ($p<0.05$) higher for SSH (241 mM TE/100 mg d.w.) compared to RSH (210 mM TE/100 mg d.w.).

We have analyzed the correlation between the antioxidant activity and TPC values. A linear, positive correlation between TEAC and CUPRAC antioxidant activity and the polyphenolic content of SSH ($r^2 = 0.848$) and RSH ($r^2 = 0.929$), respectively, was observed (Figure 3). These data confirmed that the presence of polyphenolic compounds, in particular of flavonoids, could ensure numerous hydroxyl groups and, accordingly, a significant antioxidant activity.

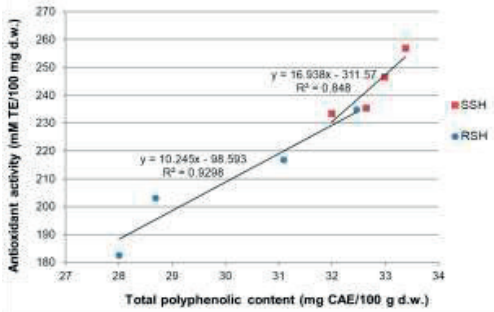

Figure 3. The correlation degree between TEAC and CUPRAC antioxidant activity and the polyphenolic content of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation

The antioxidant activity of sage hydrosols was also determined as their capacity to inhibit DPPH free radicals. The variation of the inhibition percentage as a function of hydrosol concentrations is presented in Figure 4.

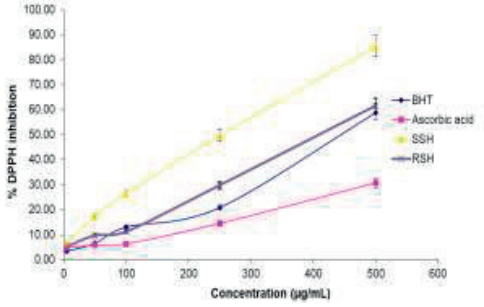

Figure 4. Inhibition of DPPH free radicals in the presence of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation, BHT and ascorbic acid

The inhibition of DPPH radicals increased in a dose-dependent manner in the presence of sage

hydrosols. The slope was linear between 100-500 µg/mL range of concentrations and values of 85.33% and 61.48% were recorded at a concentration of 500 µg/mL SSH and RSH, respectively. At the same concentration value, lower DPPH inhibition was registered for the synthetic antioxidant agent BHT (58.76%) and the natural antioxidant ascorbic acid (30.58%).
In the same time, the IC50 value of SSH (264 µg/mL) was significantly ($p<0.05$) lower than that of BHT (449 µg/mL) and ascorbic acid (883 µg/mL), confirming that the steam distilled hydrosol had higher antioxidant activity (Table 4). In the case of RSH, the IC50 value (429 µg/mL) was not significantly ($p>0.05$) different from that of BHT, but it was lower than that of ascorbic acid, indicating better capacity to inhibit DPPH free radicals.

Table 4. IC50 values of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation, BHT and ascorbic acid determined for DPPH free radicals inhibition and acetylcholinesterase (AChE) activity

Sample	IC50 DPPH (µg/mL)	IC50 AChE (mg/mL)
SSH	264.14 ± 9.23	9.79 ± 0.68
RSH	429.33 ± 12.27*	18.83 ± 2.05*
BHT	449.80 ± 17.44*	ND
Ascorbic acid	883.67 ± 23.26*	ND

ND - not determined; *$p<0.05$, compared to SSH value

Pesticide potential of sage hydrosols

The pesticide potential of sage hydrosols was assessed *in vitro* using an experimental model mimicking their neurotoxic effect on aphides. The inhibition of AChE in the presence of sage hydrosols is presented in Figure 5, while the calculated IC50 values are given in Table 4.

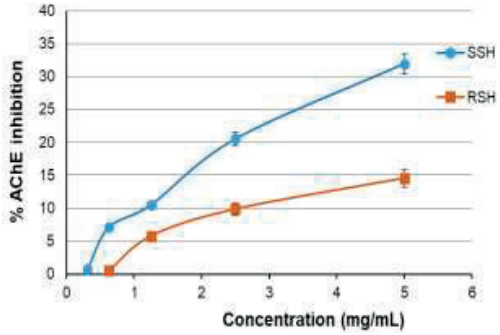

Figure 5. Capacity of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation to inhibit the activity of acetylcholinesterase (AChE)

The results showed that both sage hydrosols inhibited AChE activity, but SSH in a higher proportion (32%) than RSH (15%). No AChE inhibition activity was recorded for BHT and ascorbic acid, indicating that an important role was that of the polyphenolic compounds content. The IC50 value of SSH (9.79 mg/mL) was significantly ($p<0.05$) higher than that of RSH (18.83 mg/mL) (Table 4).
In a recent study, the IC50 value of the methanolic extract of *S. officinalis* was 24 mg/mL in the same model of AChE activity *in vitro* (Sharma et al., 2020), confirming the neurotoxic activity of sage and its potential use as biopesticide. At the same time, rutin present as main flavonoid in sage extracts exhibited an IC50 value of 2.5 mg/mL against AChE activity (Neagu et al., 2015).
In regard of volatile compounds, it was previously showed that they did not present AChE inhibition and neurotoxic capacity (Lopez & Pascual, 2009). However, 1,8-cineole also present in sage hydrosols exhibited pests toxicity (Abdelgaleil et al., 2009). It was proposed a mechanism of neurotoxic action from hydrosol compounds based on changes at hormonal level, neurologic effect by gustatory effects and olfactory responses, and imbalance of pro-oxidant/antioxidant reactions, all of these processes leading to disturbances in pests development (Spochacz et al., 2018).

CONCLUSIONS

Two sage hydrosols with different composition were prepared by steam (SSH) and reflux (RSH) distillation methods. GC-MS results indicated that RSH contained 15 types of volatile compounds, unlike SSH presenting traces. In turn, significantly higher polyphenolic content and correlated antioxidant activity were recorded for SSH.
Both hydrosols presented DPPH and AChE inhibition variation in a dose-dependent manner, but SSH demonstrated better radical scavenging and neurotoxic activity than RSH, with IC50 values lower than those of known antioxidant agents, like BHT and ascorbic acid.
The hydrosol obtained by steam distillation of *S. officinalis* aerial parts presented higher flavonoid and lower volatile compounds content, in comparison to that of the reflux

obtained sage hydrosol. A positive correlation was found between the polyphenolic composition and the antioxidant activity of sage hydrosols.
The AChE inhibition activity of sage hydrosols varied in a dose-dependent manner and was higher for the steam-distilled product, rich in flavonoids.
All these results demonstrated that steam distillation of sage could provide a product with practical applications not only in food industry, but also as functional ingredient of novel biopesticide formulas for pest control in eco-agrosystems.

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REVOLUTIONARY METHODS IN FERTILIZATION PROCESSES OF AGRICULTURE OF ROMANIA: BACTERIAL BIOPREPARATIONS – A `GREEN REVOLUTION`

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Abstract

The paper aims to present the use of new fertilization technologies in agricultural crops in Romania. At the same time, the paper presents the results obtained after testing these new fertilizer products (ecological fertilizers based on bacterial cultures) in parallel with the fertilization of crops with chemical fertilizers. These researches were carried out in two prestigious research institutions, the first being the Suceava Research-Development Station for Agriculture and the second being the Buzau Research-Development Station for Vegetable Culture. Within the two research institutions were established the crops to which both chemical and biological fertilizers were applied, during the experiments analyzing a series of biological parameters of the plant, soil and agricultural production. Following the completion of the experiments, it was observed that the physiology and anatomy of the plants showed changes as well as the production of the crops themselves, the yields showing an increase of up to 57% (in lots fertilized with organic fertilizer, compared to lots that were fertilized with a chemical fertilizer).

Key words*: plant growth-promoting, biofertilizers, phytohormones, sustainable agriculture, nitrogen fixation, bacterial inoculants, nutrient solubilisation.*

INTRODUCTION

Bacteria in the soil structure have a role in the processes carried out at the soil level as well as in the growth and development of plants. Bacterial cultures can be used as biological/organic fertilizers, bioinsecticides, biofungicides in agriculture so as to ensure an increase in crop yield and maximization of their production (Khalid et al., 2009).

The use of these bacteria as biological fertilizers in agricultural crops leads to better plant growth through various mechanisms that these bacteria produce, mechanisms such as the synthesis of nutrients and phytohormones, (which are absorbed and used by plants), the mobilization of compounds in soil structure (decomposition of complex soil compounds into soluble compounds that are used in the basic processes of plants-photosynthesis and chemosynthesis), role in plant protection (stress conditions, pest attack, disease, etc.) (Malusá & Vassilev, 2014).

The use of these revolutionary processes and methods of fertilization in agricultural crops in Romania have proven a great potential in growing, developing and maximizing plant production, as well as in restoring the soil structure and its beneficial fauna. The use of these biofertilizers based on live bacterial cultures has the role of greening the soil, opening a new way for farmers to approach a sustainable, sustainable agriculture, and obtaining much healthier products rich in nutrients.

Thus, the use of PGPR mechanisms in agricultural crops in Romania brought with it the obtaining of additional productions for

farmers, productions which, for them, represented a substantial profit. Also, the PGPR mechanism is a simple one, but based on which plants receive a much higher amount of nutrients as well as a certain resistance to certain factors (Figure 1).

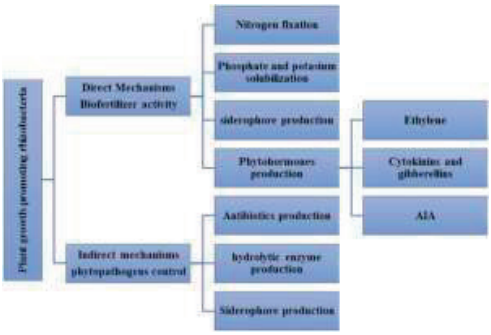

Figure 1. Mechanisms of Plant Growth-Promoting Rhizobacteria (Sansinenea, 2019)

Nitrogen is the basic element in the processes carried out inside the plants and in the soil structure. Nitrogen is needed in the formation of amino acids and proteins, it is the main element for plant growth and development.

Atmospheric nitrogen is combined into organic forms, forms that can be assimilated by plants (Tejera et al., 2005).

The most representative bacteria that act as atmospheric nitrogen fixers are those of the genera *Azospirillum*, *Azotobacter* and *Bacillus* (symbiotic bacteria associated with cereals and legumes), *Frankia* (actinorizal plants) (Ravikumar et al., 2007; Ininbergs et al., 2011).

Through this process of hydrolysis, ammonia and α-ketobutyrate will be obtained. These obtained sources can be used as carbon and nitrogen sources in plant growth and development processes (Yang & Hoffman, 1984).

Through this process of hydrolysis, ammonia and α-ketobutyrate will be obtained. These obtained sources can be used as carbon and nitrogen sources in plant growth and development processes (Yang & Hoffman, 1984).

The use of these biological fertilization technologies, based on bacterial cultures, was an important step in Romanian agriculture. Thus, these technologies were an alternative to classical chemical fertilization, the bacteria in the composition of fertilizers having a role in the growth and development of plants, the restoration of beneficial soil fauna and an increase in plants and agricultural production.

MATERIALS AND METHODS

In order to prove the effectiveness of a biological fertilization product, in relation to a chemical fertilization product, into Buzău Research-Development Station for Vegetable Growing and Suceava Research-Development Station for Agriculture, a series of experiments were performed, as follows:

SCDA Suceava - corn crop, millennium variety:

V1 lot - unfertilized lot,

V2 lot - chemically fertilized NPK 20: 20: 0,

V3 lot - biological fertilizer lot Rom-Agrobiofertil NP.

SCDL Buzău - culture of pepper, superior yellow variety:

V1 lot - Biological fertilizer Rom-Agrobiofertil NP (based on live cultures of *Azotobacter chrooccum, Azospirillium lipoferum* and *Bacillus megaterium*),

V2 lot - Biological fertilizer Azoter (based on *Azotobacter chroococcum, Azospirillum brasilense, Bacillus megaterium* bacteria).

V3 lot - Chemical fertilizer NPK 16:16:16.

The study was carried out in 2019-2020 period.

The data, collected from those two research-development stations, have been statistically processed and interpreted, based on the obtained results and interpretations proving the effectiveness of the biological fertilizer.

RESULTS AND DISCUSSIONS

In SCDA Suceava, the chemical fertilizer Complex 20: 20: 0 (V2-300 kg/ha - control lot) was tested in parallel with the organic fertilizer Rom-Agrobiofertil NP (V3-15 liters x 2 applications/ha), referring to an unfertilized lot (V1), for the cultivation of corn, the Millennium variety. In carrying out the experiments (Table 1), a series of parameters were followed, with the following results obtained into the three groups (Figure 2), as follows (Table 1, Table 2):

Table 1. Corn crop production data

Variant	No. of grains / row	MMB/ g	Grain yield%	Hectolitre mass /kg	Production / kg / ha	Humidity/ %
V1- unfertilized lot	31	251	81.7	72.7	4587	16.6
V2-chemical fertilizer 300 kg / ha 20: 20: 0	36	274	89.0	78.7	6478	16.4
V3- organic fertilizer Rom-Agrobiofertil NP	37	303	98.9	80.4	7315	16.6

Table 2. Corn crop production data-Anova test

Anova: Single Factor						
SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
V1- unfertilized lot	6	5040	840	3376633		
V2- chemical fertilizer 300 kg / ha 20: 20: 0	6	6972.1	1162.02	6790709		
V3- organic fertilizer Rom-Agrobiofertil NP	6	7850.9	1308.48	8669230		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	689247.7	2	344624	0.05489	0.947	3.682
Within Groups	94182862	15	6278857			
Total	94872110	17				

Figure 2. Corn crops (V1 - batch not fertilized, V2 - batch chemically fertilized, V3 - batch biologically fertilized)

Between the three groups, a series of differences were observed in the predators noticed during the experiments (Figure 3).

Among the most important differences are the production of seed material (production per hectare), plant height and the number of plants per lot. Following the experiments, it was found that the plants in the group fertilized with the biological product Rom-Agrobiofertil NP showed a higher growth in height, compared to the two groups (chemically and non-fertilized). The fructification as well as the other parameters followed gave a higher economic yield than the other two lots (Table 1 and Figure 3).

Figure 3. Corn plants (V1 - unfertilized lot, V2 - chemically fertilized lot, V3 - biologically fertilized lot)

Biometric determinations were performed using the Anova test to highlight the effects of organic fertilizers relative to chemical fertilizer product (Table 2).

The second experiment was carried out at SCDL Buzău, on superior yellow variety of pepper culture. Following the experiments on the pepper culture, a series of parameters were settled such as plant height, number of fruits per plant, total production, etc., as well as aspects related to chlorophyllmetry or dry matter mass. Biometric determinations were performed using the Anova test to highlight the effects of organic fertilizers relative to chemical fertilizer product (Table 3).

Table 3. Pepper crop production data

Objectives	**V1-Rom-Agro**	**V2-Azoter**	**V3-NPK**
Plant height (cm)	46.33	49.33	45.22
Plant diameter (cm)	44.89	42.67	39.89
Number of leaves	142.00	120.11	108.11
Leaf length (cm)	17.68	17.48	16.58
Leaf width (cm)	6.88	7.08	6.89
Number buds	25.56	19.22	18.56
Number of fruits	6.89	5.44	6.56
Number of flowers	7.78	5.44	6.33
Stem diameter (cm)	1.11	1.11	1.16
Total production (t/ha)	20.5	18.7	12.3

The production per biologically lots showed an increase of up to 66.67% (V1 lot compared to V3 lot) and 52.03% (V2 lot compared to V3 lot). This experiment showed that the production of biologically fertilized lots was much higher than the chemically fertilized lot, due to bacterial activity of the organic fertilizers (Table 4).

Table 4. Pepper crop production data-Anova test

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
46.33	9	273.29	30.365556	1927.9028		
49.33	9	237.25	26.361111	1391.0202		
45.22	9	216.38	24.042222	1122.4135		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	184.1921	2	92.096048	0.0622083	0.9398381	3.4028261
Within Groups	35530.693	24	1480.4455			
Total	35714.885	26				

Figure 4. Differences between pepper plants, the upper yellow variety: a. Rom-Agrofertil NP; b. Azoter; c. NPK chemical fertilized plant

Some differences were identified between the three lots, the most visible being the biologically fertilized lots. Thus, in the two biologically fertilized groups there was a much higher plant growth than the control group, chemically fertilized. At the same time, the number of fruits per plant also showed a variation of growth between the three groups (Figure 5).

From Figure 4 we can deduce that the effect of organic fertilizers applied in pepper culture (Rom-Agrobiofertil NP and Azoter) had a beneficial effect on plant growth, shoot grow and, implicitly, agricultural production.

Experiments performed with *Azospirillum lipoferum* inoculated seeds, revealed the bacterium ability to produce antioxidant enzymes such as catalases, glutathione, and peroxidases, which have different roles (establishing plant protection in their stress phases, protection of plants from reactive oxygen- superoxide, hydroxyl ion, hydrogen peroxide) (Mittler, 2002).

Figure 5. Superior yellow bell pepper plant (organic fertilized lot produced Rom-Agrobiofertil NP)

From the research performed with *Azospirillum lipoferum* bacterium, it was found that an action of bacterial colonies on the soil surface, lower the level of ethylene in order to increase plant growth in stress conditions (Glick et al., 2007).

The role of these bacteria in the content of the two biological fertilizers, in addition to the processes they carry out in the soil, also have the role of producing certain antioxidant enzymes to allow the plant optimal growth (both plant and production) under stress (pedo-climatic) (Fu et al., 2010).

The application of organic fertilizers in agricultural crops has had the effect of a substantial increase in the plants themselves, an increase in the amount of nutrients in the soil and bacterial recolonization of soil structure. The use of organic fertilizers was an important step in Romanian agriculture as well as globally. The fact that chemical fertilizers can be substituted with bacterial biopreparations was an important factor for farmers to move towards sustainable, organic agriculture and to obtain much healthier products, with a large amount of nutrients, proteins necessary for human and animal food.

CONCLUSIONS

Recently, many emerging technologies have been implemented in the agricultural field in Romania, such as the use of drones, the use of

robots to measure the degree of pest attack, the occurrence of diseases, the use of precision sensors and the use of bacterial biopreparations (bacterial based biofertilizers).

The replacement of chemical fertilizers with bacterial biopreparations was a great success in Romanian agriculture because plant-beneficial bacteria applied on the soil surface ensure a recolonization of the soil, a restoration of its texture and pH and an increase in agricultural production, these aspects representing an alternative to chemical fertilization technologies. At the same time, the growth and maximization of agricultural production has brought with it an additional profit for farmers as well as a growing demand for agricultural products obtained from these crops (Revillas et al., 2000).

The use of excess chemical fertilizers, the use of plant protection products in increasing doses has led to lower soil fertility, acidification and poor productivity of crops but especially to pollution of soil, groundwater and of the environment. The use of biological fertilizers based on live bacterial cultures have the role of reducing these effects given by chemical fertilizers as well as restoring nutrients from the soil, recolonizing the soil with beneficial bacteria and obtaining healthier production for humans and animals (Mahanty et al., 2017) .

The use of plant beneficial bacteria in agriculture have had the effect of stimulating the seed material and protecting it from soil pests, stimulating plant growth, growth and development of agricultural crops and obtaining a high yield of agricultural production. Bacteria play a role in the production of enzymes, proteins, acids and even the solubilization of insoluble compounds in the soil into soluble compounds. At the same time, the use of biopreparations in agriculture is a significant reduction in costs for farmers.

The microorganisms in the composition of organic fertilizers have the role of balancing the processes inside the soil, these bacteria through certain physico-chemical processes, provide nutrients for the growth, development and production of plants. The decomposition of insoluble elements into soluble elements will lead to an increase in soil nutrients, increased soil fertility and obtaining healthy crops, with a higher production than conventionally fertilized lots.

At the same time, on the research that was carried out on the corn and sunflower seeds (inoculation of seeds in bacterial cultures) it was found that the activity of bacteria from the Rom-Agrobiofertil NP product has a beneficial effect in the processes of stopping and creating a plant defence against *Tanymecus dilaticollis* attack.

Another important aspect in using bacterial cultures in Romanian agriculture it is the beneficial effect that they have on the ground. The use of the decomposer *Azospirillum lipoferum* aims to decompose insoluble compounds in the soil. The decomposition of these compounds will lead to the release of soluble compounds necessary for plant growth and development.

The soil becomes richer in the elements necessary for the plants (nitrogen, phosphorus, potassium, etc.). A large amount of elements will lead to a better assimilation of them by plants.

Soil microorganisms are an important component of agricultural ecosystems. These microorganisms play an important role in soil fertilization, plant growth, development and production, as well as maintaining a much healthier ecosystem.

The population of soil microorganisms includes bacteria, cyanobacteria, acetomycetes, fungi and a certain category of viruses. The most important microorganisms are rhizobacteria. These bacteria have the role of promoting plant growth (PGPR) as well as an effect of protecting plants against diseases and pests.

The use of chemicals (fertilizers, pesticides, herbicides, insecticides, etc.) has led to a decrease in the soil microbial community. As such, the entire soil ecosystem has been disrupted. This disorder has led to a low yield of agricultural production as well as favoring pest attack and disease.

An important aspect of chin is that certain bacteria (*Pseudomonas syringae*) have the ability to protect plants from frost by producing proteins (INA proteins, produced by INA genes). The use of these proteins in the plant cycle has the role of forming films called "antifreeze proteins" that have the role of protecting the plant from low soil temperatures and the environment (Sajid et al., 2016).

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THE EVOLUTION OF THE QUALITY PARAMETERS ON THE STORED WHEAT

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Abstract

As one of the main grains consumed throughout the world, wheat is very important for food security. Harvesting and storage periods may have negative effects on physical and physiological quality and increased fungal incidence in the silo, especially when certain essential conditions for carrying out these processes are not complied with. Quality of wheat is changing during storage and this can influence the quality of bread and bakery products. During storage, the moisture of wheat must be keep at a safety level to prevent germination process and also the insect infestation have to be under control. This work aimed to monitoring the changes in the quality parameters of the wheat stored for a period of at least two years. The analysis carried out on the basis of which the observations were made were the following: moisture content, protein content, hectometer mass, falling number, gluten content, and E-nose analysis. The results of the analysis have shown that in the stored cereals certain quality properties have been modified, but over time the deterioration occurs more and more aggressively.

Key words*: wheat, storage, physical- chemical analysis, quality parameters.*

INTRODUCTION

One of the most important grains grown around the globe is wheat. This cereal is a rich source of nutrition in carbohydrates and proteins. Wheat is collected in a short period of time, but then it is processed and used throughout the year. Therefore, wheat must be stored in adequate conditions to maintain the nutritional and rheological properties necessary for use in the milling and bakery industry (Tipples, 1995). Wheat is considered to be the essential crop, with an overall production of around 771 million tons in 2019. The world's largest wheat supplier is considered to be the European Union. Romania is in the top five largest wheat producers in the European Union, with a growing area of 2.106.813 hectares and an annual production of 10.144.000 tons. More than half of the annual wheat production in Romania is exported to different countries around the world (USDA, 2015). On the other hand, the Romanian population has a high consumption of wheat and wheat products (364.6 g/day), more than the European average (295.4 g/day) and twice the world average (178.8 g/day) (FAOSTAT, 2015). Wheat crop in Romania is a traditional and fundamental feature for national agriculture. The essential area where most wheat is grown is the Danube plain in the south of the country. Other important areas of wheat growth are Transylvania, the northern part of Moldova in the north-east of Romania and the Banat region in the south-west (Bălan, 2015). The wheat is grown only for a limited period during the year; therefore, it must be stored safely for consumption throughout the year. Infestation is a hard blow to food security, as it causes a huge amount of losses suffered from feeding insects inside the grain, as well as a deterioration in the quality of the stored wheat grains. The main cause of insect and pest attacks is due to poor storage conditions and lack of a quick method for quality analysis during storage. Insects are the most adaptable forms of life; once they proliferate over the grain, it begins to degrade in a logarithmic progression. Of these, two

major insects which are the main cause of infestation in stored wheat are *Sitophilus oryzae* and *Rhizopertha dominica*. Both insects propagate eggs into wheat grain and start to grow by feeding on the starch until they appear as adult insects (Jarruwat et al., 2014). Wheat grains have a moisture content of between: (9-5%), dry matter (80-90%) and crude protein (7-17%). The economic value of wheat is determined on the basis of its protein content, as it indicates the final quality of the flour to the consumer class. The moisture content of the wheat grain is given by its relevance for long-term storage (NZFMA, 2014). The main agents of wheat quality characteristics are tested by analytical quantification of a range of parameters such as wet gluten content, protein concentration, sediment value and falling number. All these parameters have their own distinct role in anticipating the quality of wheat flour. The protein content plays an essential role in describing the characteristics of the flour, while the way gluten is formed and shown after hydration highlights the ability of the flour and its efficiency to retain the gas during fermentation and ensures the successful completion of the final product (Pareyt et al., 2015).

The purpose of this study was to observe the development of certain essential parameters expressing the quality of the wheat during two years of storage. The samples were analyzed during 2019 and were collected in the same year and analyzed again in 2020.

MATERIALS AND METHODS

Raw materials

Twelve samples of wheat (*Triticum aestivum* L.) have been taken for this work. The wheat from which the samples were taken was grown on the territory of Romania in the south-east part of the country. Its storage was in raffia sacks, in proper grain storage. Sampling has been carried out by a qualified person. The samples were packed in plastic bags weighing 2 kg each. During the analysis the samples were stored under optimal storage conditions. It is well known that the maximum storage temperature should be 15ºC to avoid the deterioration of wheat quality (Fletcher, 2010). In this study, low temperatures were taken into account to maintain quality during grain storage. All functional properties were measured at room temperature (23 ± 2ºC). Weighing of the samples was carried out with the analytical scales. This study was carried out in Bucharest, Romania.

Moisture content

The determination of the moisture content was carried out by rapid methods using two devices: Aquamatic AM 5200-A and Inframatic IM 9500 (Perten Instruments, Hudinge, Sweden). For the accuracy of the results, the equipment shall be checked and calibrated annually.

Protein content

The Inframatic IM 9500 equipment (Perten instruments, Hudinge, Sweden) was used for this method. Analysis has been conducted according to the working instructions for the equipment provided.

Hectolitre mass

The analysis for the determination of the hectomical mass was carried out using the Nilematic tools (Tripette & Renaud tools for results 128, France) and Aquamatic AM 5200-a (Perten instruments, Hudinge, Sweden). Sample testing for this determination was carried out on both equipments.

Gluten content

Wet gluten has been determined according to ISO method 21415-2: 2016 using Glutomatic 2200 equipment (Perten instruments, Hudinge, Sweden). The determination of gluten verifies the physical properties of the dough (Kashta, 2014). The gluten content gives nourishment to the bakery products. This is a very important protein due to vasco-elastic properties (Kaushik et al., 2015).

Falling Number

The determination of the drop index was carried out using Perten FN 1000 equipment and automatic stirrer for Shakematic viscometer 1095 (Perten instruments, Hudinge, Sweden), according to SR EN ISO 3093:2010. The grinding of the samples was carried out by means of laboratory mallets Perten LM 120. The grain size check was carried out with sieves with dimensions of holes of 0.2 mm, 0.5 mm and 0.710 mm, ISO3310-1:2016 Vibitarion

filter. The mill-check groats was sieved using the Vibitarion filter vibratory base for 10 minutes. The samples obtained from grinding have been kept in optimum conditions of temperature and humidity.

E-nose analyze

The principle of the method is to generate head space in the capsule vial in which the sample is contained, to extract a quantity from it (µl, amount determined in the method) by means of syringe and to inject into the System Array Sensor (SAS). This gives the sensory print with the primary data, which is then analyzed statistically and the differentiation index is obtained. From the received samples, 2 g were weighed in 10 ml glass vials and subsequently sealed. 3 identical vials were prepared from each sample. The samples have been heated and agitated in the oven at 70°C for 500 seconds at a shaking speed of 500 rpm, the syringe being heated to 80°C. From the volatile part generated inside the vial, 2000 µl were injected into the 18-sensor system for recording the total volatile print and comparing them between the samples (Figure 1).

Figure 1. Common wheat samples harvested since 2019

Figure 2. A-Prometetheheus Multisensor System (E-nose)

Equipment and materials used: A-Prometheus multi-sensor system (Figure 2); 10 ml vials; caps; vial sealer; analytical balance; spatula/tweezers; calibration standards.

RESULTS AND DISCUSSIONS

Moisture content of the samples (Table 1)

Table 1. Moisture of wheat samples over two years of storage

Samples	Results obtained	
	2019	2020
S_1	13.4 %	13.1%
S_2	13.4%	12.8%
S_3	13.3%	13.0%
S_4	13.0%	13.2%
S_5	12.9%	13.1%
S_6	13.5%	13.7%
S_7	13.3%	13.8%
S_8	13.3%	13.4%
S_9	12.8%	13.1%
S_{10}	13.4%	12.4%
S_{11}	12.7%	13.4%
S_{12}	12.9%	13.5%

The results of the humidity analysis of the twelve samples are given in this table. As it can be seen, the wheat does not change its moisture in two years, and there are only small changes in moisture, which remain almost unchanged. It can be observed that the moisture content values are not exceeded, resulting in optimal preservation.

Variation in protein content over time (Table 2)

Table 2. Protein of wheat samples during two years of storage

Samples	Results obtained	
	2019	2020
S_1	12.9 %	13.4%
S_2	12.6%	12.7%
S_3	12.6%	12.6%
S_4	12.4%	12.8%
S_5	12.2%	13.0%
S_6	12.6%	12.6%
S_7	12.2%	12.7%
S_8	12.2%	12.0%
S_9	12.0%	12.9%
S_{10}	12.1%	12.4%
S_{11}	12.7%	12.0%
S_{12}	12.5%	12.2%

It can be observed that the results obtained for protein content are very close in value, so the protein content has not undergone any major changes as a result of storage conditions during those two years.

Evolution of the hectolitre mass (Table 3)

Table 3. The hectolitre mass of the two-year storage park

Samples	Results obtained	
	2019	2020
S_1	77.8 kg/l	79.4 kg/l
S_2	78.9 kg/l	77.0 kg/l
S_3	78.4 kg/l	77.8 kg/l
S_4	76.7kg/l	77.0kg/l
S_5	77.6 kg/l	77.1 kg/l
S_6	78.9 kg/l	78.3 kg/l
S_7	78.6 kg/l	77.8 kg/l
S_8	78.6 kg/l	77.8 kg/l
S_9	76.7 kg/l	77.0 kg/l
S_{10}	81.2 kg/l	79.8 kg/l
S_{11}	79.0 kg/l	79.6 kg/l
S_{12}	80.8 kg/l	79.8 kg/l

The difference between the two years in the case of a hectolitre mass is not significant, which means that storage has not affected wheat in this respect, although the values obtained are not favorable to a high quality wheat.

Wet gluten (Table 4)

Table 4. Wet gluten content

Samples	Results obtained	
	2019	2020
S_1	26.4 g	25.9 g
S_2	24.1 g	25.8 g
S_3	24.2 g	25.5 g
S_4	24.0 g	24.7 g
S_5	24.4 g	25.3 g
S_6	24.8 g	23.8 g
S_7	26.2 g	25.0 g
S_8	24.0 g	22.6 g
S_9	26.3 g	25.9 g
S_{10}	24.2 g	23.5 g
S_{11}	27.2 g	25.7 g
S_{12}	26.9 g	27.4 g

Data from wet gluten analysis were recorded in this table. Wet gluten did not vary widely between the years in which it was stored. No lower gluten content than 22.0 g was recorded, no high gluten value above 28.0 g was recorded, the highest value obtained was 27.2 in 2019 and the lowest value recorded for 22.6 in 2020. Comparing the two years the evolution of gluten was not dramatic to be able to say that the storage factors had a negative or positive impact on this property.

Falling Number (Table 5)

Table 5. Falling Number results

Samples	Results obtained	
	2019	2020
S_1	338 s	407 s
S_2	374 s	420 s
S_3	385 s	357 s
S_4	274 s	293 s
S_5	299 s	272 s
S_6	361 s	402 s
S_7	322 s	359 s
S_8	406 s	448 s
S_9	394 s	421 s
S_{10}	408 s	455 s
S_{11}	372 s	390 s
S_{12}	269 s	298 s

When comparing the two years there was no data with very large differences between the values obtained.

Data obtained indicate low amylolytic activity, only sample 4 and 12 fall into a normal value of amylase activity (220-280 s).

The results obtained indicate that the bread to be made from this flour will not develop enough, the kernel will be small and too dry.

Storage conditions have not significantly influenced the drop index, although it can be observed that in most samples it is increasing, resulting in very low and impy amylasic activity to flour that will have to be improved to achieve the desired result.

E-nose

Volatile compound analysis results (Figures 3-15):

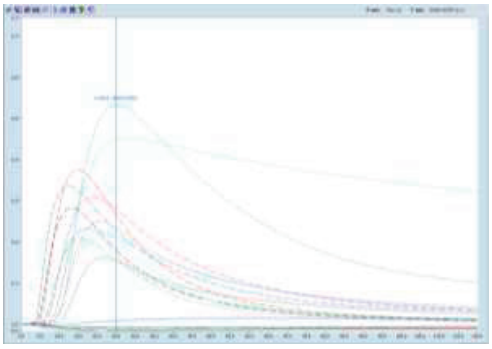

Figure 3. Sample 1 - common wheat

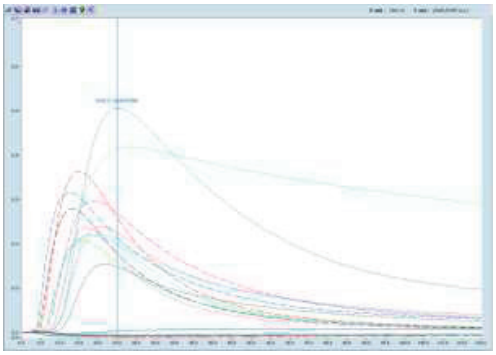

Figure 4. Sample 2 - common wheat

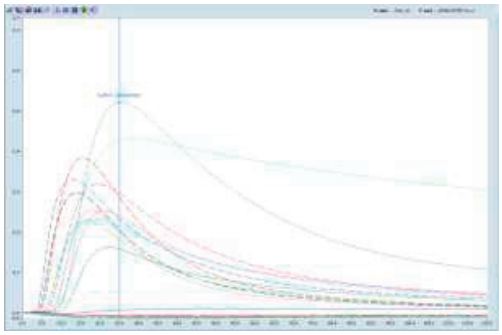

Figure 5. Sample 4 - common wheat

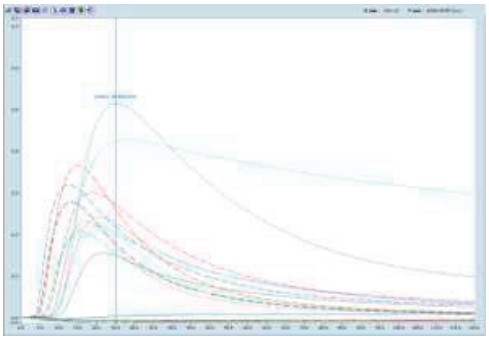

Figure 6. Sample 3 - common wheat

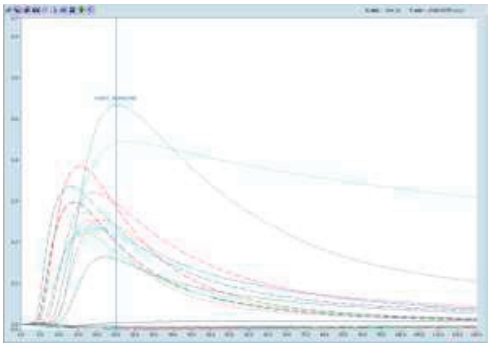

Figure 7. Sample 5 - common wheat

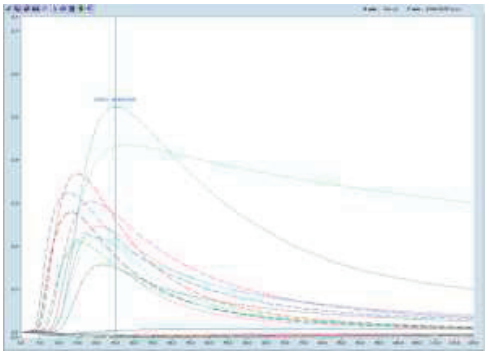

Figure 8. Sample 6 - common wheat

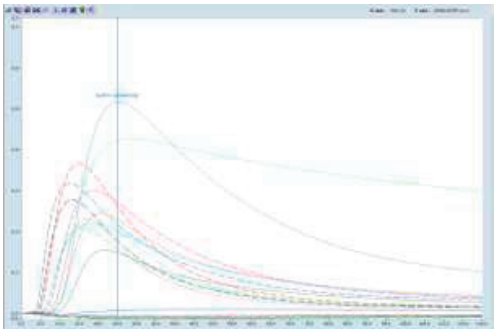

Figure 9. Sample 7 - common wheat

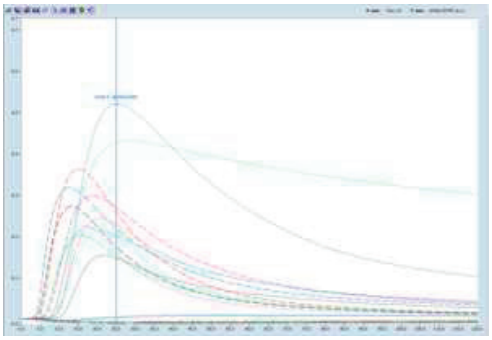

Figure 10. Sample 8 - common wheat

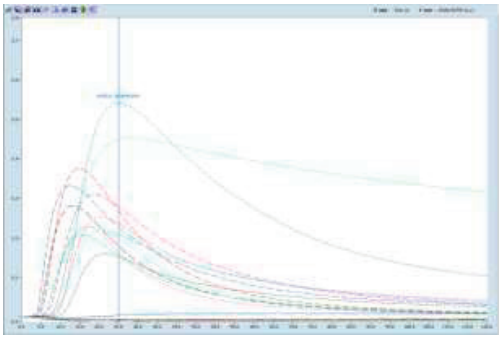

Figure 11. Sample 9 - common wheat

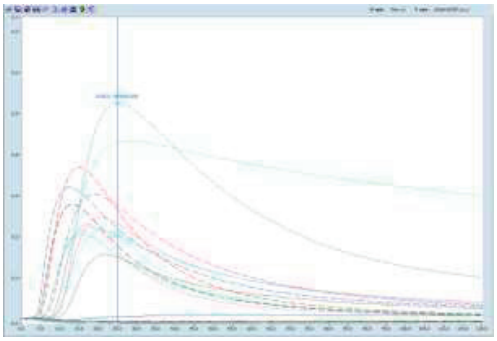

Figure 12. Sample 10 - common wheat

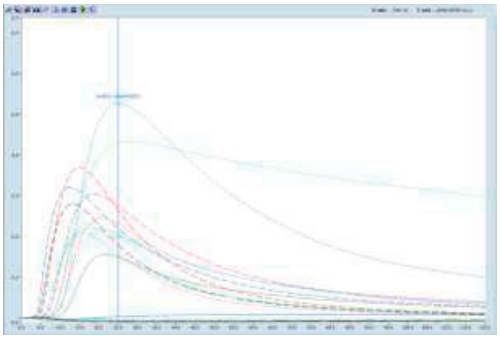

Figure 13. Sample 11 - common wheat

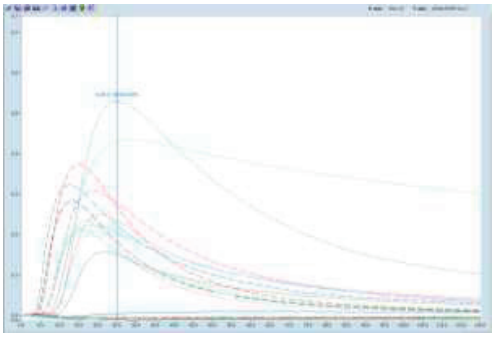

Figure 14. Sample 12 - common wheat

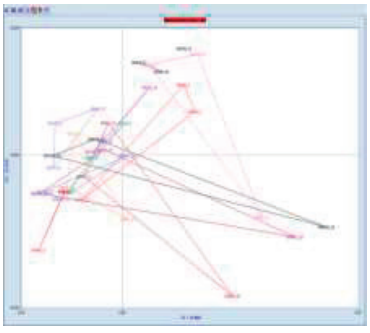

Figure 15. Differentiation of the 12 common wheat samples

In these charts the response of the 18 sensors of the α-Prometheus multisensor system for each sample is shown. Following the sensory analysis of volatile compounds in the twelve common wheat samples collected since 2019, the response given by the 18 sensors did not show any significant differences in the way that any contamination of certain samples could be concluded.

CONCLUSIONS

The results from this study show that moisture, protein, hectolitre mass, gluten content and falling number have not been significantly altered under the storage conditions under which the wheat was kept. In the two years the wheat was stored, the parameter values did not have any semi-operative variations. In the case of Falling number analysis where most of the results obtained show a low amylasic activity because the drop index values are higher than 280 s, certain factors such as temperature variations may lead to wheat germ, and this process leads to even lower alpha-amylasic activity. The samples were differentiated using PCA-type statistical analysis (analysis of main components). Following the interpretation of the data, a discrimination index was obtained using the PCA which provides an assessment of the quality of the discrimination on the selected plan. This assessment is given by the area between the groups and the size of a group, the maximum value for the differentiation index is 100 if the samples are different. The wheat samples analyzed had a negative differentiation index, which leads to the conclusion that the samples are very similar, no significant differences in volatile smell composition can be identified.

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EFFECTS OF EXTRACTS FROM *PASSIFLORA CAERULEA* LEAVES TREATED WITH A *TRICHODERMA* BIOSTIMULANT CONSORTIUM ON LACTIC ACID BACTERIA

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Abstract

The alcoholic extracts from Passiflora caerulea leaves protect prebiotic lactic acid bacteria (LAB) against oxidative stress induced by (micro) aerobic conditions. This prebiotic activity is mainly related to the polyphenols accumulated in P. caerulea leaves. Polyphenols are used by prebiotic LAB as electron acceptors of their carbohydrate catabolic chains and promote LAB adaptation to aerobic conditions. Foliar treatment with 10^8 cfu per ml chlamydospores of a Trichoderma plant biostimulants consortium enhances the accumulation of polyphenols in the P. caerulea leaves. The Trichoderma plant biostimulant consortium includes two strains, T. asperellum T36b, and T. harzianum Td50b. Enhanced levels of polyphenols are related to the enhanced prebiotic effect of the extracts from leaves treated with Trichoderma consortium, compared with the control leaves from P. caerulea non-treated with plant biostimulants. The results are discussed in relation to the known physiological effects of P. caerulea leaves extracts on mood and sleep disorders, which could also be explained by the probiotic bacteria's postbiotic effects, promoted by polyphenols from leaves extracts.

Key words*: Passiflora caerulea, Trichoderma-based plant biostimulants, lactic acid bacteria, polyphenols, leaves extracts*

INTRODUCTION

Extracts from plant tissues rich in polyphenols usually exert significant antibacterial effects. There were describes several mechanisms of action of polyphenols on bacteria: disruption of the integrity of cell wall (Nohynek et al., 2006; Zhao et al., 2001) and cellular membranes (Ollila et al., 2002; Perumal et al., 2017); interactions with ion channels and membrane receptors (Alvarez-Martinez et al., 2020; Taylor et al., 2005); inhibition of final stages of catabolism and energy production (Chinnam et al., 2010; Liu et al., 2017); precipitation of the functional proteins (Kang et al., 2006; Nakayama et al., 2013); inhibition of DNA synthesis (Mickymaray et al., 2020; Wang et al., 2017); interferences with quorum-sensing signals and biofilm formation (Gopu et al., 2015; Hengge, 2019), complexation of metabolic substrate and essential metal micronutrients (Daglia, 2012; Rajakovich & Balskus, 2019). However, polyphenols were proven in the last decade to enhance the development of lactic acid bacteria (Chan et al., 2018; Filannino et al., 2016). The main mechanisms of action are related to the lactic acid bacteria (LABs) ability to metabolize polyphenols (Gaur et al., 2020; Ricci et al., 2019). It was demonstrated that lactic acid bacteria could utilize the polyphenols as electron acceptors of their carbohydrate catabolic chains (Filannino et al., 2014). Also, LABs metabolize polyphenols to more bioactive compounds (Rodríguez et al., 2009). Lactic acid bacteria fermented plant materials with high polyphenols contents have an enhanced functionality due to mutual interactions between polyphenols and LABs (Piekarska-Radzik & Klewicka, 2021).

LABs' ability to metabolize the polyphenols differentiate the effects of the polyphenols to

gut microorganisms (Pacheco-Ordaz et al., 2018; Ziarno et al., 2021). Nowadays is primarily accepted that polyphenols promote the development of probiotic bacteria and are included in the new definition of prebiotics (Bindels et al., 2015), as a (metabolic) substrate for probiotic bacteria.

The aerial parts from *Passiflora* genus plants are a functional food (Zeraik et al., 2010), consumed as infusions, extracts, or tinctures. The main active ingredients of *Passiflora* leaves, which are involved in the passion flowers' effects against neuropsychiatric and metabolic disorders, are polyphenols (Angel-Isaza et al., 2021; Saravanan & Parimelazhagan, 2014). Our group recent work demonstrated that treatment with a *Trichoderma* consortium, which has an established plant biostimulant activity, enhances polyphenols' accumulation in the aerial parts of passion flowers (Şesan et al., 2020).

This work investigates the influence of extracts from *Trichoderma* treated and non-treated passionflower leaves on the development of lactic acid bacteria cultivated in aerobic conditions.

MATERIALS AND METHODS

Biological material. *Lactobacillus reuteri* DSM 20016 was cultivated in MRS (de Man, Rogosa, Share) broth and MRS Agar media (Oxoid, Thermo Fischer, Hampshire, UK), at 37°C, in microaerophilic conditions. A *Trichoderma* consortium was used for the treatment of the passionflower. This consortium includes two plant biostimulant strains from the INCDCP-ICECHIM collection, *T. asperellum* T36 NCAIM F 001434 and *T. harzianum* Td50b NCAIM F001412. These strains were selected because they produce bioactive compounds which stimulate plant growth (Oancea et al., 2016; Raut et al., 2014; Răut et al., 2016). *Passiflora caerulea* plants (blue passionflower) were grown in the Hofigal experimental field. This experimental field is established on a reddish preluvosol, and it is located south of Bucharest at 44°25'15" N, 26°1'34" E, altitude 84 m.

Application of treatment with the Trichoderma plant biostimulant consortium. A suspension of 10^8 cfu/mL chlamydospores was prepared (Şesan et al., 2020). Briefly, the fungal consortium was cultivated for two weeks days in a medium that promotes the chlamydospore accumulation in the presence of light. The composition of this medium is > 34.2 g/L glucose, 0.37 g/L ammonium sulfate, 0.8 g/L yeast extract, 2.7 g/L soymeal, 1.2 g/L K_2HPO_4, and 1.7 g/L KH_2PO_4 (Zamfiropol-Cristea et al., 2017). After two weeks, the chlamydospores were separated from the culture media in aseptic conditions and quantified by spore counting in a Thoma counting chamber (Aberkane et al., 2002). The experiment was organized with two treatments: Trichoderma 10^8cfu/mL and one treatment with water. Each treatment was applied in four repetitions, and each repetition consisted of 20 passion flowers plant selected to have a similar development.

The *Trichoderma* chlamydospores suspension was applied at the beginning of June 2019. A backpacker spraying unit (Solo SG71, Waiblingen, Germany) with an extension tube and a fan jet brass nozzle TeeJet. The spraying volume for each treatment was equivalent to 400 $L.ha^{-1}$. At the moment of treatment application, the *P. caerulea* plants were in the phenological phase of leaf development and flower primordium formation. After 30 days, 250 grams of leaves and sprouts with leaves (fresh weight) were randomly collected from each repetition.

The multi-annual average values for the experimental area where the experimental field is located are the following: temperature - 11.5°C, total precipitation: 615 mm; wind speed: 3.2 m s^{-1}; daily sunshine duration - 6.8 h. During the experimental period of 2020, the average monthly temperatures and precipitations were the following: May - 19.5°C and 72.4 mm; June - 23.5°C and 88.6 mm; July - 27.2°C and 22.7 mm. The soil was maintained at 80% water capacity by irrigation during the entire experiment.

Plant material extraction and determination of the total polyphenols and flavonoids. The plant leaves and sprouts with leaves were dried at 50°C. The dried passionflower material was ground in a laboratory mill Retsch SM2000 (Retsch GmbH, Haan, Germany) fitted with a 1

mm sieve. The resulting ground plant material was extracted in ethanol, 10 grams of dried, and ground plant material was extracted in 300 mL solution ethanol-water, for 60 min, at room temperature. Two ultrasound treatments (VCX 130, Ultrasonic Processor, Sonics, Newtown, CT, USA) of 5 min were applied, one of the beginnings of extraction treatment and the other after 30 min of extraction. The extract was separated by centrifugation at a relative centrifugal force of 3028 *x* g (Universal 320R, Hettich, Tuttlingen, Germania). The resulting extract was stored at 4°C in dark bottles till further use.

In the ethanolic extracts, we used the Folin-Ciocâlteu method (Huang et al., 2005), with some modifications (Craciunescu et al., 2012), for the total polyphenols determination. Briefly, 750 µL of Folin-Ciocâlteu reagent, 4 mL of 15% Na_2CO_3, and distilled water were added to 150 µL of the sample. The final volume was 15 mL. The incubation was done at room temperature. The optical density was measured after 2 h at λ = 756 nm, in a microplate, by using a multimode microplate reader (CLARIOstar Plus, BMG Labtech, Ortenberg, Germania). The total phenolic compounds (reacting with Folin-Ciocâlteu reagent) were expressed as gallic acid (GA) equivalents based on a calibration curve. This calibration curve was done with known concentrations of gallic acid.

We used the aluminum chloride colorimetric method to determine the total flavonoids (Chang et al., 2002). Briefly, 0.5 mL of sample was mixed with 1.5 mL ethanol, 0.1 mL of 1 M potassium acetate, 0.1 mL of 10% aluminum chloride, and 2.8 mL of distilled water. The incubation was done for 30 min at room temperature. The optical density was determined at λ = 415 nm in a microplate, using a multimode microplate reader (CLARIOstar Plus, BMG Labtech. The flavonoid content was expressed as quercetin (Q) equivalents, using a calibration curve constructed with known quercetin concentrations.

All of the analyses were done in triplicate. The reagents used were analytical-grade reagents purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany).

Antioxidant activity assay in the extracted plant material. Two different assays determined the antioxidant activity: DDPH• radical (2,2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging and TEAC (Trolox equivalent antioxidant capacity). The alcoholic extract of *P. caerulea* leaves and sprouts with leaves was evaporated while using a Rotavapor® R-300 (Büchi, Flawil, Switzerland). The exact quantities were re-solubilized using absolute ethanol.

We used for the DPPH• radical scavenging activity the method of Re et al. [55], with slight modifications. Briefly, 150 µL DPPH ethanolic solution (0.25 mM) was vigorously mixed with 15 µL of the sample (re-solubilized in absolute ethanol) and 90 µL of 0.1 M Tris-HCl buffer. We incubated in the dark the resulting mixture at 37 °C for 30 min. Butylated hydroxytoluene (BHT) was used as a positive control. The sample absorbance (A_{sample}) was read using a microplate multimode reader (CLARIOstar Plus, BMG Labtech) at λ = 520 nm, against a blank with ethanol (A_{blank}). DPPH inhibition (%) was calculated using the following equation:

$$\% \, Inhibition = (1 - A_{sample} / A_{blank}) * 100. \quad (1)$$

We measured the antioxidant capacity (TEAC) using the method of Re et al. [55], with slight modifications. Briefly, the ABTS [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical was generated by the reaction of 7 mM 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution with a 2.45 mM potassium persulfate solution (1:1, *v*/*v*). We incubated the mixture in the dark at room temperature for 16 h. The initial optical density of the ABTS radical solution was equilibrated to a value of 0.7 ± 0.02 at λ = 734 nm. (CLARIOstar Plus, BMG Labtech). Next, a 0.1 mL test sample was mixed with 1 mL of the ABTS radical solution and then incubated for 6 min. After incubation, the optical density was measured at λ = 734 nm (CLARIOstar Plus, BMG Labtech). A calibration curve of Trolox (0–250 µM) was used to convert the absorbance into the equivalent activity of Trolox per mL sample (µg Trolox/mL).

All the assays were performed in triplicate. All of the reagents used were analytical-grade reagents purchased from Sigma-Aldrich (Merck Group).

Influence of passion flower extracts on L. reuteri growth. We inoculated 10^7 ufc/ml of *L. reuteri* in 5 mL test tubes with 2 mL MRS broth. Each of the inoculated tubes contained 5% ethanolic extracts from aerial parts of *P. caerulea*, treated or not treated with *Trichoderma*. The extracts were sterilized by filtration on a 0.2 µm filter before being added to the MRS broth. The test tubes were incubated for 48 h at 37°C, in (micro)aerobic conditions. The number of bacteria was determined by a cultural method. Serial dilutions were done in fresh MRS broth, and 0.1 mL aliquots were aseptically spread in MRS agar plates. MRS agar plated were incubated at 37°C for 48 hours, and the number of colonies was counted. We used the following controls: MRS medium without extract and bacteria, MRS medium with corresponding quantities of alcohol and bacteria, MRS medium and 5% ethanolic extracts from *Trichoderma* treated and not treated aerial parts of the passion flowers without bacteria.

Statistical analysis. The statistical analysis was performed using SigmaStat (Systat Software, San José, CA, USA). Averages and standard deviations of the data from each set of replicates were calculated. The results were expressed as means ± SE (standard error). The Student's t-test tested statistical differences between groups. The means are considered to be significantly different at $P < 0.05$.

RESULTS AND DISCUSSIONS

Foliar Treatments with chlamydospores from our *Trichoderma* plant biostimulant consortium stimulated the accumulation of polyphenols and flavonoids from passion flowers aerial parts (Figure 1).

This result confirms our group's previous report, which demonstrated a concomitant chloroplast proliferation and polyphenols accumulation in *P. caerulea* aerial parts treated with the *Trichoderma* plant biostimulant consortium (Şesan et al., 2020).

This effect of enhanced polyphenols accumulation is related to the plant defense's activation and related secondary metabolism pathways - i.e., the phenylpropanoid pathway (Sharma et al., 2019).

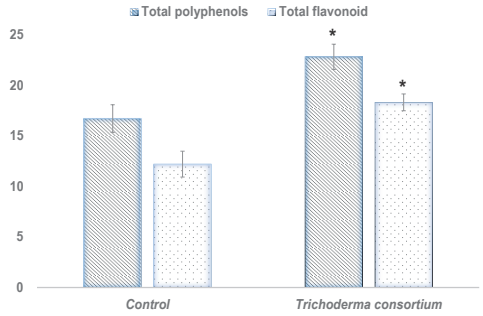

Figure 1. Enhanced accumulation of polyphenols and flavonoids following treatment with the plant biostimulant consortium *T. asperellum* T36 NCAIM F 001434 and *T. harzianum* Td50b NCAIM F001412

Activation of this phenylpropanoid pathway after *Trichoderma* treatment was demonstrated in the last years for bread wheat, *Triticum aestivum* (Singh et al., 2019), tea, *Camellia sinensis* (Shang et al., 2020), tomatoes, *Solanum lycopersicum* (Coppola et al., 2019; Yan et al., 2021), strawberry, *Fragaria x ananassa*, and corn, *Zea mays* (Agostini et al., 2021).

A higher level of polyphenols accumulated in the passionflower aerial parts following the *Trichoderma* plant biostimulant consortium is related to an enhanced antioxidant activity (Table 1).

Table 1. The antioxidant activity of the passionflower leaves treated with the *Trichoderma* plant biostimulant consortium

Specification	Control	Treated with the *Trichoderma* consortium
Antioxidant activity, DPPH method, %	72.24±6.24	54.32±4.82*
Antioxidant activity, TEAC method, µg Trolox equiv./mL	22.69±2.74	30.74±3.53

The higher level of the polyphenols and flavonoids from passionflower l is also related to an enhanced prebiotic activity, i.e., stimulating the growth of lactic acid bacteria in aerobic conditions (Figure 2).

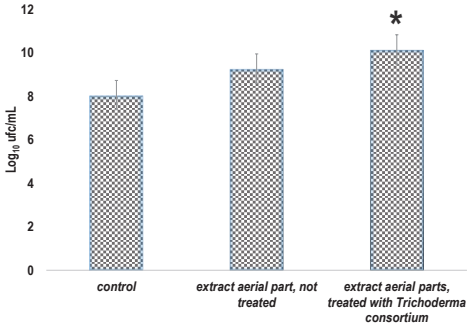

Figure 2. Stimulation of the growth of *L. reuteri* DSM 20016 by the extract of *P. caeruluea* leaves, treated and not treated with the *Trichoderma* plant biostimulant consortium

Increased content of polyphenols and flavonoids and a higher level of antioxidant activity after treatment with *Trichoderma* plant biostimulant preparations were also reported for others cultivated plants. Treatment with *Trichoderma* strains was demonstrated to increase the polyphenolic content of various plants-grape (Pascale et al., 2017), artichoke (Rouphael et al., 2017), and tomatoes (Alwhibi et al., 2017). Flavonoids and polyphenols contents were shown to increase in the edible parts of onions (Ortega-Garcia et al., 2015) and cucumbers (Nawrocka et al., 2018) following treatments with *Trichoderma* strains. Foliar application of *T. harzianum* T22 strain on grape leaves increased polyphenols content and antioxidant activity in grape fruits (Pascale et al., 2017). The same plant biostimulant strain, *T. harzianum* T22, enhances the antioxidant activity of plum tomatoes fruits (Carillo *et al.*, 2020). Foliar stimulation with the *T. atroviride* P1 strain increased the level of flavonoids, lignans, and oleuropein from olive leaves (Dini et al., 2020).

Treatment with *Trichoderma* consortium plant biostimulant strains has multilevel effects, leading to agronomic benefits (plant more resistant to biotic and abiotic stress, higher marketable yield) and enhanced health effects. Such enhanced health effects are also due to the increased prebiotic effect of the polyphenols on probiotic bacteria, including lactic acid bacteria (Figure 3).

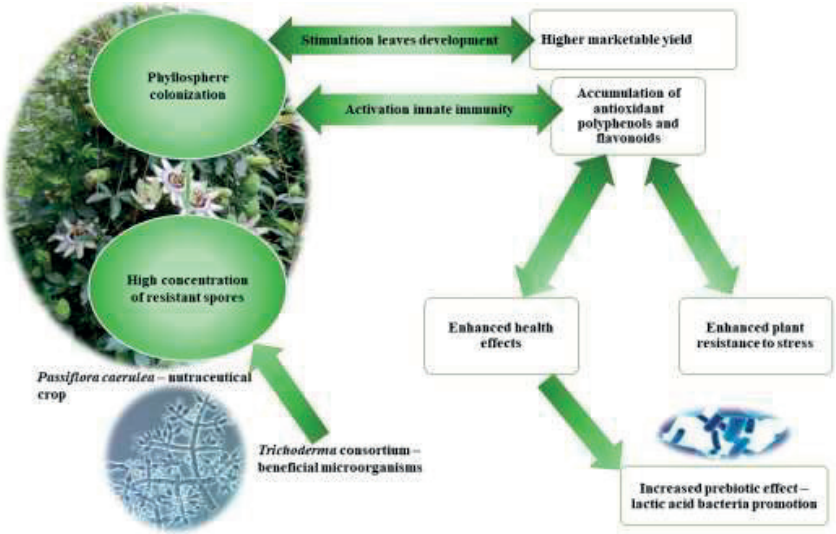

Figure 3. Multilevel effects of foliar treatments with *Trichoderma* consortium plant biostimulant strains. Modified from our previous *open-access* paper related to the effect of *Trichoderma* consortium on *P. caerulea* (Șesan et al., 2020). Licensee MDPI AG, Bassel, Switzerland

The activation of the phenylpropanoid pathway determines not only polyphenol accumulation, but also enlargement and reinforcement of the plant cell walls. Our group also demonstrated enlargement of the passionflower cell wall, especially in the palisade tissue, after treatment with *Trichoderma* consortium spores (Sârbu et al., 2018). This effect results from enhanced lignin biosynthesis (Vogt, 2010) and the accumulation of hydroxycinnamic acids

(Carrington et al., 2018). Hydroxycinnamic acids are polyphenols that anchor the hydrophilic components of the cell wall (i.e., cellulose and hemicellulose) to the hydrophobic lignin (Mnich et al., 2020).

Accumulation of the polyphenols enhances plant resistance to biotic and abiotic stress. At the same time, polyphenols from edible parts of the cultivated plants, especially from nutraceutical plants, are related to proven health benefits. (Bendini et al., 2006; da Silva et al., 2013; Lugato et al., 2014). Such health benefits also result from the mutual interactions between polyphenols and probiotic lactic acid bacteria (Piekarska-Radzik & Klewicka, 2021). The phenolic acids present in mango fruits (catechin and gallic, vanillic, ferulic, and protocatechuic acids) were reported to stimulate the growth of two probiotics, *Lactobacillus rhamnosus* GG ATCC 53103 and *Lactobacillus acidophilus* NRRLB 4495 (Pacheco-Ordaz et al., 2018). Grape pomace polyphenols induced a significant biomass increase on *Lactobacillus acidophilus* CECT 903 (Hervert-Hernández et al., 2009). Resveratrol isolated from grape pomace promotes biofilm formation and adhesion of the probiotic strain *Lacticaseibacillus paracasei* subsp. *paracasei* ATCC334 (Al Azzaz et al., 2020).

Our previous work demonstrated that treatment with plant biostimulant *Trichoderma* consortium enhances polyphenols accumulation. The findings from this paper confirm such enhanced accumulation in another year, with different climatic conditions, and demonstrates an increase in the prebiotic effect of extracts from leaves and sprouts treated with the *Trichoderma* consortium. This prebiotic effect is correlated with the enhanced polyphenols accumulation in the leaves of treated *P. caerulea* plants. Polyphenols were recognized in the last years as prebiotics (Alves-Santos et al., 2020; Bindels et al., 2015; Moorthy et al., 2020).

Prebiotic effects of the polyphenols from *P. caerulea* plants could also be related to the effect of the preparation from this plant on mood disorders. Probiotic microorganisms from digestive systems were described as a "neglected endocrine organ" (Clarke et al., 2014), with a contribution to the normal brain function – "melancholic microbes" (Dinan et al., 2019).

CONCLUSIONS

Foliar treatment with a suspension of chlamydospores from a *Trichoderma consortium* with plant biostimulant properties enhance polyphenols accumulation and antioxidant activity

Enhanced polyphenol accumulation and antioxidant activity are correlated with stimulating lactic acid bacteria *L. reuteri* DSM 20016 in aerobic conditions. Such a prebiotic effect could be related to the known efficiency of passionflower extract on mood disorders. Probiotic bacteria produce short-chain fatty acids, which assure proper brain function.

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EXTRACTION AND IDENTIFICATION OF THE MAIN ALLERGENS FROM FRIED PEANUTS

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Abstract

Food allergy (FA) is a relatively new food safety issue with an increasing prevalence worldwide. Although FA-related diseases are restricted to a small category of individuals, the number of patients concerned is continuously growing in the last years. A total of eight big ingredients have been identified for most of the allergic reactions: peanuts, milk, egg, wheat, soy, tree nuts, fish, and shellfish. The peanuts allergenicity is affected by the whole ingredient composition rather than by a single allergenic protein. In general, Ara h3 accounts for approximately 28% of the total protein fraction, followed by Ara h1 (11%), Ara h2 (10%) and Ara h6 (8%). The aim of our study was to extract the main allergens from peanuts using different methods of extraction. First the fried peanuts samples were defatted by stirring with pre-cooled acetone and 0.07% β-mercaptoethanol and then extracted with different buffers. The identification of proteins was made by Biuret and BCA assay and also by electrophoretic examination.

Key words*: food allergy, peanuts, peanuts extraction, allergens*

INTRODUCTION

Food allergy (FA) is a public health problem that has consequences on a personal, social, economic and nutritional level, being a growing global problem (Loh and Tang, 2018). A recent study conducted in the United States indicates that up to 8% of children and 10.8% of adults have FA (Gupta et al., 2018). Unlike milk and eggs allergies, which are usually overcome by the age of 5-10 years, the allergy to peanuts and nuts is often lifelong, persisting into adulthood in 80% of cases (Ballmer-Weber, 2011). In the United States, peanut allergy affects 2.2% of children and 1.8% of adults. Among children with FA, those with peanut allergy report the highest rate of anaphylaxis and side effects. Moreover, compared to other allergies, the one for peanuts results in more visits to the emergency department for anaphylaxis and 1 in 4 children requires at least one visit each year to the hospital to manage allergic reactions (Parlaman et al., 2016; Motosue et al., 2018).

Avoidance of peanuts is inherently difficult because of their widespread consumption as roasted peanuts and snack products, use in food industry for oil and peanut butter production, cake decoration, extenders in meat products, soups and desserts formulations (Zhao et al., 2012). Studies designed to determine the minimum dose of peanut proteins that may cause an allergic reaction have reported subjective symptoms at a dose up to 100 µg and objective symptoms at 2 mg of ingested proteins (Al-Muhsen et al., 2003). Nowadays, some therapies have been introduced to reduce the prevalence of peanut allergy, including strict avoidance, oral immunotherapy, rescue medication upon accidental exposure to peanuts and modifying or removing allergens from foods (Wood, 2016; Togias et al., 2017).

Peanuts contain major proteins, such as albumin, globulin (arachin, conarachin), but also several minor functional proteins with a metabolic role. To date, 17 types of peanut allergens, referred to as Ara h1 - Ara h17, have been published and named by the World Health Organization and International Union of Immunological Societies (WHO/IUIS). Among these allergens, Ara h2, Ara h6 and Ara h7 are 2S albumins, while Ara h1 and Ara h3 are 7S/11S globulins (Pi et al., 2019).

Proteins have to be efficiently extracted to ensure an accurate representation of allergens from the source material. Therefore, the selection of an adequate method of extraction represents a major step for further investigations. In order to obtain an optimized peanut extraction, several parameters and buffers were tested and their effect on protein characteristics was investigated (Ma et al., 2010; Mihai (Draghici) et al., 2020).

The aim of our study was to extract the main allergens from fried peanuts using several protocols of extraction at different pH and temperature values. The quantification of proteins was performed by Biuret and BCA assays and their identification by electrophoretic examination. The data regarding the optimization of peanut protein extraction will be valuable for medical researchers and peanut breeders.

MATERIALS AND METHODS

Materials

Acetone, disodium phosphate, monosodium phosphate, boric acid, sodium hydroxide, sodium bicarbonate, sodium carbonate (anhydrous), bicinchoninic acid (BCA), sodium hydroxide, copper(II) sulfate and potassium sodium tartrate were purchased from Sigma-Aldrich (Germany). Bovine serum albumin (BSA) (synthesis grade, ≥95%) was purchased from Merck (Germany).

Peanut sample preparation

The sample (fried peanuts) was purchased from a local supermarket, washed in distilled water and dried at 40°C, in an oven.

Protein extraction and analysis

Protein extraction

The peanut sample was milled to a fine flour using a laboratory grinder. The peanut flour was defatted by stirring in pre-cooled acetone (1:5 ratio, w/v) at 4°C, for 3 h and then filtered using a vacuum filter. The defatting process was repeated three times. The defatted flour was dried in an oven at 40°C and stored at –20°C until further use. Peanut protein was extracted from the defatted flour using six different protocols (Protocols 1-6) by varying two parameters: the extraction buffer type and the temperature of extraction.

Protocol 1: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.03M phosphate buffer, pH 7.2, in a ratio of 1:10 (w/v), at 25 °C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 2: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.03 M phosphate buffer, pH 7.2, in a ratio of 1:10 (w/v), at 4°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 3: Peanut protein extract was obtained by mixing the defatted peanut flour with 1 M borate buffer, pH 8.5, in a ratio of 1:10 (w/v), at 25°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 4: Peanut protein extract was obtained by mixing the defatted peanut flour with 1 M borate buffer, pH 8.5, in a ratio of 1:10 (w/v), at 4°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 5: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.05 M carbonate buffer, pH 9.6, in a ratio of 1:10 (w/v), at 25°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protocol 6: Peanut protein extract was obtained by mixing the defatted peanut flour with 0.05 M carbonate buffer, pH 9.6, in a ratio of 1:10 (w/v), at 4°C, for 4 h. The crude extract was cleared by centrifugation at 10,000 rpm, at 4°C, for 30 min and the supernatant was collected.

Protein quantification

Peanut protein content was investigated by Biuret and BCA assays. A standard curve was built using BSA in the range of concentrations 0.1-10 mg/ml.

Biuret assay: The principle of the method is based on the formation of a purple complex between the peptide bonds from a polypeptide chain and Cu^{2+} ion, in alkaline conditions. The intensity of the developed color is proportional to the sample protein content (Zhenga et al., 2017). Biuret reagent was prepared from an aqueous solution of potassium sodium tartrate treated with cupric sulfate and sodium hydroxide. In Biuret assay, 2 ml of sample

extract were incubated with 3 ml Biuret reagent, at 37°C, for 10 min. The absorbance was measured at 540 nm against blank (water instead of sample) using an UV-Vis spectrophotometer (Jasco V650, Japan).

BCA assay: The BCA assay determines the total concentration of protein using two step procedure by reducing Cu^{2+} to Cu^{+} ions, under alkaline conditions. Then, Cu^{+} ions react with BCA to form a colored complex (Walker, 1994). BCA reagent was prepared by mixing BCA with 4% cupric sulfate solution in a ratio of 50:1 (v/v). In BCA assay, 20 µl of sample extract were incubated with 160 µl BCA reagent, at 37°C, for 30 min. The absorbance was measured at 562 nm using the microplate reader SPECTROstar Nano (BMG Labtech, Germany).

Electrophoresis in tricine-SDS polyacrylamide gradient gel

Peanut proteins were analyzed by tricine-SDS-polyacrylamide electrophoresis, according to Schagger method (1987). The sample was denatured at 90°C, for 3 min and loaded on 10-20% tricine gels. Migration was carried out at 100 V, for 3 h using a BIO-RAD source. Wide range molecular weight marker (6.5-200 kDa) was used as standard. Gels were stained with Coomassie Brilliant Blue solution.

Statistical analysis

Experiments of extraction and protein content determination were carried out in duplicate and triplicate, respectively. All data are expressed as the mean ± standard deviation (SD). Statistically significant differences were considered at $p<0.05$.

RESULTS AND DISCUSSIONS

Extraction of the main allergens

A great challenge when studying peanut allergens is to find the most appropriate method of extraction with the highest yield and reproducibility. Several factors affect the extractability of these proteins, including the type of buffer and the extraction temperature. In our study, the allergens were extracted from fried peanuts varying the working parameters, like buffer type and temperature, in order to select the optimal ones. The extraction conditions are presented in Table 1.

Table 1. Peanut allergens extraction conditions

Sample	Extraction conditions
A I.1	0.03 M phosphate buffer; pH 7.2; 1:10 (w/v); 25°C; 4 h
A I.2	0.03 M phosphate buffer; pH 7.2; 1:10 (w/v); 4°C; 4 h
A II.1	1 M borate buffer; pH 8.5; 1:10 (w/v); 25°C; 4 h
A II.2	1 M borate buffer; pH 8.5; 1:10 (w/v); 4°C; 4 h
A III.1	0.05 M carbonate buffer; pH 9.6; 1:10 (w/v); 25°C; 4 h
A III.2	0.05 M carbonate buffer; pH 9.6; 1:10 (w/v); 4°C; 4 h

Protein quantification by BCA assay

One of the most used methods for quantifying proteins is based on their reaction with BCA, as proposed by Smith et al. (1985).

The BCA assay results for protein quantification in six extracts obtained from fried peanuts are showed in Table 2.

Table 2. Protein concentration in fried peanut extracts determined using BCA assay. The results are expressed as mean ± SD.

Sample	Protein concentration (mg/ml)
A I.1	2.66 ± 0.01
A I.2	1.50 ± 0.02
A II.1	14.61 ± 0.04
A II.2	11.77 ± 0.01
A III.1	26.49 ± 0.02
A III.2	21.74 ± 0.04

The results obtained by BCA assay showed that the amount of protein in fried peanut extracts varied between 1.50 and 26.49 mg/ml. The highest protein content was found in the case of A III.1 (26.49 mg/ml) and A III.2 (21.74 mg/ml) samples, while the lowest values were registered in the case of A I.1 (2.66 mg/ml) and A I.2 (1.50 mg/ml) samples. The samples A II.1 and A II.2 presented average values of protein content of 14.61 mg/ml and 11.77 mg/ml, respectively.

Protein quantification by Biuret assay

Compared with other methods for protein quantification, the Biuret assay has the advantages of easy operation, excellent precision.

The Biuret assay results for protein quantification in six extracts obtained from fried peanuts are showed in Table 3.

Table 3. Protein concentration in fried peanut extracts determined using Biuret assay. The results are expressed as mean ± SD

Sample	Protein concentration (mg/ml)
A I.1	7.04 ± 0.01
A I.2	4.66 ± 0.03
A II.1	21.14 ± 0.04
A II.2	14.00 ± 0.01
A III.1	36.69 ± 0.02
A III.2	29.88 ± 0.01

The results obtained by Biuret assay showed that the amount of protein in fried peanut extracts varied between 4.66 and 36.69 mg/ml. The highest protein concentration values were found in A III.1 (36.69 mg/ml) and A III.2 (29.88 mg/ml) samples, the lowest protein content values were registered in A I.1 (7.04 mg/ml) and A I.2 (4.66 mg/ml) samples. An average protein content was found in A II.1 (21.14 mg/ml) and A II.2 (14.00 mg/ml) samples. The results obtained in the case of Biuret assay indicated slightly higher values of protein content in fried peanut extracts, as compared to those obtained for the same extracts analyzed by BCA assay. Due to similar variation observed in both methods, the effect of extraction parameters was further discussed in relation to BCA assay. In addition values of protein content were expressed as arbitrary units (A.U) for a better comparison of the results.

Effect of the extraction buffer composition and pH on total protein content

Our study was conducted in three directions: influence of buffer pH (there is a difference of 2.4 pH units), the influence of ionic strength (the variation is between 0.03 and 1.0M) and the influence of buffer type.

The effect of buffer composition on the protein content of fried peanut extracts is presented in Figure 1.

The results show that a 10 to 14.5-fold increase of protein content was obtained in the case of fried peanut extraction using carbonate buffer, pH 9.6, compared to the case of extraction in phosphate buffer, pH 7.2. In the case of fried peanut extraction using borate buffer, pH 8.5, a 5.5- to 7.8-fold growth of protein content was observed, compared to the case of extraction in phosphate buffer, pH 7.2.

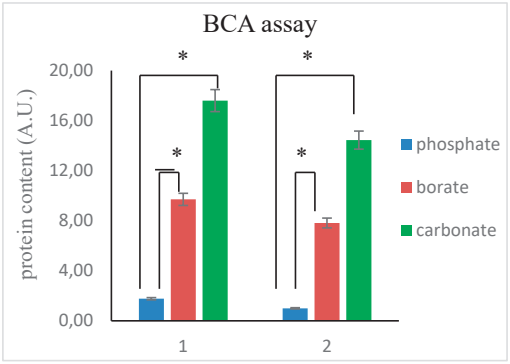

Figure 1. Effect of the extraction buffer composition on total protein content in fried peanut extracts determined using BCA. The results are expressed in arbitrary units (A.U.), as mean ± SD, *p<0.05

Statistical analysis showed significantly ($p<0.05$) higher values of protein content in extracts obtained in carbonate buffer, pH 9.6, compared to those in extracts obtained in phosphate buffer, pH 7.2. Similar, significantly ($p<0.05$) higher values of protein content were observed in borate buffer, pH 8.5, compared to those in phosphate buffer, pH 7.2.

Effect of the extraction temperature on total protein content

The effect of the temperature on the protein content of fried peanut extracts is presented in Figure 2.

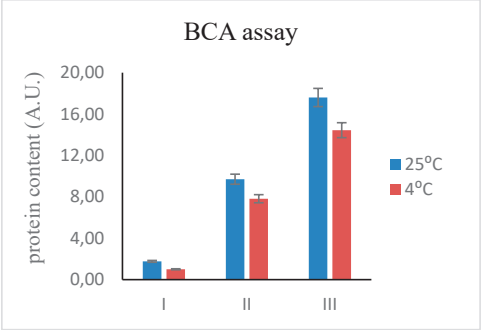

Figure 2. Effect of the extraction temperature on total protein content in fried peanut extracts determined using BCA. The results are expressed in arbitrary units (A.U.), as mean ± SD, *p<0.05

It was observed that the temperature of extraction has induced great variation of the protein content in the six fried peanut extracts. Thus the extract obtained in phosphate buffer, pH 7.2 (I), at 25°C had an 1.77-fold higher protein content than that observed in the case of fried peanut extract at 4°C. A slightly lower increase of 1.24-fold was recorded for the peanut extract in borate buffer, pH 8.5 (II), at 25°C, compared to 4°C. Similar increase of 1.22-fold was calculated for the protein content in the peanut extract obtained in M carbonate buffer, pH 9.6 (III), at 25°C, compared to 4°C.

Statistical analysis showed significantly ($p<0.05$) higher amount of protein obtained at a temperature of 25°C than that at 4°C, regardless of the used buffer type for peanut extraction.

In other studies Tris-HCl was frequently used for peanut protein extraction at different pH and molarities. It was observed that pH values higher than 8 increased the extraction of peanut allergens similar to results our present study (Mihai (Draghici) et al., 2020; Masuyama et al., 2018).

Identification of allergens from fried peanuts by electrophoresis

The main allergens from fried peanuts were identified by electrophoresis in tricine-SDS-polyacrylamide gradient gel. The migration patterns of six protein extracts from fried peanuts are presented in Figure 3. Based on the molecular weight, the main bands were assigned to Ara h6 at 15 kDa, Ara h2 at 17-19 kDa, Ara h3 at 28 kDa and 42 kDa and Ara h1 at 64 kDa. The protein extracts in borate (A II.1, A II.2) and carbonate (A III.1, A III.2) buffers presented all types of allergens, although the peanuts were subjected to high temperature processing. In turn, faint bands corresponding to identified allergens were extracted in phosphate buffer, in particular at 4°C (A I.2).

Previous studies identified and quantified the main allergens in peanuts as Ara h1 in a percentage of 12-16%, Ara h2 10%, Ara h3 3.7-4.3% and Ara h6 in a proportion of 6-9% of the total peanut proteins content (Ma et al., 2010). Due to the presence of multiple disulfide bonds, Ara h2 and Ara h6 are more resistant to high temperatures and proteases than Ara h1 and Ara h3 (Pi et al., 2019).

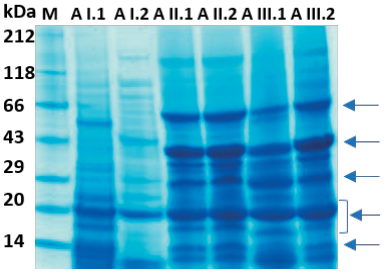

Figure 3. Tricine-SDS-polyacrylamide electrophoresis of fried peanut extracts A I.1-A III.2. The molecular weight marker (M) was migrated in the same gel

CONCLUSIONS

In this study, the influence of two extraction parameters, the buffer type and temperature was assessed, in order to identify the conditions that improved peanut allergens extraction. The protein content significantly increased in the case of using carbonate buffer at pH 9.6 as extraction solvent, compared to that obtained in phosphate buffer. The temperature also played an important role, the best results being obtained in the case of extraction at room temperature. The main allergens from fried peanuts were identified using tricine-SDS-polyacrylamide electrophoresis. Ara h1 (64 kDa), Ara h2 (17-19), Ara h3 (28 kDa, 42 kDa) and Ara h6 (15 kDa) appeared as main bands in all analyzed samples, excepting for phosphate buffer extracts. All these results presented valuable information for the optimization of allergens extraction from fried peanuts and their identification and could be valorized within medical and food industry studies.

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ROMANIAN CONSUMER BEHAVIOUR REGARDING PASTA CONSUMPTION DURING THE COVID 19 PANDEMIC

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Abstract

Since the onset of the COVID-19 pandemic and the associated lockdowns, many events occurred, such as supply chain disruption, shielding and working from home restrictions along with diminished incomes and people's extra time spent in their own homes; all these have led to broad changes in consumer attitudes and behaviour. Therefore, people spent more time at home in all countries, and there was a significant increase in the number of people who enjoyed experiencing cooking at home, having a regular schedule for their meals. Pasta represents one of the most consumed food products in the world, their consumption increasing from year to year, experiencing a boost demand during the pandemic, because it has many important advantages such as: low cost, easy to cook, high nutritional value and long shelf life. Properly cooked pasta has a low potential to increase blood sugar, it slows down digestion and give a feeling of satiety for a longer period. The purpose of this study was to obtain information on consumer perceptions towards pasta consumption in order to develop new products that are adapted to current consumer requirements and preferences.

Key words*: pasta consumption, consumer behaviour, Covid 19 pandemic.*

INTRODUCTION

The global crisis generated by the COVID-19 pandemic has devastated the global economy, the medical system, causing panic, anxiety and uncertainty among people.

More than a year after the global crisis caused by the COVID 19 pandemic began, humanity is facing a new wave of infections, despite all the restrictions imposed and the protection measures taken. According to data collected on March 29, 2021, from the World Health Organization (WHO) website regarding the evolution of the COVID 19 pandemic, 126,890,643 cases were confirmed and 2,778,619 confirmed deaths were recorded (WHO, 2021). The third wave of infections comes with new restrictions for the population, due to the appearance of new strains of SARS-COV 2 virus that have proven to be much more virulent. Presently, billions of people around the world are under lockdown.

In Romania, the new restrictions imposed by the government to limit the spread of infections are, among others, limiting the operating hours of economic operators, which implicitly affects the operating hours of grocery stores. We are witnessing a new form of panic expression that is quite increased and widespread among consumers, which has led to the overcrowding of these stores.

Panic buying has become a global phenomenon reflecting that loss of control among consumers in the era of Coronavirus lockdown (Islam et al., 2021). According to Oxford (2020), panic buying is "the action of buying large quantities of a particular product or commodity due to sudden fears of a forthcoming shortage or price increase". For instance, when consumers start panic buying dry pasta, eventually, the whole supply chain with raw and auxiliary materials, involving eggs, flour, wheat, is affected (Nikolopoulos, 2021).

The impacts of COVID-19 pandemic on food systems can be divided between direct impacts of the virus outbreak, and indirect impacts derived from containment measures (e.g. lockdown, mobility restrictions, shops closure) adopted at different levels, from local to global. While all food systems across the globe have been affected by the pandemic, it is argued that vulnerability is different for different types of

food systems. Long food supply chains have been particularly affected by COVID-19 crisis; however, it is important to avoid universalization of impacts and responses as agri-food systems are characterized by a huge diversity and heterogeneity (Rivera-Ferre et al., 2021).

Despite declining sales during lockdown, they remained above average for non-perishable food. For example, depending on the week, in March 2020 rice showed sales between +27% and +208% and flour between +105% and +139% as compared to the averages of the previous six months. Pasta and strained tomatoes showed a similar pattern of sales (Statistisches Bundesamt, 2020).

The filled fresh pasta market is expected to register a Compound annual growth rate (CAGR) of over 4% during 2020-2024 (Business Wire, 2020).

As the number of people infected with SARS-COV 2 continues to rise, consumers are being advised to ensure that they have enough food to last for two weeks in case they experience symptoms or have come into contact with people who have been diagnosed positively and need to self-isolate. This has led to a rising demand for non-perishable goods like dried pasta. The closure of many restaurants to prevent the spread of the virus has also led to more consumers cooking at home. In March 2020, UK sales of dried pasta increased by 55%. Another factor that has caused increased sales of dried pasta is the impact the global crisis has had on consumer's finances, with many looking for economical ways to feed their families such as pasta dishes, casseroles and stews. The spike in demand for dried pasta and noodles has also boosted demand for wheat, leading to rising wheat prices across the US and Europe (Globe Newswire, 2020).

The significant increase in the average energy density of solid foods consumed by participants was not reflected in changes in fruit and vegetable intake, expected to decrease, or snack food intake, expected to increase. However, intakes from other food categories that we did not analyse might have changed, including bread, pasta, meat, and prepared salads (Poskute et al., 2021).

It may be possible that non-perishable food was not more consumed than usual as, for instance, typical meals which were previously eaten in restaurants or canteens (e.g., pasta) were now cooked at home (Lehberger et al., 2021).

Traditional pasta dishes have always been a part of the human diet, and they are comprised from different types of cereals. One of the most important and used crops in the world is represented by cereals, and *Triticum vulgare* and *Triticum durum* are the most important species of wheat used to produce pasta (Biernacka et al., 2021).

Refined flour or semolina are the main ingredients from which pasta is made because they have a low quantity of vitamins, fibre, and bioactive compounds. Therefore, these products are usually fortified with products that increase the technological and nutritional quality (Nilusha et al., 2019)

Fresh pasta dishes are widely consumed all over the world, being a good substituent for the traditional dry pasta products. Fresh pasta is used as a carrier of functional ingredients such as β-glucans, because of their ability to limit blood cholesterol levels and in the same time, not affect the organoleptic proprieties of the fresh pasta products (De Santis et al., 2020). There are several kinds of new raw materials used in order to make pasta products. Zarzycki et al. (2021) studied the use of Moldavian dragonhead defatted flour in order to produce new pasta products, and the study revealed that the Moldavian dragonhead defatted flour pasta had a better nutritional content and sensory acceptance than the traditional pasta.

In this context, the aim of the present study was to obtain information on consumer perceptions towards pasta consumption in order to develop new products that are adapted to current consumer requirements and preferences.

MATERIALS AND METHODS

In order to obtain information on consumer preferences and perceptions towards pasta consumption during the COVID-19 pandemic a survey in form of an online questionnaire was designed and uploaded on “Google Forms” platform, this being distributed in the online environment on different social networks. The target population were persons over the age of 18, who are responsible for the shopping activities within their household (i.e. food

shoppers), from both the urban and rural regions of Romania. The questionnaire-based interview method is one of the most widely used information collection techniques. The questionnaire has a short completion time of several minutes and consists of a small number of logical questions that aim to provide information about buying habits, preferences, behaviour and even the profile of consumers (Colibaba, 2001).

In order to conduct the study on pasta consumption, a questionnaire was developed containing 20 questions, 13 of which refer to consumption habits and consumer preferences, and the other 7 questions are aimed at obtaining socio-demographic information about consumers such as: age, gender, income, environment from which they come, etc. The questions are closed, simple, clear, some with a single answer and others with multiple answers. In the first part, the respondents were asked if they consume pasta, what types of pasta do they consume, how often do they buy pasta products, their method of preparation and questions regarding the price/manufacturer of the pasta products. The second part of the questionnaire included questions about the age, professional status, education and monthly earnings.

This study was conducted in 2020 and following the completion of the questionnaire a total sample of 178 answers was gathered 78.6% women and 21.4% men. The data were collected, processed and interpreted, thus drawing the final conclusions of the study.

RESULTS AND DISCUSSIONS

In the first part of questionnaire, the participants were asked if they consume pasta products (Figure 1).

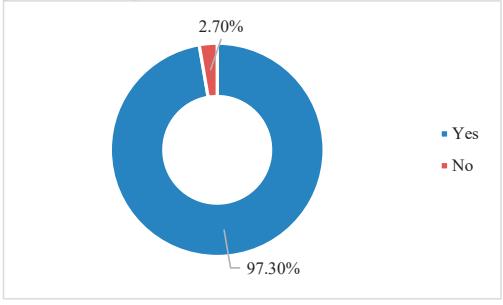

Figure 1. Q1 - Are you a pasta consumer?

The results indicated that 97.3% of the respondents stated that they consume pasta, while 2.7% mentioned that were not among the pasta consumers.

Pasta dishes seem to be the favourite products of Romanian household consumption registering an important increase in recent years. This increasing trend in the pasta consumption could be observed in the first half of 2020, amid the crisis and the new rules imposed during the state of emergency caused by the Covid 19 pandemic, consumers being concerned about food products purchasing with increased validity in order to limit the supply frequency used to reduce the risk of contamination with the new coronavirus.

The next question (Figure 2), a multiple answer question, asked the respondents what types of pasta products they consume regularly. According to the obtained results, the most consumed types of pasta are spaghetti (70.7%) and vermicelli (42.4%), followed by macaroni and penne, both with a percentage of 38%, then noodles (29.3%), tagliatelle (18.5%), lasagne (13.6%), tortellini (12.5%), and the last places in the top of consumer preferences were ravioli (9.2%) and couscous (3.8%).

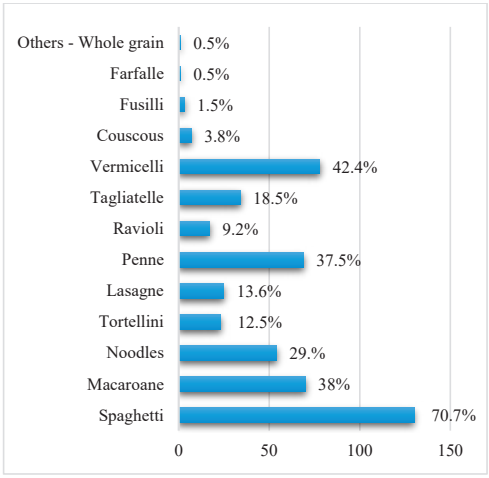

Figure 2. Q2 - What types of pasta do you consume regular?

In the next question (Figure 3), also a multiple answer question, respondents were asked how they consume the pasta. Of those who participated in the study, 62.2% consumed pasta as a stand-alone dish, followed

immediately by their consumption in soups or broths by 56.2% of respondents, while 49.2% of respondents prefer to eat pasta as a dessert. Only 5.9% of respondents choose to eat pasta as an addition to various salads, and 7% in other forms.

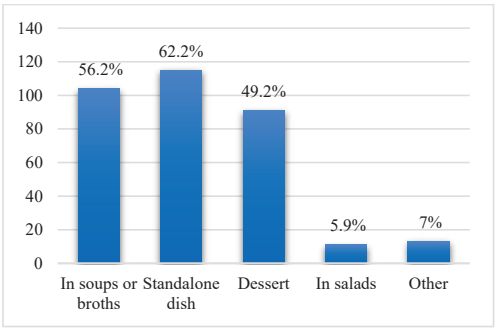

Figure 3. Q3 - In what form do you consume the pasta products?

Analysing the answers of the question "How often do you eat pasta" it can be seen that 38.9% of respondents eat pasta once a week, 18.9% consuming them even 2-3 times a week. There are a small number of people who eat pasta daily (1.6%), but there are also people who say that they only rarely eat pasta (8.6%). 22.7% of the respondents eat pasta once every two weeks, and 9.2% eat only once a month (Figure 4).

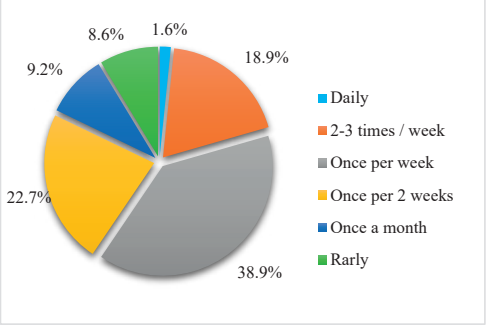

Figure 4. Q4 - How often do you consume pasta?

The next question was about the amount of pasta consumed per month by the respondents. The results showed that 65.9% of them consume 500 grams, 20% consume 1000 grams, and 14.1% of them consume more than 1500 grams per month, according to the data presented in Figure 5.

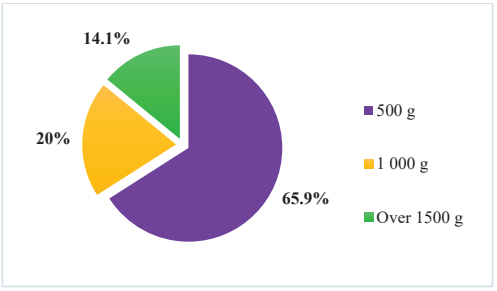

Figure 5. Q5 - What amount of pasta do you consume on a monthly basis?

A question regarding the criteria that determines the respondents to buy pasta was asked (Figure 6).

The main criteria that are taken into account when purchasing pasta are quality (64.9%) and taste (42.7%). The following important attributes are price and brand / manufacturing company, registering the same number of answers, respectively 27.6%. Of those surveyed, 13% take into account the ingredients contained in pasta, and for 10.8% of respondents the type of flour from which pasta was made is important. The least important criteria for choosing pasta was packaging, only 8.1% of respondents taking it into account.

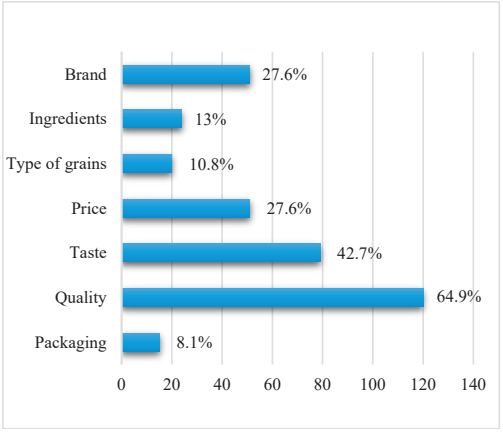

Figure 6. Q6 - What are the criteria that determine you to buy pasta?

In terms of price, more than half of the participants in the questionnaire (60.3%) stated that they are always looking for the best value for money. About 16.3% of consumers buy pasta depending on the brand, regardless of price, while 7.1% choose the most expensive

pasta, which are better in terms of quality according to them. 4.9% of consumers choose pasta that has the lowest price, and 11.4% do not take into account either price or brand, which is a good thing when it comes to launching new products (Figure 7).

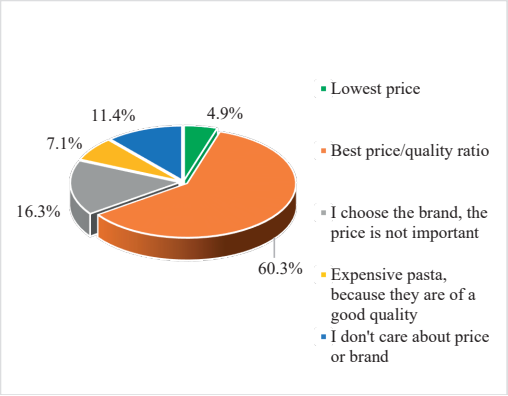

Figure 7. Q7 - In terms of price, how do you choose your pasta?

Regarding the availability of respondents to consume different pasta types depending on the used flour, they indicated the most common pasta known, namely those with white wheat flour gluten (77.2%) and gluten-free pasta made from whole wheat semolina (17.4%). A certain importance was also given to gluten-free pasta made from corn flour, 16.3% of the respondents being willing to consume it, respectively to gluten-free pasta made from rice flour (10.9%) and gluten-free pasta made from 100% brown rice (9.2%).

The next places in the top of consumer preferences are pasta with golden flax flour (7.1%), gluten-free pasta with pea flour (6%) and gluten-free pasta made from rice flour and corn flour (5.4 %). The pasta type with smallest percentage of responses are gluten-free pasta made from buckwheat flour (4.3%), gluten-free pasta made from chickpea flour (3.8%), gluten-free pasta from black bean flour (3.8%), gluten-free pasta made from lentil flour (2.2%) and red lentil flour (2.2%), which means that the consumers showed a lower interest for these products.

The respondent's socio-demographic profile is presented in Table 1. 48.6% of the participants in this study are between 18-25 years old, while 17.3% of the interviewees are between 35-45 years old. Respondents aged 45-60 years represent 17.8% of the total participants, while respondents aged 25-35 represent 16.2%. The vast majority of the respondents were employed (54.9%), and another important part of them were students (38%). Regarding the monthly net income, 41% have low incomes below 1500 RON, 39.3% have average incomes in the range of 1500-3000 RON, while 19.7% of consumers earn more than 3000 RON per month. Regarding the question "What is the last form of education you completed?" it was observed that 58.7% of the participants graduated high school, 29.3% have higher education (graduated from college), 10.9% graduated from vocational schools, while only 1.1% of respondents graduated from primary school. At the end of the questionnaire, consumers were asked to specify their environment, so 58.9% of them mentioned urban areas, and 41.1% represent the number of those from rural areas.

Table 1. The socio-demographic profile of the respondents

Age group	48.6% - 18-25 years 16.2% - 25-35 years 17.3% - 35-45 years 17.8% - 45-60 years
Gender	21.4% - male 78.6 - female
Highest level of education completed	58.7% - High school 29.3% - University 10.9% - Professional School 1.1% - School
Household size	54.6% - four or more members 27.6% - three members 16.2% - two members 1.6% - one member
Professional status	54.9% - employed 38% - student 4.9% - unemployed 1.6% - pensioner 0.5% - entrepreneur
Household's monthly net to income	41% - under 1500 RON 39.3% - 1500-3000 RON 19.7% - over 3000 RON
Where do you live?	58.9% - urban area 41.1% - rural area

CONCLUSIONS

Following this study, the profile of Romanian pasta consumers was identified, as well as their

habits, preferences, purchase and consumption criteria, in order to ensure to consumers, the availability of their favourite products in the grocery/store, as well as to successfully launch new products on the market.

The study showed that almost all participants are consumers of pasta, there are people who consume them even daily, which leads us to believe that pasta is an important category of human food products. Most of the interviewees consume pasta as an independent dish, being exceeded their consumption in soups or broths hence resulting in increased consumption of pasta.

The results obtained revealed that the most consumed types of pasta are spaghetti, and the least consumed are farfalle. The majority of respondents were female, aged between 18-25 years, coming from urban areas. The main criteria taken into account when purchasing pasta are quality and taste, so producers must adopt specific procedures to improve these two features. A very small number of respondents choose the pasta with the lowest price, which is an advantage in terms of launching new products, as they can start from a higher price.

Studies have shown that due to the COVID-19 pandemic, most food companies have experienced a significant drop in orders. The difficulties they faced consisted in finding suppliers of raw and auxiliary materials, as a result of the cancellation of events, respectively fairs, in which partners could meet and sign contracts. Thus, the whole agro-food chain was negatively affected. In particular, the transport of foodstuffs has been difficult to carry out in some countries that have temporarily closed their borders, subsequently establishing much stricter rules and fixed transit routes. This has led to an increase in consumer panic buying, which has been reflected in increasing demand for non-perishable goods, such as dried pasta.

ACKNOWLEDGEMENTS

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SOLUBLE PROTEIN CONTENT ASSESSMENT IN DRY PET FOOD RAW MATERIALS: COMPARISON BETWEEN FRESH MEAT AND MEAT MEAL FORMULATIONS

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Abstract

Long shelf life and ease of use make dry pet food a popular choice among pet owners, inasmuch as it represents the majority of pet food on the market today. Two kinds of raw materials are commonly employed for the production of dry pet food, namely fresh meats (FMs) and particularly meat meals (MMs). These raw materials, before coming onto the market as dry pet food, undergo production processes, transportation, and, when it comes to MMs, industrial transformations, which may result in unwanted modifications of such ingredients, especially as far as their protein content is concerned. The goal of this study is to analyze the protein content of the raw materials regularly used in the production of dry pet food. Different formulations of white, red, and fish FMs and MMs have been prepared and analyzed. The protein concentration of both FM and MM mixes was assessed by the Bradford assay, with the aim being to evaluate the soluble protein content, which represent also a convenient digestibility index. Subsequently, the quality of proteins was evaluated through the characterization of the electrophoretic profile assessed by SDS-PAGE followed by staining with Coomassie Blue dye. The results proved that the formulations made of FMs, compared to the ones based on MMs, have a higher soluble protein content and a better-defined protein profile, thus making the former the best choice as raw materials for dry pet food production.

Key words*: Digestibility, Dry Pet Food, Fresh Meats & Meat Meals, Raw Ingredients, Soluble Protein Content.*

INTRODUCTION

The rate of growth of the dry pet food market is continuously increasing, and new formulations are always proposed. The need for a thorough evaluation of the quality of the raw materials used in the production process thus becomes urgent (Montegiove et al., 2021; Zicker, 2008).
Most dry pet foods found on the market today are made of two different types of raw materials, which differ in their protein content. They consist of fresh meats (FMs) and, in particular, meat meals (MMs) (Montegiove et al., 2021; Montegiove et al., 2020a; Thompson, 2008). FMs derive from wastes of meat intended for human consumption, whereas MMs are meat by-products obtained during meat processing. FMs mainly consist of animal parts that are not suitable for human consumption but have shown no signs of disease that can be transmitted to humans. MMs, on the other hand, according to the Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21/10/2009, may also include other parts of the animals, such as bristles, feathers, hooves, and horns. These MMs are largely used by pet food suppliers to obtain complete feedstuffs, by enhancing the protein and amino acid (AA) content in pet kibbles; on the other hand, MMs undergo intensive industrial processes which may determine unfavorable effects on their

digestibility (Montegiove et al., 2021; Murray et al., 1997). Further to this, the onset of oxidation processes and the partial degradation of MM raw materials can lead to the loss of protein content bioavailability (Montegiove et al., 2021; Ribeiro et al., 2019; van Rooijen et al., 2013). The handling processes also play a key role in maintaining the organoleptic properties of proteins, as during the transport and storage of raw materials the protein component could be altered by microorganisms, whose proliferation can lead to the decarboxylation of some AAs with the formation of biogenic amines, responsible for numerous toxic effects on the body (Brozić et al., 2019; Carter et al., 2014; Learey et al., 2018; Montegiove et al., 2021; Montegiove et al., 2020b; Piergiovanni & Limbo, 2010).

Therefore, the quality of the final product is strictly dependent on the initial choice of the raw materials used, which thus becomes a crucial point for the manufacturing companies in the dry food production process for dogs and cats. Hence, this study aims at carefully analyzing the protein component of the raw materials typically employed in dry pet food production. Mixes of white (*i.e.*, chicken, turkey, and rabbit), red (*i.e.*, pork, beef, and lamb), and fish (*i.e.*, salmon, tuna, and anchovy) FMs and MMs were investigated in this study as representative examples of different kinds of animal protein sources that are commonly found in the pet food industry (Figure 1) (Aldrich, 2006; Thompson, 2008; Yathavamoorthi et al., 2020).

Protein content analysis was carried out using the Bradford assay (Bradford, 1976) in order to quantify the soluble protein content, which represents a convenient digestibility index and, at the same time, give an estimate of the total protein content as highlighted by a recent study (Montegiove et al., 2021).

Protein quality was evaluated through the electrophoretic protein profile assessed by SDS-PAGE followed by Coomassie Blue staining.

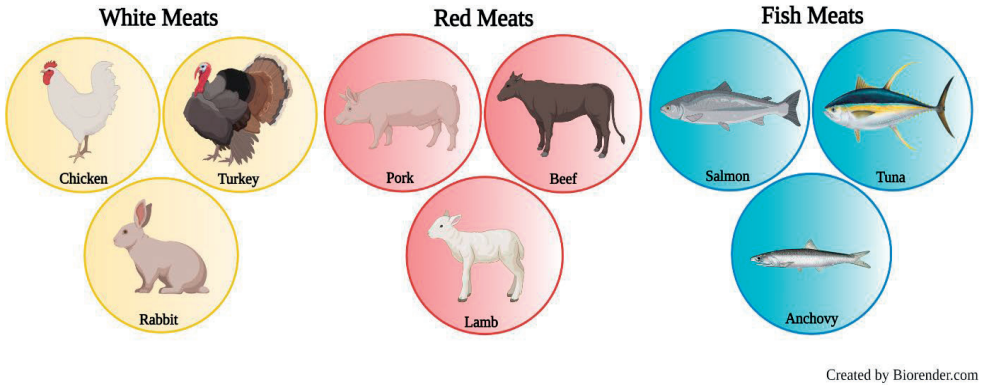

Figure 1. Protein sources for pet food raw materials analyzed in this study

MATERIALS AND METHODS

Raw Materials

The raw materials analyzed in this study are listed in Table 1 and they consist of a mix of white fresh meats (WFMs), white meat meals (WMMs), red fresh meats (RFMs), red meat meals (RMMs), fish fresh meats (FFMs) and fish meat meals (FMMs). Each formulation was prepared by mixing in equal parts each of the three animal raw materials considered for white, red, and fish FMs and MMs. All raw materials were provided by an Italian pet food company.

Determination of Moisture content

The AOAC's official method for animal feed moisture analysis was used to measure the moisture content of raw material formulations (Latimer, 2016).

An exact amount of each raw material mix (2 g) was uniformly distributed on a dish and dried at 135°C for 2 hours in an oven (Termaks TS 8136, Bergen, Norway).

The samples were weighed using an OHAUS Pioneer™ Analytical Balance (OHAUS Corporation, Parsippany, NJ, USA), after cooling at room temperature in a desiccator containing silica gel, until a constant and stable weight was achieved. The difference between the initial and final weight was used to measure the water content.

Table 1. List of raw materials used in this study

Raw Materials		
White Meats	Chicken	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Turkey	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Rabbit	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
Red Meats	Pork	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Beef	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
	Lamb	3 batches of FMs from Italian farms
		3 batches of MMs from Italian manufacturers
Fish Meats	Salmon	3 batches of FMs from Norwegian farms
		3 batches of MMs from European manufacturers
	Tuna	3 batches of FMs fished in the Pacific Ocean
		3 batches of MMs from European manufacturers
	Anchovy	3 batches of FMs fished in the Mediterranean Sea
		3 batches of MMs from European manufacturers

Protein Solubilization

The sample preparation was performed according to the protocol described by Montegiove et al. (2021). Raw material mixes were homogenized for 90 seconds at 4 °C in a hypotonic solution (10 mM Tris-HCl pH 7.5) at the concentration of 30 g/L (w/v) using ULTRA-TURRAX T25 (IKA®-Werke GmbH & Co. KG, Staufen, Germany).

In order to promote protein release from the organic matrix, 0.1% (v/v) IGEPAL® CA-630 (Sigma-Aldrich, Saint Louis, MO, USA), a non-denaturing detergent for satisfactory solubilisation of membrane protein complexes, was then applied. After that, with the purpose of removing the insoluble material, samples were sonicated for 30 seconds at 4°C with an ultrasonic disintegrator (Soniprep 150, MSE, Heathfield, East Sussex, UK) and centrifuged at 10,000 × g for 5 minutes at 4°C (5804 R, Eppendorf, Hamburg, Germany). The soluble protein fraction was recovered for the Bradford assay and SDS-PAGE coupled with Coomassie Blue staining.

Determination of Soluble Proteins

Soluble protein content in the three formulations was determined with the Bradford assay (Bradford, 1976) using Quick Start™ Bradford 1× Dye Reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The Coomassie Brilliant Blue G-250 dye (Bio-Rad, Hercules, CA, USA), which has an absorption peak at 595 nm in the protein-bound form, was employed for the quantitative analysis. A Shimadzu UV-160A UV-Visible Recording Spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) was used to measure the absorbance at 595 nm. The soluble protein concentration of

the samples was calculated from the absorbance values using a calibration curve previously prepared with known concentrations of bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA). Data were normalized taking into account the different water content of the samples and expressed as g of soluble protein per 100 g of dry sample.

SDS-PAGE and Coomassie Blue Staining Method

The electrophoretic profile of the samples was evaluated according to Laemmli's protocol (Laemmli, 1970). An exact quantity of soluble protein extract was mixed with sample buffer (0.1 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue, and 25 mM dithiothreitol; Sigma-Aldrich, Saint Louis, MO, USA). Samples were boiled for 5 minutes and electrophoresed on 10% acrylamide gel (Mini-PROTEAN® 3 Cell, Bio-Rad, Hercules, CA, USA) at 40 mA. Gels were then stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data shown in this study are reported as mean values of the three analyzed formulations ± standard error of the mean (SEM). Student's t-test was used to assess the significance of the differences between the means of the protein content of each type of FM and its relative MM formulation (WFM vs. WMM; RFM vs. RMM; FFM vs. FMM) evaluated by the Bradford assay. The level of significance for the data was set at $p < 0.05$. All statistical tests were performed using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSIONS

Raw material soluble protein content, which, as highlighted by a recent study, represents also a convenient digestibility index and gives an estimate of the total protein content (Montegiove et al., 2021), was evaluated through the Bradford assay (Bradford, 1976), a fast, reproducible and cheap method for the quantification of proteins based on the use of the Quick Start™ Bradford 1x Dye Reagent. This method indeed turns out to be a simple and quick way to estimate the bioavailable protein content compared to the official methods (*e.g.*, the Kjeldahl and Dumas methods) usually used for this type of analysis (Latimer, 2016; Montegiove et al., 2021; Nielsen, 2017). These official methods, on one hand, take into account all the protein content, but, on the other hand, are much more time consuming, potentially hazardous for the workers, because of the reagents and high temperatures required, and more expensive for the manufacturing companies (Conklin-Brittain et al., 1999; Sáez-Plaza et al., 2013a; Sáez-Plaza et al., 2013b). Ultimately, these kinds of methods, estimating the nitrogen content, could overestimate the real protein content (Liu et al., 2015; Mariotti et al., 2008; Mæhre et al., 2018; Peng et al., 2014). However, as previously mentioned, a recent study showed how a method that assessed the soluble protein content can also give an estimate of the total raw material protein content allowing the problematics correlated to the traditional and official methods to be overcome (Montegiove et al., 2021).

Before assessing the soluble protein content by means of the Bradford assay, each raw material mix was investigated as for its moisture level. Figure 2 reports the water content in the different formulations.

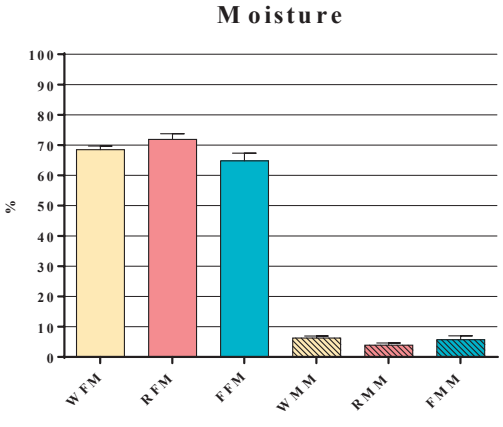

Figure 2. Water content in white fresh meat (WFM), red fresh meat (RFM), fish fresh meat (FFM), white meat meal (WMM), red meat meal (RMM), and fish meat meal (FMM) mixes. Data are reported as mean ± SEM, n = 3

It becomes clear that FM formulations feature much higher water contents compared to MM formulations. The moisture levels in the FM formulations span from about 65% in the case of FFMs to 72% in the case of RFMs; while all MM formulations exhibit a water content level lower than 10%. This feature is the result of the high-temperature treatment and dry processes used for the preparation of MMs through the rendering process (Montegiove et al., 2021; Murray et al., 1997).

Taking into account the different moisture level in the FM and MM mixes, the soluble protein content was subsequently evaluated by performing the Bradford assay. As it is apparent from the results shown in Figure 3, the soluble protein content is almost halved in the case of MM formulations with respect to the concentration found in all FM formulations. WFM mix has a content in soluble proteins about 1.9 times higher (16.2 g/100 g of dry sample) compared to the WMM mix, similar to what observed for the FMM mix, where the content is about 1.7 times higher (13.8 g/100 g of dry sample); while the RFM mix has a soluble protein content about 2.2 higher (15.3 g/100 g of dry sample) compared to the relative RMM mix.

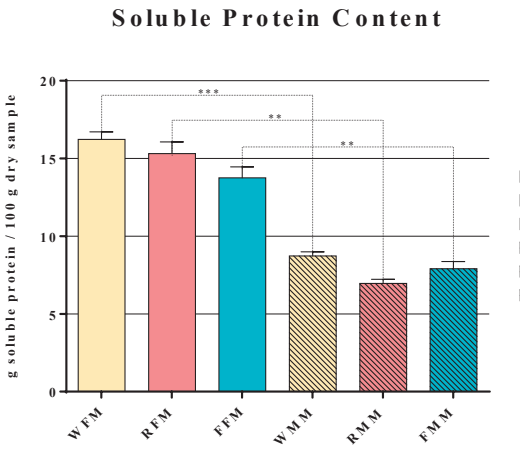

Figure 3. The soluble protein content of white fresh meat (WFM), red fresh meat (RFM), fish fresh meat (FFM), white meat meal (WMM), red meat meal (RMM), and fish meat meal (FMM) mixes determined by the Bradford assay. Data are reported as mean ± SEM, n = 3, ** $p < 0.01$, *** $p < 0.001$

These findings could be justified by the fact that MMs are also composed of bristles, feathers, hooves, and horns, containing large amounts of collagen, elastin, and keratin, which are fibrous proteins known to be insoluble or poorly soluble. In fact, globular proteins are fairly soluble and exhibit relatively high digestibility features, whereas fibrous proteins tend to be resistant to digestion (Kies, 1981; Liu et al., 2015).

The soluble protein content, assessed by the Bradford assay, was strongly in favor of FM formulations, regardless of the type of raw material taken into account, highlighting how these ingredients may be considered the best choice to be used in dry pet food production from a protein point of view, in that they have a greater quantity of soluble proteins which exhibit high digestibility and bioavailability features (Montegiove et al., 2021; Kies, 1981; Liu et al., 2015).

Afterward, in order to evaluate the quality of the protein content in the samples, the electrophoretic profile of both FM and MM raw material mixes was acquired and compared (Figure 4).

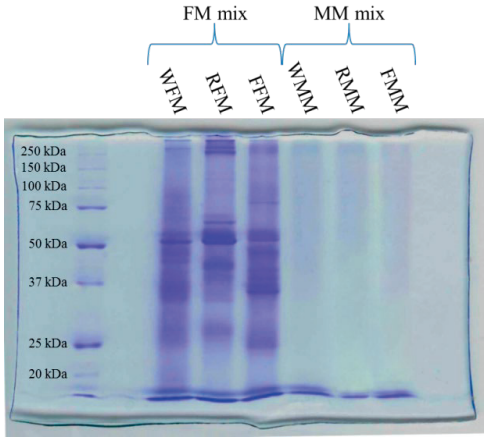

Figure 4. Protein profile of white fresh meat (WFM), red fresh meat (RFM), fish fresh meat (FFM), white meat meal (WMM), red meat meal (RMM), and fish meat meal (FMM) mixes evaluated by SDS-PAGE, followed by the Coomassie Blue staining method

The results shown in Figure 4 demonstrate how there is a substantial difference in the electrophoretic banding pattern between the two kinds of raw material formulations, suggesting a potential partial degradation of

proteins in all MM mixes analyzed. As a matter of fact, the lanes of MM mixes are characterized by smears rather than net bands, as instead expected in the presence of intact or slightly degraded proteins (Fischer, 1983). However, the smears disappear in the lower part of the gel, where small peptides would be found. This finding implies that the dimensions of the formed peptides are so small that they cannot be retained by the gel during the electrophoretic run. These results well correlate with the intensive rendering processes undergone by MMs, which may cause severe degradation and deterioration of the raw materials (Brozić et al., 2019; Carter et al., 2014; Learey et al., 2018; Montegiove et al., 2021; Montegiove et al., 2020b; Ribeiro et al., 2019; van Rooijen et al., 2013). *In vitro* and *in vivo* studies have indeed demonstrated how rendered raw materials, *i.e.* MMs, are more difficult to digest than meats (Montegiove et al., 2021; Murray et al., 1997). In addition, a degradation of the protein content, combined with inappropriate transport conditions could lead to the proliferation of some microorganisms as a result of decarboxylation processes would form biogenic amines, toxic compounds for the organism, which being heat-stable could be also found in the final product (Brozić et al., 2019; Carter et al., 2014; Einarsson et al., 2019; Learey et al., 2018; Montegiove et al., 2020b).

This study has thus shown how the various raw materials usually employed for dry pet food production effectively differ in their soluble protein content. These differences could significantly affect the quality of the final products, as the soluble protein content is closely related to the digestibility and bioavailability of the protein component (Montegiove et al., 2021; Kies, 1981; Liu et al., 2015).

These results could help the manufacturing companies to guide the choice toward the best raw materials to be used for the production of healthier dry foods for companion animals.

CONCLUSIONS

In conclusion, the reported investigation has demonstrated that the different kinds of raw materials generally used in the production of dry pet food, *i.e.*, FMs and MMs, have a quantitatively and qualitatively different protein composition.

As opposed to MM mixes, FM formulations appear to be the best kinds of raw materials that can be chosen when it comes to the production of dry food for pets, in terms of both protein bioavailability, as demonstrated by the higher soluble protein content evaluated through the Bradford assay, and better protein quality, as revealed by the electrophoretic analysis, which instead showed a marked degradation of proteins in MM formulations.

These findings can therefore provide a new approach in order to both produce better-quality pet food and assess the protein content in the starting raw materials. Companies could collect novel information on how to proceed in the formulation of new quality-improved products.

In light of these results, further and more in-depth studies may be carried out in order to correlate these preliminary results with the properties of the final products in terms of protein content, also evaluating the possibility of performing *in vitro* and *in vivo* tests.

Finally, this study has clearly shown how raw materials composed of FMs appear to be the best choice as ingredients for the production of dry food for dogs and cats.

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SOURDOUGH FERMENTATION IN GLUTEN-FREE BREAD: A SHELF-LIFE IMPROVEMENT

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Abstract

Nowadays, the demand for high quality gluten-free (GF) products is growing, especially for bread. New approaches are necessary, such as finding new combinations of GF flours or different fermentation processes. Sourdough is one of the fermentation methods used in baking industry which showed an improvement to the quality of GF bread (texture, palatability, aroma, shelf life). The research aimed to improve the GF bread characteristics based on buckwheat flour, quinoa flakes and pea protein powder using two types of lactic acid bacteria (LAB). Three samples of GF formulation were developed: S1 with the addition of Lactobacillus plantarum (0.6%), S2 with Lactobacillus sanfranciscensis (0.6%) and a control without LAB. Breads were evaluated for their physico-chemical, microbiological and sensory characteristics. The best acceptance score was for S2 followed by S1 and control. The colour measurement showed similar values, while the crumb texture recorded diversified levels with S1 being the hardest. The microbiological analysis showed an increase in the bread shelf life from 3 days (control) to 7 days (S2). This study underlined that both shelf life and taste of GF bread have been improved by using LAB.

Key words*: gluten-free bread, lactic acid bacteria, sourdough, buckwheat, quinoa.*

INTRODUCTION

Celiac disease is an inflammatory condition induced by the ingestion of gluten to people who are genetically susceptible and affects approximately 1% of the global population (Catassi & Fasano, 2008). Celiac disease has no treatment yet, the only thing to keep the disease under control is following a strict gluten-free (GF) diet. Gluten is an essential element which forms the structure of wheat bread and confers high quality cereal-based goods. This also offers the dough its unique viscoelasticity, cohesiveness and elasticity, so, the gluten absence causes a liquid batter and several defects in baked products such as low loaf volume, dry crumbling texture, poor mouth feel and poor flavour (Gobbetti et al., 2008). Besides these, following a strict gluten-free diet leads to an unbalance input of the main nutrients (proteins, fats, carbohydrates) and the lack of some macro- and microelements and vitamins (Ozkan et al., 2012; Simpson & Thompson, 2012). The patience could also have a lack of dietary fiber, iron, unsaturated fatty acids, calcium and vitamins (such as B12, A, D, E, K) (Hopman et al., 2006). Therefore, researchers face multiple challenges to make gluten-free products and try in multiple ways to overcome any difficulties. One of these is the diversification of raw materials replacing rice flour and corn flour, which are the most used with others such as cereals (sorghum), pseudo-cereals (buckwheat, quinoa, amaranth), minor cereals (teff, millet) and legumes (soybean, chickpea, lentil, pea). Besides these, other ingredients are used to diversify and balance nutrients in gluten-free diet, such as: seeds flour (flax seeds, chia seeds, pumpkin seeds), nut flour (almonds, hazelnuts, chestnuts, walnut, cashew nut) and tubers flour (arrowroot, tapioca, jicama, taro, potato). Another issue in developing gluten-free products is the taste and the flavour. According to Kenny et al. (2001), the incorporation of dairy ingredients increased calcium content and protein efficiency ratio and also improved the flavour and the texture. The addition of milk

proteins in GF bread formulations showed an improvement in loaf volume and crumb texture and also delayed the staling of the bread (Ahlborn et al., 2005; Gallagher et al., 2003; Moore et al., 2004). The addition of protein from eggs is also used for improving the quality. The use of enzymes (cyclodextrin glycosyl, glucose oxidase) for improving the quality of GF bread has also been attempted. The addition of enzymes improved the functional properties of the flour and also, the crumb structure. However, these should be avoided or used in a low concentration. Dairy ingredients, due to the incidence of lactose intolerance, together with egg proteins intolerance, may cause allergies among celiac patients (Poulsen et al., 2001; Ojetti et al., 2005). In addition, consumers are skeptical about the use of enzymes or additives in food industry. These do not meet the consumers' requirements for natural products. Thus, the researchers had to find an alternative technology for producing high quality GF bread (Moroni et al., 2009). They discovered that sourdough solves many problems related to the production of high quality GF bread. Sourdough is a mixture of flour and water fermented with lactic acid bacteria (LAB) and yeasts (Hammes and Ganzle, 1998).

Lactic acid bacteria (LAB) represent a heterogeneous group of industrially important bacteria which contributes producing fermented food and beverages (Mozzi, 2016). LAB belongs to the *phylum Firmicutes*, class Bacilli, order *Lactobacillales* according to the current taxonomic classification. The most important advantage of using LAB is that these are recognized as being safe (Zamfir & Grosu, 2014).

LAB has been used empirically for preservation and production of fermented foods of plant or animal origin since ancient times and since 1930s, LAB started to be used as lactic starter cultures in the fermented food industry and later as probiotics (Mozzi, 2016).

De Vuyst & Vandamme shown the improvement of the shelf life of fermented foods due to lactic acid bacteria since 1994. The improvement is due to production of a large variety of compounds such as organic acids, ethanol, hydrogen peroxide, bacteriocins, antibiotic-like peptides. They act in a synergically way to prevent or remove microbial contamination. Besides antimicrobial effect, LAB also contributes to the improvement of organoleptic qualities offering a characteristic flavor to the fermented products. Furthermore, using beneficial microorganisms leads to increased health benefits (Leroy & De Vuyst, 2004; Mozzi, 2016).

Sourdough production and consumption have been documented Before Christ (Adrrario, 2002). Egyptians were the first who mixed flour and water, left it ferment and then added it to fresh dough before baking (Cappelle et al., 2013). After them, ancient greek took the technique in 800 BC (Moiraghi, 2002).

Sourdough is a mixture of flour usually from wheat (*Triticum* spp.) or rye (*Secale cereale* L.), water, salt with the addition of lactic acid bacteria (LAB) and yeasts which lead to the fermentation process. Besides wheat and rye other cereals such as maize (*Zea may*s L.), spelt (*Triticum aestivum* subsp. *spelta* L.), barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) are used nowadays in baking industry (De Vuyst et al., 2017).

Therefore, this study aimed to investigate the influence of sourdough on gluten-free bread and the reaction of *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* to buckwheat dough.

MATERIALS AND METHODS

Materials

Both quinoa flakes and pea protein powder were bought from Paradisul Verde (Romania) while buckwheat flour was purchased from Eurokalis. The LAB starters (*Lactobacillus plantarum* and *Lactobacillus sanfranciscensis*) used in this study were provided by Millbo (Italy).

Bread formulations

The main ingredients used for developing gluten-free breads were quinoa flakes, buckwheat flour and pea protein powder. Besides these, two samples were prepared with LAB: sample 1 (S1) with *Lactobacillus plantarum* and sample 2 (S2) with *Lactobacillus sanfranciscensis*.

The bread developing process consisted of the following: day 1-mixing the ingredients (quinoa flakes, buckwheat flour and pea protein powder) for sourdough preparation. After that water and LAB starter were added to the mixture (Table 1). The composition was incubated at 35°C, 80% relative humidity (RH) for 24 h; day 2-weighing and mixing the ingredients for dough making (mixture, salt, yeast, sourdough) (Table 2). The ingredients were mixed using DOMOCLIP DOP150R stand mixer. In addition to S1 and S2, a control sample (without sourdough) was considered. The composition (500 g) is placed in trays covered with baking paper and then were proofed in a proofer (leavening cell M.C.E. Meccanica) at 35°C, 80% RH for 20 min. Four bread were made from each batch. In addition, there was an extra amount of dough left. The final step consisted in the bread baking in a preheated oven at 180°C for 30 min. After cooling, breads were stored in plastic bags at room temperature.

Table 1. The ingredients for sourdough preparation

Ingredients	S1	S2
Buckwheat flour	100 g	100 g
Quinoa flakes	50 g	50 g
Pea protein powder	50 g	50 g
Water	300 ml	300 ml
Lactobacillus plantarum	3 g	-
Lactobacillus sanfranciscensis	-	3g

Table 2. The main ingredients of bread formulations

Ingredients	Control	S1	S2
Sourdough	-	200 g	200 g
Buckwheat flour	500 g	500 g	500 g
Quinoa flakes	250 g	250 g	250 g
Pea protein powder	250 g	250 g	250 g
Water	1500 ml	1350 ml	1350 ml
Salt	20 g	20 g	20 g
Yeast	25g	25g	25g

Microbiological analysis

Yeasts and molds

The method for number of yeasts and molds determination was performed according to SR ISO 21527-1: 2009. This method involves the determination of microbiological contamination with yeasts and molds of food products with water activity greater than 0.95. The analysis method was used to determine the microbial load of the packaged bread samples. Using a sterile pipette, inoculate 0.1 ml of the sample from the initial dilution of the sample into a Petri dish with Dichloran Rose-Bengal Chloramphenicol (DRBC) agar then a quantity of 0.1 ml of the 10^{-2} dilution was performed. The liquid was distributed on the surface of the Petri dish with agar using a sterile stick until the liquid is completely absorbed into the medium. A control dish, with an average of 15 ml, to check sterility was prepared. The inoculated plates were placed with the lid down in the thermostat at 25°C ± 1°C for 5 days.

Between two and five days of incubation, the colonies in each Petri dish were counted. After five days, those dishes containing less than 150 colonies were retained. The determination of the number of yeasts and molds per gram of product (N) after reading the colonies raised on selective media was performed by applying the formula:

$$N = \frac{\Sigma C}{(n_1 + 0{,}1 n_2) \times d}$$

in which:

- ΣC = sum of colonies counted in all retained dishes;
- n_1 = number of dishes retained at first dilution;
- n_2 = number of dishes retained at the second dilution;
- d = dilution from which the first counts were made.

The result is expressed as a number between 1.0 and 9.9 multiplied by 10^{X}. If there is no colony in the dishes corresponding to the initial suspension, where the initial product was solid, the number of yeasts and molds per gram of product is reported as less than 10. The following equipment was used to prepare the sample Automated Diluters- Dilumat START (US), Peristaltic homogenizer - Stomacher 400 Circulator (UK) and a bacteriological hood with vertical laminar flow-FASTER VS - 4 for Petri dish incubation.

Sensory analysis

Sensory analysis was performed by 18 people (11 females and 7 males, 25-60 years old) from National Institute of Research and Development for Food Bioresources - IBA

Bucharest. The sensory analysis followed the attributes such as exterior and interior appearance, aroma, taste and aftertaste using a descriptive test with a 5-point scale. Marks consisted on ratings between to 0 (unnoticed, slight, irregular) to 5 (intense attribute, colorful, flavoured). After each sample people cleaned up their mouth with water.

Colour measurement

Colour analysis of the bread crumb was performed using CM-5 Konica Minolta colorimeter. This method is based on the interpretation of three parameters such as parameter L* which measures the sample brightness on a scale from 0 to 100 (0 value represents black and value 100 represents white); parameter a* represents the sample color on the scale from pure green to pure red (the negative values are green, the positive values are red and 0 is neutral) and parameter b* represents the sample position on a scale from pure blue to pure yellow (the negative values are blue, the positive values are yellow and 0 is neutral).

Texture measurement

Textural analysis was determined using an Instron Texture Analyzer (5944, Illinois Tool Works Inc., SUA). First, 2 cm slices were cut from the middle of the bread. After that, the texture device was calibrated, then a test was ran by placing the bread slice sample on the platform of the texture analyzer. Determination were performed four times. This method consists of a cycle of compression in the middle of each slice of bread up to a distance of 50% from the height of the slice. The setting parameters were: compression speed: 12 mm/min; load cell: 50 N. The Bluehill 3.13 program calculated the texture parameter firmness (or hardness) which represents the maximum force (expressed in N) during the bread compression.

Physico-chemical analysis

Chemical composition of bread

Chemical composition of bread was determined according to the Association Official of Analytical Chemists (AOAC, 2005) methods for ash, fat, protein and total dietary fiber content. These analysis were performed to calculate the energy value. For the energy value calculation, the following conversion factors were taken into account: 9 for fat, 4 for carbohydrates, 4 for protein and 2 for fibre for kcal/100 g value, 17 for protein and carbohydrates, 37 for fat and 8 for fiber for KJ/100 g determination.

Water activity

The value of water activity (aw) for food is an essential criterion for the microbiological control of products. Water activity is defined as follows: when a hygroscopic material is placed in a closed chamber, a balance will be achieved between the material and air above it. Relative humidity, which occurs at a constant air temperature, corresponds to the value of water activity multiplied by 100 (aw = relative humidity (%)/100). Aquaspector AQS-2-TC was used for water activity determination. The sample was placed in a polypropylene box and was introduced in the special place of the device. The measured values of the samples were read to 3rd decimal place.

Moisture content

This procedure consists in sprinkling 5 grams of sample on the entire surface of the moisture analyzer tray without pressing. The equipment used was METTLER TOLEDO, model HE73 at 130°C.

pH values

WTW inoLab 7110, pH-meter and SenTix Sp-T 900 were used to measure the dough pH values. The pH-meter was calibrated using three standard pH buffer solutions (pH 4, pH 7 and pH 10).

Bread Volume

The volume was determined using the Fornet method as follows: the volume of rapeseed displaced by the bread product was measured and then it was reported per 100 g of product. After placing the sample the container was sealed and than basculated. The zero point position was verified 3 times and the maximum differences of 3 successive measurements have to be less than 30 cm^3.

The product volume (cm^3) at 100 g product was calculated using the formula:

$$V = \frac{V_1}{m} * 100 \text{ } (cm^3/100 \text{ g product})$$

where:
V_1 - the measured volume of the analyzed sample, in cm^3;
m - the bread sample mass in grams;
The result was calculated using one decimal and rounds up to a whole number (SR 91, 2007).

RESULTS AND DISCUSSIONS

Microbiological analysis

Yeasts and molds

Each bread sample was microbiological analyzed following the presence of yeasts and molds, a microbiological parameter specific to bakery products. The samples were analyzed at 24 h, 3 days, 5 days and 7 days after manufacture. The results of the initial microbiological analysis showed that the samples were compliant. At 24 h after manufacture, neither of bread samples showed yeasts and molds. On the 3rd day, the control presented a contamination of 6.2 x 10^2 cfu/g degree (Table 3), while the maximum limit allowed according to Order no. 27/2011 of ANSVSA is 1 x 10^2 cfu/g. S1 was compliant until the 5th day of storage. S2 recorded the most satisfactory results during the storage period (7 days shelf life). The differences between the samples and control are due to the lactic acid bacteria used in the formulations.

Table 3. The presence of yeast and molds

Bread samples	1 day	3 days	5 days	7 days
Control	<10	6.2 x 10^2	1.5 x 10^3	2.2 x 10^3
S1	<10	<10	2.0 x 10^1	1.2 x 10^2
S2	<10	<10	<10	<10

Sensory analysis

After performing the sensory analysis, the following results were obtained: the crust and crumb colour were similar, with small differences between them. Regarding the crust texture and crumb elasticity, no significant differences were registered. Considering the uniformity of the pores the most appreciated bread was S2 with the addition of *L. sanfranciscensis* sourdough followed by S1 with *L. plantarum*. Also, Jagelaviciute and Cizeikiene (2020) reported a higher porosity on sourdough bread. The following attributes were considered for flavor evaluation: yeast and cereal aroma. The cereal aroma registered a medium level. An important fact that can be observed is that the yeast flavour has low scores which means that it does not negatively influence the GF bread taste (Table 4). The results obtained for the sweet and salty attributes are similar. This fact demonstrates that the lactic acid bacteria used in this study do not influence the formulation for sugar and salt taste. However, we can observe that the LAB slightly influenced the sour taste (the most intense for S1). LAB improved the GB bread taste. The bitterness of quinoa flakes and the predominant taste of buckwheat were hidden due to the addition of LAB. For sensory analysis at the first bite, the following sensory attributes were taken into account: firmness, chewiness and moisture crumb. The results had similar values but it should be mentioned that a larger amount of water was added to develop the control because S1 and S2 compensated by adding liquid sourdough. Also, probably the differences would have occurred if all the samples had the same amount of water. The amounts of water were different in order to maintain the same texture of the dough. Moore et al. (2007) discovered that the chemical acidification led to a more fluid-like gluten-free dough than the control dough, whereas the use of a biological acidifier (dough obtained using sourdough) led to a significant increase in dough firmness (Moore et al., 2008). The following attributes, cohesiveness and mass adhesion were analyzed during chewing and referred to the mouth agglomeration degree and crumb teeth adhesion. All three samples had similar scores; moreover, the control and S2 had identical results. The last analyzed attribute was the aftertaste. All the results were similar but the highest value belonged to S2 (2.77). When people were asked which sample they preferred, 9 people have chosen S2, 5 people S1 and 4 of them answered the control is their favourite GF bread sample.

Table 4. The sensorial attributes analysed in GF bread sample

Sensorial attributes		Sample scores		
		234	**567**	**890**
EXTERIOR APPEARANCE	Crust colour	3.00	3.11	2.86
	Crust texture	3.02	3.16	2.80
INTERIOR APPEARANCE	Crumb colour	3.33	3.16	2.88
	Pores uniformity	2.93	3.50	4.21
	Crumb elasticity	2.61	2.27	2.63
AROMA/FLAVOUR	Cereal	2.61	2.94	2.63
	Yeast	1.16	1.22	1.36
TASTE	Sweet	0.88	0.94	0.91
	Salty	1.44	1.52	1.38
	Sour	0.61	2.16	1.80
	Bitter	3.25	1.50	0.91
FIRST BITE	Firmness	2.27	2.55	2.63
	Gumminess	2.33	2.38	2.50
	Crumb moisture	3.86	3.75	3.86
CHEWING	Cohesiveness	2.94	2.77	2.94
	Adherence	2.00	1.94	2.00
	Aftertaste	2.58	2.52	2.77

*The sample names have been modified for not influencing the tasters so it has been chosen 234 for control, 567 for S1 (*Lactobacillus plantarum*) and 890 for S2 (*Lactobacillus sanfranciscensis*).

Colour measurement

The color results of the analyzed samples showed similar values. The raw materials were the same for each formulation excepted the sourdough addition, so it proves that sourdough did not impact on the bread colour (Table 5).

Table 5. The colour of developed gluten-free bread

	L*(D65)	a*(D65)	b*(D65)
Control	59.05	1.88	13.06
S1	59.58	1.75	14.19
S2	60.34	1.71	14.88

Texture measurement

The results indicated an increase in GF bread hardness with the addition of sourdough. There was a significant increase between control (6.08 N) and the other samples which contain sourdough. S1 and S2 recorded values of 10.4 N and 9.2 N, respectively. Moroni et al. (2011) showed a substantial increase in crumb hardness and crumb chewiness with the amount of sourdough in buckwheat gluten-free bread. The values increased from 7.85 for control to 12.12, 13.01 and 15.94 for a sourdough addition of 20%, 35% and 50%, respectively. According to Rozyło et al. (2015a, 2015b), the crumb hardness in buckwheat and amaranth sourdough bread had a significant decrease. They also showed that the hardness of control rice bread was much lower compared to the bread with buckwheat and amaranth addition (Rozyło et al., 2016). In research presented by Novotni et al. (2012), breads with 15 and 22.5% sourdough had the lowest value of initial firmness and reduced firming compared to the control bread.

Physico-chemical analysis

Chemical composition of bread

The addition of sourdough did not greatly influence the physico-chemical properties, so the values were quite close. The energy value of S1 was the highest compared to control which was the lowest, but this difference is insignificant (Table 7).

Water activity

According to Flückiger and Cleven (1978), the level of water activity for white bread should be 0.92. Despite with the fact that the water activity result was higher (Table 6), the gluten-free bread samples showed good results in terms of contamination with yeasts and molds. Although water activity values were similar, the yeasts and molds concentration was different between samples. Control was first contaminated, on the 3rd day. It was noticed that the addition of lactic acid bacteria has slowed down the contamination process. *Lactobacillus plantarum* extended the shelf life up to 5 days and *Lactobacillus sanfranciscensis* up to 7 days. According to Moroni et al., (2011) the addition of 20% sourdough on gluten-free buckwheat bread positively influenced the staling rate. Otherwise, the incorporation of 50% sourdough led to a slight increase in staling rate during storage.

Table 6. The water activity values

Bread samples	1 day	3 days	5 days	7 days
Control	0.967	0.988	0.986	0.972
S1	0.985	0.987	0.985	0.989
S2	0.986	0.984	0.986	0.998

Moisture content

All the analyzed samples (gluten-free buckwheat bread) have a higher value of moisture content than normal bread (maximum 45%). The highest level was recorded for control (61.54%), followed by S2 (59.65%) and then S1 (58.03%). These high moisture values resulted due to the addition of a higher water content for a suitable dough developing.

pH value

The sourdough influenced the pH dough, this decreased compared to the control (6.00) to 5.65 for S1 and 5.59 for S2. According to Jagelaviciute and Cizeikiene (2020), the same trend was noticed for all the fermented sample. All the fermented sample (fermented chia dough; fermented hemp dough; fermented quinoa dough) have lower values than controls (control chia dough; control hemp dough; control quinoa dough;) with the following values 5.45, 5.21, 4.46 for fermented dough and 5.55, 5.75, 5.60 for control dough. According to another study on sourdough buckwheat bread, the pH also significantly decreased with the increasing level of sourdough (20%, 35%, 50%). After proofing, the control batter pH was 5.96 and the sourdough bread recorded lower values such as 5.15, 4.68 and 4.39, respectively (Moroni et al., 2011). Rozyło et al. (2016) also recorded differences between control and samples with sourdough. The pH decreased more with the amount of sourdough from 5.31 to control to 4.98 with 10%, 4.49 with 20%, 4.22 at 30% and 4.13 at 40%. Moore et al., (2008) reported a significant increase in crumb hardness during storage for all GF breads. However, the increase was higher for the chemically acidified and lower for *L. plantarum* and *L. sanfranciscensis* gluten-free breads, which means that sourdough helped in delaying the staling of gluten-free breads. Also, Corsetti et al. (1998) reported that fermentation by sourdough LAB, microbial hydrolysis of starch, and proteolysis influenced physicochemical changes during bread storage including a positive effect in delaying both bread firmness and staling.

Table 7. Chemical composition of gluten-free bread

Parameter	Control	1-*L. Plantarum*	2-*L. Sanfranciscensis*
Energetic value (kcal/100g)	**152**	**166**	**161**
Energetic value (kJ/100g)	**645**	**705**	**680**
Protein	12.74	12.69	12.70
Fat	1.18	1.26	1.30
Carbohydrates which:	22.28	25.49	24.12
-sugars	<1.0*	<1.0*	<1.0*
Fiber	0.77	1.14	0.75
Salt	1.20	1.20	1.10
Ash	1.49	1.39	1.48
Moisture	61.54	58.03	59.65

*quantification limit for sugars analysis
**all values are reported %

Bread volume

It has been noticed that LAB had a different influence on volume samples. S1 with the addition of *Lactobacillus plantarum* sourdough has a negative impact on bread volume compared to control. This decreased from 130 cm^3/ 100 g for control to 127 cm^3/ 100 g for S1. The greater volume was obtained for the bread S2 with *Lactobacillus sanfranciscensis* (135 cm^3/100) (Figure 1).

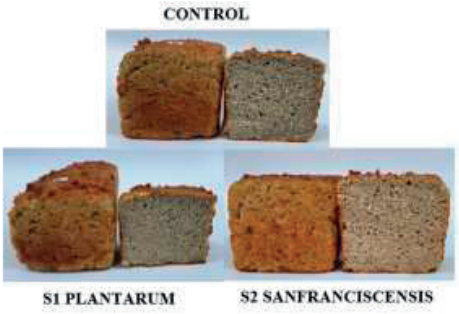

Figure 1. Gluten-free bread samples with buckwheat flour, quinoa flakes and pea protein

Rozylo et al. (2016) reported a volume increase from 167 cm^3/100 g for control to 170 cm^3/ 100 g for the sourdough sample. Novotni et al. (2012) and Moore et al. (2007, 2008) observed favourable changes in the volume of bread after sourdough addition.

CONCLUSIONS

The sourdough addition had a major impact on shelf life and taste of gluten-free bread. Thus, the bread shelf life was extended from 2 days (control) to 7 days (S2). Sourdough also improved the taste and flavour: reduced the bitter taste that comes from quinoa flakes and diminished the intensive buckwheat taste that felt too strong in case of control bread.

Thus, the use of *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis* in bread formulations showed an improvement on both taste and extension of shelf life, but S2 with *L. sanfranciscensis* sourdough showed the best results and a better acceptance. Moreover, *Lactobacillus sanfranciscensis* had the best effect in increasing the gluten-free bread volume.

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IDENTIFYING SYNTHETIC COLORANTS FROM WINE BY UPLC

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Abstract

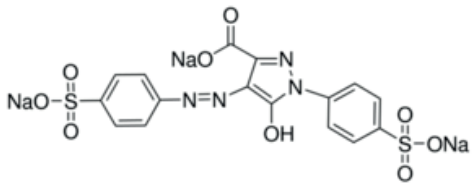
Legislation in force about wine quality states that concealing defects and alterations of the wines by introducing into their natural content of something that could determines changes in natural composition, aroma and taste could be a counterfeit product. This constitutes fraud and shall be punished according to the law. Synthetic colorants are a kind of additives, which although they are forbidden, may be found in wines. Consumption of them may affect the health of consumers with numerous side effects and toxicity, at both medium and long-terms, allergic reactions, behavioral and neurocognitive effects. To reduce consumers inconveniences and to avoid fraud in the wine sector, sensible analytical methods are required. Identification and quantification of some commonly used synthetic colorants (tartrazine - E102, amaranth - E123, sunset yellow- E110 and erythrosine - E127) is presented in this paper by ultra-performance liquid chromatography (UPLC) with UV detection in an adapted method for wine matrix. The method proves all of specific parameters for validation.

Key words*: food safety, quality, synthetic colorants, UPLC, wine.*

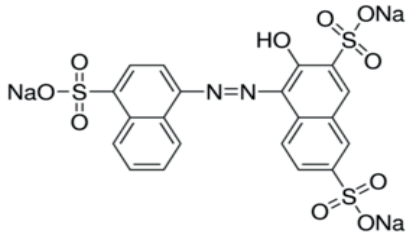
INTRODUCTION

The food colour is strongly associated with consumer choices (Zeece, 2020). Colour is the first important intrinsic sensory to consumers expectations regarding the flavour and taste of food and beverages. Natural alternatives are increasingly important for replaceing artificial colorants all over the world (Vinha et al., 2018). Using synthetic colorants in food becomes a major issue and this is the reason because food producers are trying to satisfy today's consumer demands with natural, and safe food products (Gebhardt et al., 2020). Consumers have more and more demands for natural plant-derived alternatives and also there are several scientific reports about harmfulness of synthetic colorants in food. (Vinha et al., 2018). Although there are currently allowed established acceptable daily intake (ADI) for food colorants and are very used, however there have been gradually substituted by those from natural origins. A lots of side effects and toxicity, at both medium and long-terms, behavioral and neurocognitive effects and allergic reactions have been related with their consumption. Otherwise, naturally-derived food colorants proves high quality, efficiency and organoleptic properties, and also have an important role as health promoters (Martins et al., 2016). Colorants are widely used in the food industry for improving food quality and food safety during processing, packaging and storage. Sourcing of these molecules is mainly done by three means: extraction from natural sources, chemical synthesis and bioproduction, the first two being the most utilized (Sun et al., 2021). A sensitive dispersive solid-phase extraction (D-SPE) method for the extraction and enrichment of four important synthetic colorants using high performance liquid chromatography was introduced by Chai W. et al in 2016, when the limits of detection (LODs) for the established d-SPE-HPLC method were 0.20-0.25 $\mu g\ L^{-1}$, which were lower than other chromatographic methods earlier reported for amaranth, ponceau 4R, sunset yellow and allure red. The method was also successfully applied to determination of colorants in samples of beverage with satisfactory results. Also, a new MSPE-HPLC method was developed for simultaneous determination of four synthetic colorants (amaranth, ponceau 4R, sunset yellow and allure red) in food samples (candy, jelly and carbonated drink) by Chen et al in 2019. The MSPE-HPLC method was simple and effective and can be used for the analysis of colorants in real samples.

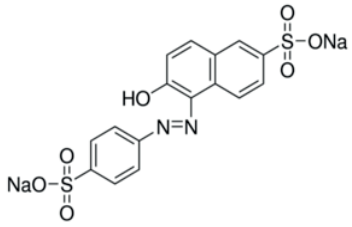
The most commonly synthetic colorants in food are: tartrazine or yellow acid 23 (E102), amaranth (E123), sunset yellow (E110) and erythrosine (E127) (Figure 1).



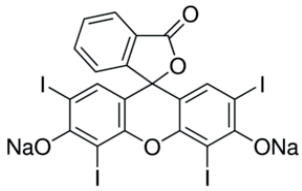
Tartrazine



Amaranth



Sunset Yellow



Erythrosine

Figure 1. Chemical structure of the four artificial colorants analysed in this study

The aim of present study was to find a reliable UPLC-UV method for analysing tartrazine, amaranth, sunset yellow and erythrosine in wine and to validate it. Assessed method parameters were: selectivity, linearity, sensitivity, accuracy, repeatability, reproducibility, limits of detection, limits of quantification, linear range and recoveries. After validation, the method was used to analyse the four artificial colorants in 20 wine samples.

MATERIALS AND METHODS

Materials

For this study the analytical standards of tartrazine (TZ) (trisodium (4E)-5-oxo-1-(4-sulfonatophenyl)-4-[(4-sulfonatophenyl)hydrazono]-3-pyrazolecarboxylate), amaranth (AM) (trisodium (4E)-3-oxo-4-[(4-sulfonato-1naphthyl)hydrazono]naphthalene-2,7disulfonate), sunset yellow (SY) (disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate) and erythrosine (ER) (acid 2-(6-hidroxi-2,4,5,7-tetraiodo-3-oxo-xanten-9-il)benzoic) were purchased from Dr. Ehrenstorfer. Ammonium formate (100%) were supplied by Thermo Fisher, acetonitrile (99.99%) and methanol (99.98%) were purchased from Honeywell and the ethanol (99.9%) were purchased from Merck, all of them being HPLC grade. Samples of bottled wine were purchased from local markets and were classified as red, rosé and white wines and also as dry, medium dry, medium sweet and sweet. The wine samples were stored at 4-8°C until analysis.

Standard solutions and sample preparation

Stock standard solutions were prepared with 1000 mg/L concentration in ultapure water for each of analytical standard. Then from these stock solutions was prepared a mixed working standard solution containing 100 mg/L TZ, 100 mg/L AM, 100 mg/L SY and 100 mg/L ER in 10% methanol. For obtaining the calibration curve were prepared five standard levels by diluting the mixed working standard solution with 10% methanol. Stock standard solutions and mixed working standard solution were stored at 4-8°C prior to use.

Wine samples have removed it's possible gases by sonication, then were properly diluted with ultrapure water. After dilution stage, the samples were pH adjusted to 6.5 ± 0.1, filtered using nonsterile hydrophobic PTFE syringe filter with 0.45 µm pore size and placed in an UPLC vial for instrumental analysis.

The fortified test samples were prepared by spiking at the LOQ level, respectively at 5 mg/L TZ, 5 mg/L AM, 5 mg/L SY and 5 mg/L ER.

Chromatographic method

For identifying and quantifying the four synthetic colorants in wine samples by liquid chromatography method, it was used Waters Acquity UPLC equipment (with binary solvent manager, thermostatic column compartment, heater/cooler sample organizer) with UV detector. The separation was performed with a Kinetex EVO C18 column from Phenomenex (1.7 µm, 100Å, 150 mm x 2.1 mm), at 40°C, by isocratic elution with 0.2 mL/min flow rate. The mobile phase was 10 mM ammonium formate prepared in ultrapure water. All solvents were sonicated before using. The injection volume was setted at 1.3 µL and the run time at 15 minutes. Our synthetic colorants detection was performed at 420 nm wavelength for yellow compounds, like tartrazine and sunset yellow and at 530 nm wavelength for red ones, like amaranth and erythrosine. Data were collected and processed using Empower 2 software.

RESULTS AND DISCUSSIONS

Presented chromatographic method was applied for identification and quantification of the four synthetic colorants. After diluting stock solutions stage, each analyte was injected in turn for determining their sequence. All the four artificial colorants were identified in the obtained chromatogram for the mixed standard solution, in the following order: at 420 nm wavelength TZ (2.26 min retention time) and SY (5.11 min retention time) and at 530 nm wavelength AM (3.82 min retention time) and ER (7.25 min retention time). The analysis proved a good separation of all four compounds to each wavelength, with resolution and symmetry and also peaks shape (Figures 2.1. and 2.2.). Retention times determinated and peak width proved that resolution values were calculated at 2.5 for TZ, 3.8 for SY, 4.3 for AM and 4.5 for ER.

The purpose of the method was validation it for demonstrating that its performance characteristics are adequate (Barwick et al., 2014). There were setted and confirmed all of specific validation parameters like selectivity, limit of detection and limit of quantification, working range, trueness, analytical sensitivity, precision, ruggedness, uncertainty measurement (Barwick et al., 2014).

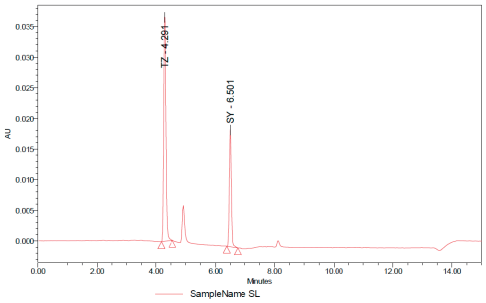


Figure 2.1. Chromatogram of mixed standard solution (SL) at 420 nm wavelength

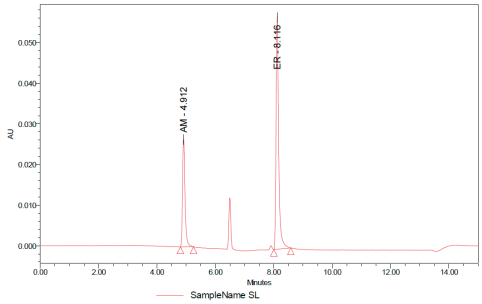


Figure 2.2. Chromatogram of mixed standard solution (SL) at 530 nm wavelength

Selectivity is about a method's ability to highlight the differences of analysis determination in presence of other compounds that could be similarly (Rusea, 2016). The peaks were without overlapping, so each compound were individual registered, there wasn't interferences and it was a clear identification of them (Figures 2.1. and 2.2.).

Limit of detection (LOD) is the minimum amount of analyte that could be detected by equipment in a test sample, but this couldn't be quantified as an exact number value (Rusea, 2016). In this study it was evaluated a signal-to-noise ratio of 3 for LOD.

LOD were determined at following values: 0.034 mg/L for TZ, 0.049 mg/L for AM, 0.031 for SY and 0.072 mg/L for ER.

Limit of quantification (LOQ) is the minimum amount of analyte that could be quantitatively determined by equipment in a test sample with acceptable accuracy and repeatability (Rusea, 2016) and is about 10 x LOD. LOQ were determined at following values: 0.34 mg/L for TZ, 0.49 mg/L for AM, 0.31 for SY and 0.72 mg/L for ER.

Working range is the interval between minimum and maximum concentration of an analyte in a test sample (Rusea, 2016). In our study the working range was setted from 0.25 mg/L to 20.0 mg/L for TZ, AM and SY and from 1.0 mg/L to 20.0 mg/L for ER.

Linearity is when we have an established domain and the method proves the ability to provide a set of results that are directly proportional to analyte concentration value (Rusea, 2016). External calibration method was performed for quantitative analyzes. Calibration curve was performed with standard solutions with five concentration levels, with three injections per each level. Correlation coefficient was higher than 0.999 for all of four synthetic studied colorants. The registered values of r^2 were 0.999985 for TZ, 0.999998 for AM, 0,999966 for SY and 0.997139 for ER.

Analytical sensitivity is the modification of measuring instrument response reported to the stimulus changing (Rusea, 2016). It is when an analytical method proves minimum concentration variations of an analyte and our method proved it.

Accuracy or trueness of an analytical method is about how close to the true value is the determinated value. It express the raport between average value of analytical set results and a reference value that is accepted (Rusea, 2016). Bias is the expressed term for measuring accuracy and establishes a total systematic error. Our study proves a bias of 1.95% for TZ, 9.93 for AM, 1.74 for SY and 19.72 for ER.

Precision or *repeatability* is determined by a series of analytical determinations obtained from different aliquots of the same test sample, in same conditions (Rusea, 2016). This is a part of measurement uncertainty and is expressed as relative standard deviation (RSD%). Repeatability was established by six times injecting in a row three levels of the mixed standard solution. RSD was assessed to the following averages values: 4.87% for TZ, 6.3% for AM, 4.03% for SY and 6.46% for ER.

Reproductibility is when repeatability could be made by another analyst using the same analytical procedure for determining the same test samples or by the same analyst but with another, similar, equipment. The reproducibility in our study was assessed by analysing the same three levels of mixed standard solution, 6 times in a row by two analysts. RSD average values were following: 5.11% for TZ, 6.01% for AM, 4.95% for SY and 9.54% for ER.

Recovery is a determination of method efficiency for detecting all quantity of analyte, it is express by percentage and is about the real concentration of studied recovered substance during the analyzes (Rusea D., 2016). Recovery is a rapport between extracted samples response, obtained for three analyte concentrations, and response without the extraction stage. Our test samples ware fortified to the LOQ level, respectively with 5 mg/L of each colorant and registered recovery values were 96% for TZ, 101% for AM, 109% for SY and 93% for ER.

Measurement uncertainty is a technical parameter that could be associated with the measurement result that is associated with attributed values dispersion to the measurement. Measurement uncertainty involves to evaluate of the errors sources at each analytical stage and estimation of associated uncertainty. The global uncertainty means to have available datas, quality control and comparison tests.(Rusea, 2016). We setted a measurement uncertainty up to 8% for this analytical method.

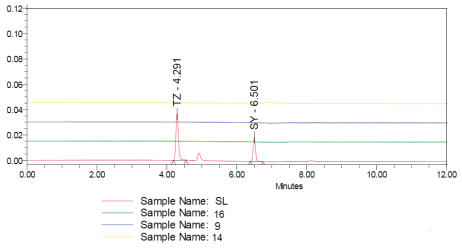


Figure 3.1. Overlay chromatogram of three negative wine samples for tartrazine and sunset yellow (at 420 nm wavelength) and mixed standard solution (SL)

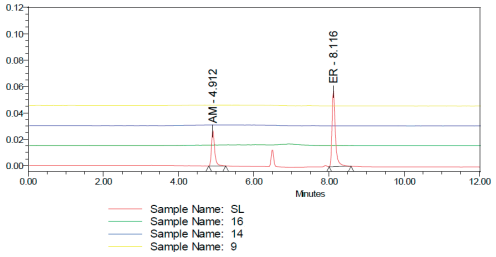


Figure 3.2. Overlay chromatogram of three negative wine samples for amaranth and erythrosine (at 530 nm wavelength) and mixed standard solution (SL)

The presented analytical method was used for assessing the possible presence and the quantify the four studied synthetic colorants in 20 different bottled wine samples from Romanian market. Although, Virtanen et al. observed in 1999 that erythrosine prepicitates when is added into wine, we can not confirm this, but also we admit that we didn't do a stability study for the addition of erythrosine in wine to be able to observe any possible precipitate in time. Assessing the obtained data, there weren't identified any of the four synthetic colorants in the analized wine samples. (Ex. Figure 3.1. and 3.2.). Tartrazine, amaranth, sunset yellow and erythrosine were not detected in any sample. We identified slight traces of amaranth in a single bottled wine sample but the value was below of LOQ and this means that it was not possible to quantified it.

CONCLUSIONS

The UPLC-UV method proves that is suitable for identifying and determining of the concentration of tartrazine, amaranth, sunset yellow and erythrosine that could be illegally added in wine. The analytical method is rather simple, with no need of special sample preparation. The reliability of this method is assured by the obtained values for the validation parameters.

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MAXIMIZING TOTAL PROTEIN EXTRACTION FROM SPENT BREWER'S YEAST USING HIGH-PRESSURE HOMOGENIZATION

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Abstract

Large quantities of spent brewer's yeast are generated as a by-product during beer production, representing a cost-effective, nutrient-rich substrate. Spent brewer's yeast is especially rich in protein and several methods have been tested in order to maximize the protein extraction and reduce the costs of this extraction. Here we present the optimization of total protein extraction from brewer's spent yeast by design of experiments (DOE) using a high-pressure piston homogenizer. The yeast was pre-treated with a β-glucanase for one hour at 50°C. A full factorial design with two levels, four factors (yeast amount, pressure, number of passes, enzyme concentration) was used. The model and the data indicated that two parameters (pressure, enzyme concentration) were highly significant ($p < 0.05$), while the other two parameters (yeast amount, number of passes) were moderately significant and not significant, respectively. The interaction between pressure and enzyme concentration was also marginally significant. Our optimization indicates that efficient extraction of proteins from brewer's spent yeast could be obtained and up-scaled with minimal costs.

Key words*: total yeast protein extract, design of experiments, piston homogenizer, enzymatic treatment.*

INTRODUCTION

Beer is a staple drink in many countries around the world. However, this high demand generate pressure on the brewing industry to produce more and more beer, which in turn creates large amounts of residues that are usually vastly under-utilized.

The industrial process of obtaining beer, as described by Carlsberg uses several simple ingredients: water, malt, hops and yeast. The first step in the process involves turning the barley into malt, then transforming the starches in the malt into fermentable sugars. Hops are added for flavour and the mixture is boiled for an hour. Afterwards, the liquid fraction is cooled and yeast is added to start the fermentation process which can last between 7 and 14 days (Carlsberg).

The driving force behind beer fermentation is the yeast, *Saccharomyces pastorianus*, which was specially selected to produce alcoholic beverages with a low alcohol content. This type of yeast is a bottom fermenting lager-type, which means that it thrives at colder temperatures, as opposed to top fermenting ale-types of yeast (such as *Saccharomyces cerevisiae*) which prefer a warmer environment. There are also differences in the taste profile of beer obtained through these two methods, lager types boasting a crisp and "cleaner" taste compared to the bitter ale-types (Bonatto, 2021). The former seems to have captured the hearts and taste buds of most consumers, with ale types having a smaller, but loyal fan base. A case in point is represented by the Romanian beer industry. An analysis from 2012 (Dobre-Baron, 2012) reveals the fact that the beer Romanians consume is 99% locally sourced with a staggering percent of it being lager-type. This amounts to an average production volume of approximately 15 million

hectolitres. For each hectolitre of beer produced, an average 3 kg of spent brewer's yeast (SBY) is produced, which in time amounts to more than 44000 tonnes of spent yeast (San Martin et al., 2018).

However, in the last years, there have been efforts to valorise this yeast through different methods. Due to its high protein content (between 45 and 60% w/w) yeast seems to be a perfect protein source and is generally regarded as safe for human consumption. Still, yeast protein extracts contain between 6 and 15% nucleic acids which in humans cause an elevation of uric acid levels in the blood (Podpora et al., 2016). Thus, spent brewer's yeast has been used as a cheap source of feed for livestock or just disposed of as a waste into the environment (Puligundla et al., 2020). Spent brewer's yeast cannot be consumed in the form released from the brewery, so the best solution is the extraction of economically valuable compounds such as yeast cell walls, aroma compounds etc. Yeast cell walls contain fibres, mainly β-glucans and α-mannans, which serve an important role in nutrition. The presence of bioactive peptides as well as other functional components, such as carotenoids, oligosaccharides, polyphenols make yeast a golden mine for nutrition (Rai et al., 2019). While they represent the basics of good nutrition, they could also be used for creating healthier crops and enhancing plant nutrition through their effects on plant metabolism.

One important trend in the last years has been the use of natural, agricultural inputs, included in the category of plant biostimulants. The EC 2019/1009 regulation has opened the market as well as given a vote of confidence to plant biostimulants while also offering some guidelines towards regulating the claims and the contaminants (European Union, 2019). Plant biostimulants are considered to be any agricultural inputs, that brings an improvement to plant nutrition, crop health or resistance to biotic or abiotic stress, however they do not fit under the umbrella of fertilizers, pesticides or biocontrol agents (du Jardin, 2015).

Type-wise, the plant biostimulants can be microbial or non-microbial. The non-microbial ones are further on split into other categories, based on the chemical composition. The most abundant biostimulants on the market are represented by humic and fulvic acids, followed by seaweed extracts and finally plant and other types of extracts among which the most important to mention are amino acids and protein hydrolysates. Protein hydrolysates can be obtained from different sources with high protein content, one such source being industrial waste from the brewing industry. The usual method of extraction involves a process called autolysis which consists of heating the yeast slurry in order to activate the intra-cellular enzymes and proteases to destroy the membrane and the cell wall and thus, release the cellular content (Takalloo et al., 2020). However, this process can be approached in a different manner by using pressure and enzymatic pre-treatment to elevate the efficiency of the process.

There is little study in the field of high pressure homogenization and enzyme assisted extraction of proteins from yeast. Most of the previously published data used high pressure homogenisation as a pre-treatment for yeast autolysis (Baldwin & Robinson, 1990; Dimopoulos et al., 2020; Verduyn et al., 1999), but there is little knowledge on the optimisation of the high-pressure homogenisation parameters for the purpose of obtaining yeast extract high in protein content. It is also worth mentioning that these studies used different intervals for the parameters, which could influence the protein yield.

The aim of this study was to develop an improved method of industrial processing of spent brewer's yeast by using a multi-factor experimental design and to determine which parameters should be carefully controlled to release high quantity of protein from the treated yeast and to allow separation of the yeast cell wall. The final goal for this yeast cell breakage separation of the two components, yeast extract and yeast cell wall, is related to agricultural applications of both resulted components.

MATERIALS AND METHODS

The spent brewer's yeast was supplied by our partners AGSIRA SRL and its provenience is from one of the beer factories in Romania. All used yeast was lager-type. The yeast was supplied in the dry-compacted form. The spent yeast slurry was previously dried, by using a

double-drum dryer (T9/30, Gouda, Waddinxveen, Netherlands), operated at 140°C and 3 rpm.

The dried yeast was resuspended in deionized water and pre-treated with a commercial β-glucanase preparation (VinoTaste® Pro, Novozymes, Bagsværd, Denmark) at 50°C for one hour under stirring. The commercial enzyme preparation include also enzymes with pectinolitic activity (Averilla et al., 2019). However, pectinases are not active against plant cell wall.

Afterwards, the mixture was homogenized using a Lab Homogenizer Panda PLUS 2000 (GEA Niro Soavi, Parma, Italy), applying different experimental settings generated by the design of experiment described below. The experimental design was created using a full factorial design with two levels and four factors, namely: yeast amount (as percent concentration), homogenizer pressure applied, number of passes and enzyme concentration. The two levels were 10 and 20% for yeast, 1000 and 2000 bar for pressure, 3 and 7 number of passes and 0.1 and 0.3 mg/L for the enzyme concentration. Three centre points were added to estimate the standard error of the design space.

The yeast extracts were stored overnight at 4°C to favour sedimentation of cell walls and remaining yeast. Further separation of the two phases was done through centrifugation. The supernatant was tested for protein concentration using a modified biuret method (Gornall et al., 1949).

The protein extract was spray-dried (by using a B-290 Mini Spray Dryer, Büchi, Flawil, Switzerland) to prevent spoilage and stored for further applications. The precipitate containing the yeast cell walls was freeze-dried and stored for further analysis.

The data were analysed using Design-Expert 11 software (Sta-Ease, Minneapolis, MN). Statistical significance of the terms was determined by ANOVA (analysis of variance).

RESULTS AND DISCUSSIONS

The experimental setup that was used, involves a set of conditions which were randomized to gain as much information concerning a possible model, from a lower number of experiments. The experimental design is presented in Table 1 and shows the combination of conditions required for each of the considered factors, in each of the 19 experimental points used into this optimization study.

Table 1. Parameters of the experimental setup

Exp. no.	Yeast concentration (%) (A)	Enzymatic pre-treatment (g enzyme per L) (B)	No. of passes (C)	Homogenizer pressure (bar) (D)
D1	10	0,1	3	1000
D2	20	0,1	3	1000
D3	10	0,3	3	1000
D4	20	0,3	3	1000
D5	10	0,1	7	1000
D6	20	0,1	7	1000
D7	10	0,3	7	1000
D8	20	0,3	7	1000
D9	10	0,1	3	2000
D10	20	0,1	3	2000
D11	10	0,3	3	2000
D12	20	0,3	3	2000
D13	10	0,1	7	2000
D14	20	0,1	7	2000
D15	10	0,3	7	2000
D16	20	0,3	7	2000
D17	15	0,2	5	1500
D18	15	0,2	5	1500
D19	15	0,2	5	1500

An experimental design was used to explore the effects and the relationship between several variables and the effect they have on the response, as well as optimise the response to certain levels presenting interest. The samples were analysed using a modified biuret method as presented by Gornall and his collaborators (Gornall et al., 1949).

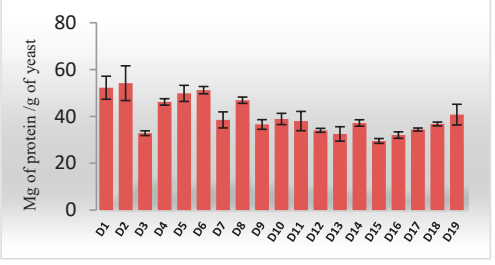

Figure 1. Protein yield measured through Biuret assay on the soluble protein obtained through enzymatic and pressure treatment of spent brewer's yeast. The bars represent the standard error

The data described in Figure 1 represent the primary response variable which was used to further investigate the relationship between the varied parameters.
The desired response is represented by the highest protein yield per gram of yeast used. The data indicates that this is achieved for the conditions applied to sample D2. Statistical significance of the terms was assessed by ANOVA (analysis of variance) using Design-Expert 11. The most appropriate model was obtained by checking iteratively the p-values of the terms starting from the design model (4 main terms, 6 secondary interaction terms and higher-level interaction terms).
Table 2 shows the results for the best-fitting model involving significant terms and marginally significant terms.

Table 2. Statistical analysis of data generated by the most appropriate model

Source	Sum of Squares	Mean Square	p-value	Significance
Model	961.82	137.40	0.0006	**S**
A-Yeast concentration	67.21	67.21	0.0525	**MS**
B-Enzymatic Pre-treatment	174.74	174.74	0.0049	**S**
D-Pressure	578.50	578.50	< 0.0001	**S**
AB	2.34	2.34	0.6926	**NS**
AD	26.37	26.37	0.2006	**NS**
BD	60.55	60.55	0.0635	**MS**
ABD	52.11	52.11	0.0820	**MS**
Residual	156.50	14.23		
Lack of Fit	113.19	12.58	0.7673	**NS**
Pure Error	43.32	21.66		
Cor Total	1118.32			

In the significance column, S = the parameter is significant, MS = the parameter is marginally significant, NS = the parameter lacks significance in the model

From the analysed parameters, some presented higher significance ($p<0.05$) while others were only marginally significant. The primary factors namely enzymatic pre-treatment (B) and pressure (D) presented significance, while yeast concentration (A) was only marginally significant ($p = 0.052$). The number of passes (C) did not present significance in this model. The secondary and tertiary interaction factors were also taken into consideration. The secondary interaction factor BD as well as the tertiary interaction factor ABD were the only ones within the marginally significant group, with $p_{BD} = 0.063$ and $p_{ABD} = 0.082$. Overall, the model had a high significance, with a value of $p = 0.006$.
Pareto charts are used to delimitate the most important factors and place them in a descending order. In our case, the Pareto chart supports the choice of parameters to include in the model, as it can be seen in Figure 2. The factor with the highest significance, pressure, drives both the values for the Bonferroni limit and that of the t-test upwards, compared to those corresponding to the marginally significant terms.
Thus, both primary factors with significance, enzymatic pre-treatment and pressure may influence the quantity of extracted protein, and the marginal significance of their interaction factor may point at a synergic effect of these two parameters.

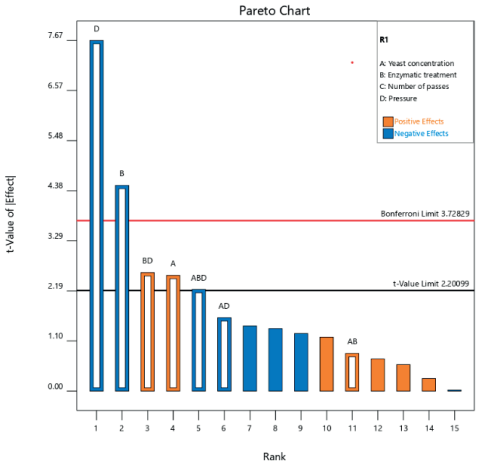

Figure 2. Pareto chart depicting the importance of the parameters in descending order. The parameters are labelled using their codification mentioned in the ANOVA analysis

The yeast concentration, which presents marginally significant p-values tends to explain the variation of data, but in a lesser degree than the other primary factors.
The equation characterising the model is described below with coded values for the factors:

$$Prot.\ Yield\ \left(\frac{mg}{g}\right) = 41.47 + 2.05 \cdot A - \\ - 3.30 \cdot B - 6.01 \cdot D + 0.38 \cdot AB - 1.28 \cdot AD + \\ 1.95 \cdot BD - 1.80 \cdot ABD, \quad (1)$$

Where:
Prot. Yield = the main response (the protein yield of the extraction)
A = Yeast concentration
B = Enzymatic Pre-treatment
D = Pressure
The combination factors = the secondary interaction factor between A and B, A and D and B and D; the tertiary interaction factor between A, B and D.

The equation (1) provides some interesting insights into how each parameter affects the protein yield. The negative terms have a negative impact, their increase leading to a decrease of the protein yield, at least in the case of the primary significant parameters, the most poignant negative effect being that of pressure, closely followed by that of enzymatic pre-treatment.
The adequacy of a model's ability to describe the interactions between the parameters is measured through the lack of fit. In our case, this is not significant which supports the fact that the model is well suited to describe the interactions of the parameters (StatEase, n.d.).
To support this, we need to take into consideration the $\mathbf{R^2}$ and the adjusted $\mathbf{R^2}$ values which indicate the amount of variation around the mean explained by the model. It is considered as a rule of thumb that the closer this value is to 1, the better the model. In our case the $\mathbf{R^2}$ = 0.86 and **adjusted** $\mathbf{R^2}$ = 0.77, which also points to the fact that the model is fitting.
A set of 3D surface plots (Figure 3) were generated using equation (1) and yeast concentration and enzymatic treatment as axis for the plots. Pressure was varied between the low level (A) and the high level (B), to observe the aspect of the curves.

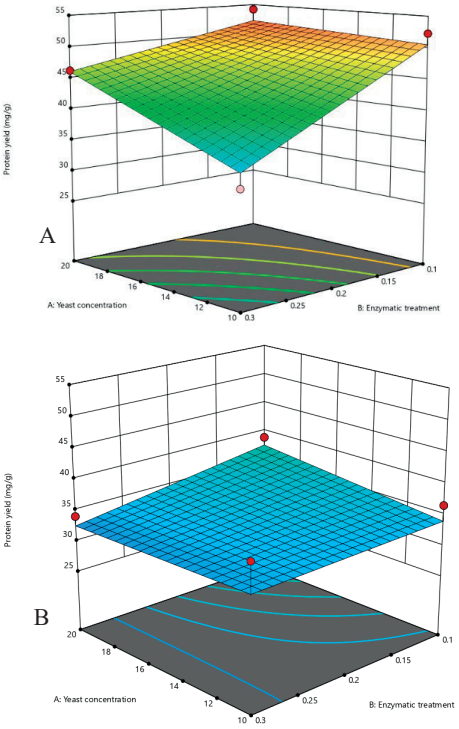

Figure 3. 3D plots of protein yield, as a function of yeast concentration and enzymatic treatment, when the pressure is 1000 bars (A) and when the pressure is 2000 bars (B). The number of passes was kept constant for the two graphs at a value of 3

In terms of soluble protein yield, our results indicate the fact that in the case of this experimental setup, higher yield was obtained for lower values for both pressure and enzyme concentration and using more pressure and more enzyme does not bring any improvement to the process.

Table 3. Highest and lowest concentrations of soluble proteins as measured through the Biuret reaction

Sample	Concentration of soluble protein (mg/mL)
D2	11.22
D15	2.95

One study (Dimopoulos et al., 2020) indicates a corelation between the obtained soluble protein concentration and combining different

approaches to this issue. Our results indicate the fact that our approach seems to be more efficient, the values exceeding by far the values mentioned in literature.

There is another matter that might be useful in understanding what happens with the intensification of processes. Higher shear force, driven by an increase of pressure translates to improved cell disintegration. However, this has a downside as well, meaning that the intracellular content of lytic enzymes is brought in contact with large amounts of suitable substrates. This might cause a drop in protein concentrations, in conjunction with a higher number of passes, as other studies relied on several passes - between 1 and 3. Considering we used much higher pressure and number of passes than the studies, it might point to a reason why with increasing pressure and increased enzyme concentration the protein yield drops.

The combination of piston homogenizer pressure and enzymatic treatment with yeast cell wall lysing enzymes proved to be very efficient in releasing yeast cell content. Separation of these two components of the yeast cell, yeast cell wall and intracellular proteins and peptides is important for further development of plant biostimulant.

Yeast cell wall acts as an elicitor of the plant innate immunity, due to its content of (1→3)-β-D-glucan (Sun et al., 2019) and chitin (Sun et al., 2018). Both (1→3)-β-D-glucan and chitin are active elicitors from the category of microbe-associated molecular patterns (MAMPs) (Boller & Felix, 2009). Till now, were registered as active ingredients for plant protection products the cell walls of the of *S. cerevisiae* strain LAS117, under common name cerevisane (EFSA, 2014) and lysate of the cell walls of *S. cerevisiae* strain DDSF 623), under common name ABE-IT 56. Yeast cell walls have a significant potential as low risk biopesticide, to control economic important plant diseases, such as downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*) and grey mould (*Botrytis cinerea*) in grape (Angelini et al., 2019). Yeast intracellular proteins and peptides could be further converted into plant biostimulants by hydrolysis with proteases, to capitalize the existence of active ingredients acting on plants, such as glutamic acid (Lee et al., 2021), or glutathione (Ur Rehman et al., 2021).

The yeast extract itself proved to be effective in enhancing plant response to abiotic stress (Abdel Latef et al., 2019). Therefore, the potential agricultural applications of spent brewery yeast compensate its lack of attractivity to food industry.

CONCLUSIONS

The goal of this study was to devise an experimental plan and find a set of optimised values for the relevant parameters. ANOVA analysis revealed that in a high-pressure homogenisation setting, combined with an enzymatic pre-treatment, pressure and enzyme concentration seem to work synergically, to release a higher amount of protein from yeast cells. However, a more intense process does not seem to have a favourable effect on the yeast protein extraction, possibly due to interaction between the intracellular lytic enzymes and the protein content of these cells.

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BIOLOGICAL ACTIVE COMPOUNDS OF THE *POLYGONACEAE* FAMILY - A REVIEW

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Abstract

The Polygonaceae family includes 800 species of plants, mainly herbaceous, annual, or perennial. These plants are cultivated in temperate regions of the globe. One of the most popular genera of this family is Rumex. About 200 species of the genus are widespread throughout the world. Some species of Rumex are grown as vegetables, while others are used for their biologically active compounds in traditional medicine or pharmaceutical industry.
The aerial parts, the leaves, or the roots of Rumex plants contain various biologically active compounds such as anthraquinones, naphthalenes, flavonoids, stilbenoids, triterpenes, carotenoids, phenolic acids, and vitamins. These biological compounds of Rumex plants are involved in certain diseases' treatment, like diabetes, infections, skin disorders, liver diseases, inflammation, etc.
This study is reviewing the biological active compounds extracted from Rumex plants, their biochemical profile, as well as medical uses.

Key words: *biologic active compounds, Polygonaceae family, Rumex spp.*

INTRODUCTION

Medicinal plants have a rich tradition in all human cultures. They can offer an alternative solution to recover from several health problems, due to easy availability, low costs, and rare side effects (Baig et al., 2011). Such plants deliver biologic active compounds capable of treating or preventing various diseases. Such biologic active compounds are at the basis of traditional medicine and have been tested in several fields of science (Bhalodia et al., 2011; Idris et al., 2019). Therefore, medicinal plants are considered a potential source of novel drugs (Bhalodia et al., 2011; Afolayan, 2003; Selvamohan et al., 2012; Idris et al., 2019). Depending on the plant species, the bioactive compounds are produced by one or more organs, such as seeds, fruits, flowers, leaves, roots, or certain stems (Borchardt et al., 2008). The major bioactive compounds secreted by plants are anthraquinones, flavonoids, phenolic compounds, tannins, polysaccharides, vegetal pigments, plant gums, phytoalexins, essential oils, hydrolytic enzymes, and vitamins (Mostafa et al., 2011).

In recent years due to the social and food habit changes, wild species of edible plants is becoming fashionable for consumption (Sánchez-Mata et al., 2012; Morales et al., 2014). New trends towards gastronomy led the search for novel flavours and textures of different vegetables and increase the appeal of wild plants as an alternative to the common cultivated ones (Spínola et al., 2018).
The *Polygonaceae* family has 56 plant genera with 5835 species, of which only 1384 are accepted species names. The Plant List includes a further 1777 scientific plant names of intraspecific ranks in the *Polygonaceae* family (Bello et al., 2019; The Plant List, 2021). One of the taxons included in this family is *Rumex* genus.

MATERIALS AND METHODS

This study aims to review the biological active compounds that could be found in one of the taxon included in *Polygonaceae* family. *Rumex* spp. and its biochemical profile, as well as its use were evaluated. All data collected in this study derives from research articles published

since 1967, featuring the importance of *Rumex* spp. biologic active compounds and their use in prevention and treatments of various diseases.

RUMEX SPP.

Rumex genus comprises around 200 plant species with worldwide distribution (Vassas et al., 2015; Spínola et al., 2018). *Rumex* plants are herbaceous, annual or perennial, with spontaneous growth (Spínola et al., 2018). It can rise up to 1 m. Its leaves are dark green, fleshy, long, with a fringed cone at the base. They have high chlorophyll content. However, some species or varieties have deep red or purple veins. The leaves alternate on the reddish grooved stem. The plants have green flowers, mostly hermaphrodite or unisexual, arranged in whorls on simple or branched inflorescences (Baig et al., 2011; Vasas et al., 2015). Fruits are trigonous nuts (Koperlainen & Pietilainen, 2020).

Rumex young leaves are harvested for fresh consumption, and used raw in salads, either boiled in soups or used as filling in pies (Spínola et al., 2018).

In Europe, species of *Rumex* are used for different treatments. In Romania, they are used to control intestinal transit, kidney disorders, or skin conditions and to reduce rashes (Butura, 1979, Dénes et al., 2013; Vasas et al., 2015,).

In other European countries, *Rumex* plants are used for the treatment of scurvy, as a "blood purifier", cancer cure, eczema, acne, sunburns, and colds, for their antiseptic activity or hypoglycaemic effect, and even for certain cancer cure (Vasas et al., 2015).

In traditional Chinese medicine, *Rumex* species are used in the therapy of different kinds of bacterial, fungal, or viral infection, dysentery, enteritis, acariasis (Zhang et al., 2012; Vasas et al., 2015).

In Africa, the aqueous extracts of *Rumex* species are used as remedies for various types of stomach disorder, hepatic and spleen diseases, and as an antihypertensive, diuretic, and analgesic (El-Hawary et al., 2011; Vasas et al., 2015).

In 1970, Hartwell reports that many plants belonging to the *Rumex* genus can be used against different types of cancer. For each treatment, plants formulations are very diverse, as powder, cataplasm, decoction, infusion, poultice, ointment, plaster, and unguent prepared from the roots, seeds, leaves, flowers, and barks of the plant or the whole plants (Vasas et al., 2015,).

Based on their biological and chemical profile, the most common species of *Rumex* used for medicinal purposes are: *R. abyssinicus, R. acetosa, R. acetosella, R. alpinus, R. aquaticus, R. bequaertii, R. chinensis, R. confertus, R. crispus, R. dentatus, R. ecklonianus, R. hastatus, R. hydrolapatum, R. hymenosepalus, R. japonicus, R. madarensis, R. maritimus, R. napalensis, R. nervosus, R. obtusifolius, R. patientia, R. scutatus, R. stenophyllus, R. steudelii, R. tuberosus, R. usambarensis, R. verticillatus, R. vescarius, R. woodii* (Hartwell, 1970). The medicinal properties of these plants are due to their biologic active compounds (Srivastava et al., 2014; Idris et al., 2019).

For chemical and biological characterization of *Rumex* species, various parts of the plants were examined, the active compounds were determined and their extraction methods were studied, their potential uses were analyzed, along with the dosage and application methods. Some aspects related to the cultivation area and its influence on the biologic compounds is also mentioned.

BIOCHEMICAL PROFILE OF *RUMEX*

To understand the importance of *Rumex* plants as food or in therapy, it is essential to know the biochemical profile of these species.

Anthraquinones

Among *Rumex* bioactive compounds, anthraquinones and their derivatives are the most common. They are mainly found in roots, followed by fruits (Fairbairn & Muhtandi, 1972; Vasas et al., 2015). Emodin, chrysophanol, and physicon are the most common and well documented (Liu et al., 1997).

Naphthalenes

Phytochemical investigation of Rumex plant parts revealed that naphthalenes are found in whole plant. From the roots were isolated naphthalene-1,8-diols depodin, nepodin monoglucoside, methoxynepodin (Berg &

Labadie, 1981), 3-acetyl-2-methyl-1,5-dihydroxy-2,3-epoxynaphathoquinol (Zee et al, 1998), torachrysone (Jiang et al., 2007), musizin, torachrysone, 2-methoxystypandrone (Nishina et al 1993), aloesin, rumexoside, orientaloside, torachrysone (Mei et al., 2009), rumexneposide A and B (Liang et al., 2010). Bioactive compounds such as musizin-8-O-β-D-glucopyranoside (Yoon et al., 2005) and nepodin were extracted from the aerial parts of plants. (Lee et al., 2013).

Flavonoids

Besides naphthalenes and anthraquinones, other important biochemical constituents of the *Rumex* plants are flavonoids, either flavonols or their O/C-glycosides (Vasas et al., 2015).

From the leaves and other aerial parts of *Rumex* spp. were isolated flavonoids such as vitexin, quercitrin, quercetin, isoquercitrin, and catechin (Tavares et al., 2010).

The roots are producing rutin, epicatechin (Jiang et al., 2007; Vasas et al., 2015), and guaijiverin (Orbán-Gyapai et al., 2014).

Studies on biochemical changes during plant cycle and compound variation, depending on the growth areas, revealed that flavonoids concentration increases throughout the plant cycle (Guerra et al., 2008; Vasas et al., 2015).

Stilbenoids

Due to their therapeutic effects, stilbenoids are considered the most important group of plant-derived polyphenols. Such an important biologic active compound is resveratrol. Studies have showed that resveratrol can prevent cardiovascular diseases due to its lipid-lowering effect (Fremont et al., 1999). It is also a potent inhibitor of tyrosine kinase and possesses antifungal properties (Jayatilake et al., 1993; Gonzales et al., 2003).

Resveratrol and its derivatives were isolated from dry roots and leaves of *Rumex* spp. in concentration between 165 μg/g and 239 μg/g (Rivero-Cruz et al., 2005).

Tannins, carotenoids, and polysaccharides

Roots and tubers of *Rumex* species are producing tannins such as leucocyanidin, leucopelargonidin, and leucodelphinidin. These compounds were studied due to their antitumor activity (Buchalter & Cole, 1967).

Important carotenoids such as lutein and β-carotene are frequently isolated from leaves of *Rumex* species (Molnár et al., 2005; Bélanger et al., 2010), and anhydroluteins I and II from stems (Molnár et al., 2005). The lutein content in *Rumex* spp. ranges from 53 μg/g fresh weight to 127 μg/g cooked weight. On the other side, the β-carotene content was 45 μg/g fresh weight to 139 μg/g cooked weight (Bélanger et al., 2010).

A high content of polysaccharides was isolated from the roots, D-glucose being the most representative (Ito et al., 1986).

Other compounds

Several acids, minerals, vitamins, and essential aminoacids were isolated from *Rumex* spp. Such compounds were obtained from all aqueous, acetone or ethanol plant extracts.

Acids were isolated from whole plants. The most abundant is the oxalic acid. However, caffeic, neochlorogenic, citric, malic, shikimic, and ascorbic acids are also present (Yoon et al., 2005; Khare, 2007; Guerra et al., 2008). Sorrel is known to contain a high level of oxalic acid (300 mg/100 g). When absorbed, this acid has a corrosive action upon the digestive tract. It also reacts with the calcium in plasma and the insoluble calcium oxalate tends to precipitate in kidneys, blood vessels, heart, lungs, and liver. This reaction can produce hypocalcemia (Farré et al., 1989). Therefore, it is important to have a moderate consumption of such plants.

However, leaves are a good source of minerals, such as calcium (2840 mg), cooper (2.5 mg), iron (36.2 mg), magnesium (1900 mg), potassium (2950 mg), sodium (1010 mg), zinc (5.4 mg), a moderate source of proteins (18.6 g/ 100 g of dry weight) and a low source of lipids (Alfawaz, 2006).

Depending on the growth conditions, the sorrel total vitamin C content is between 22.2 mg and 29.7 mg /100 g of fresh leaves (Sánchez-Mata et al., 2012).

BIOLOGIC ACTIVE COMPOUNDS

The biologic active compounds found in different plants of the *Rumex* genus and their utilization for different diseases treatments are presented in the following table (Table 1).

Table 1. Bioactive compounds, traditional use and plant cultivation area of *Rumex* species (after Vasas et al., 2015; Mishra et al., 2018)

Species	Plant part	Bioactive compounds	Traditional use	Formulation	Plant cultivation area
R. abyssinicus	roots	rhein, chrysophanol, emodin, emodic acid, aloe emodin, alizarin, physcion, damnacanthal, catenarin, anthraquinone, plamidin C, chrysophanol-8-β-D-glucoside, emodin-8-β-D-glucoside,8-C-Glucosyl-apigenin, 8-C-glucosyl-luteolin, 6-C-hexosyl-quercetin, 3-O-rutinosyl-quercetin, 7-O-rhamno-hexosyl-diosmetin	stomach disorders, diabetes, diuretic, analgesic, cancer	n.a.	East Africa
R. acetosa	leaves, roots	emodin, chrysophanol, physcion, emodin-8-O-β-d-glucopyranoside, sennoside A, sennoside B, vitexin, catechin, gallocatechin, epicatechin, epigallocatechin, procyanidin B2, gallate, vanillic acid, sinapic acid	Cataneu diseases, jaundice, warts	n.a.	Asia, Europe (Romania)
R. acetosella	leaves, seeds	emodin, crysophanol, pyscion, sennoside A, sennoside B	analgesic, diuretic, jaundice, warts, fever, dysentery	fresh leaves, decoction	North America, Europe (Britain, Ireland, Turkey, Hungary, Romania)
R. alpinus	seeds, roots	2-acetyl-3-methylnaphthalene-1,8-diol	stomach problems, dysentery, eczema	n.a	Europe (Hungary)
R. aquaticus	n.a.	anthraquinones (emodin, chrysophanol, physcion, citreorosein, chrysophanol-8-O-glucoside), flavonoids (quercetin, quercetin-3,3′-dimethylether, isokaempferide, quercetin 3-O-arabinoside, quercetin 3-O-galactoside, quercetin 3-O-glucoside catechin), stilbenes (resveratrol, piceid) and 1-stearoylglycerol	stomach problems, infection, oedema	n.a	Europe (Bulgaria, Ukraine, Turkey)
R. bequaertii	roots	n.a.	stomach disorders, cancer	n.a.	East Africa
R. chinensis	roots, leaves	n.a.	constipation, inflammation, acne, eczema	powder or decoction, maceration in alcohol	Asia
R. confertus	seeds	pyscion, sennoside A, sennoside B	diarrhoea	infusion	Europe (Hungary)
R. crispus	roots, leaves, seeds, fruits	rumicin, chrysarobin, β-sitosterol, hexadecanoic acid, hexadecanoic-2,3-dihydroxy propyleste, chrysophanol, chrysophanol-8-O-β-D-glucopyranoside, physcion, physcion-8-O-β-D-glucopyranoside, emodin, emodin-8-O-β-D-glucopyranoside, gallic acid, (+)-catechin, kaempferol, quercetin, kaempferol-3-O-α- L-rhamnopyranoside, quercetin-3-O-α-L-rhamnopyranoside	cleanser, skin diseases, icterus, astringent, eye infection, vermicide, skin diseases, rheumatism, tonic	infusion	Europe (Romania, Hungary, Britain, Ireland, Turkey) North and South Africa, North America
R. dentatus	roots	helonioside A, gallic acid, isovanillic acid, p-hydroxycinnamic acid, succinic acid, n-butyl-β-D-fructopyranoside, quercetin, hexadecanoic acid 2,3-dihydroxy propyl ester, β-sitosterol, daucosterol, anthraquinones, flavonoids, phytosterols, phytosteryl esters, free fatty acids, chromones, anthrones, kaempferol 3-O-β-galactoside, kaempferol 3-O-β-glucoside, kaempferol 3-O-rutinoside, isorhamnetin 3-O-β-galactoside, isorhamnetin 3-O-β-glucoside, isorhamnetin 3-O-rutinoside, chlorogenic acid, myricetin, vitamin C.	bacterial and fungal infections	n.a.	Asia
R. ecklonianus	roots	n.a.	sterility, purgative	n.a.	South Africa
R. hastatus	roots, whole plant	leucodelphinidin, leucopelargonidin	laxative, tonic, diuretic, skin disorders, cough, headache, fever	n.a.	Asia
R. hymenosepalus	leaves, roots	emodin, pyscion, rutin, leucopelargonidine, leucocyanidin, eticatechin, epigallocatechin, trans-resveratrol	skin irritation, “purify the blood”, astringent, cough	n.a.	Europe (Britain And Ireland)

Species	Plant part	Bioactive compounds	Traditional use	Formulation	Plant cultivation area
R. japonicus	n.a.	emodin, rutin, rumejaposide, epoxy-naphthoquinol, chrysophanol, physcion, 8-O-β-glucopyranoside.	constipation, hematemesis	n.a.	Asia
R. madarensis	n.a.	ascorbic acid, neoclorogenic acid	diuretic, "blood depurative", dermatitis	n.a.	Asia
R. maritimus	leaves, seeds, roots	n.a.	burns, purgative, tonic, analgesic,	n.a.	Asia
R. nepalensis	roots, leaves	rumexneposide A, physcion, chrysophanol-8-O-β-D-glucopyranoside, torachrysone, emodin-8-O-β-D-glucopyranoside, emodin-8-O-β-D-(60-O-acetyl) glucopyranoside, chrysophano, emodin, citreorosein, resveratrol, nepodin-8-O-β-D-glucopyranoside, torachrysone-8-O-β-D-glucopyranoside, chrysophanol-8-O-β-D-(60 -O-acetyl) glucopyranoside	stomach, haemostasis, purgative, colic, skin disorders	infusion, decoction	South Africa, Asia
R. nervosus	n.a.	n.a.	acne, diabetes, ophthalmic, antiseptic, eczema	n.a.	Europe
R. obtusifolius	aerial parts	anthracene derivatives, flavonoids, procyanidins, oxalic acid	astringent, laxative, antidote to nettle, sores, blisters, burns, cancer, tumour skin eruption, blood purifier	infusion	Europe
R. patientia	roots, seeds, leaves, shoot	emodin-6-O-β-d-glucopyranoside, flavan-3-ol, 6-chlorocatechin, 2-acetyl-3-methyl-6-carboxy-1,8-dihydroxynaphthalene-8-O-β-D-glucopyranoside, labadoside (4,4''-binaphthalene-8,8''-O,O-di-β-D-glucopyranoside), orientaloside (2-acetyl-3-methyl-1,8-dihydroxynaphthalene-8-O-β-D-glucopyranoside), patientosides A and B.	skin problems, anaemia, fever, respiratory disorders, rheumatism	juice, infusion	Europe, North America
R. scutatus	Whole plant	n.a.	antipyretic, astringent, antiscorbutic	juice	Europe, Asia
R. stenopyllus	seeds	n.a.	cough	n.a.	Europe (Romania)
R. tuberosus	Leaf	n.a.	antihypertensive,	infusion, fresh leaves	Europe
R. vescarius		flavonoids, C-glycosides: vitexin, isovitexin, orientin, iso-orientin; anthraquinones: emodin, chrysophanol, rumicin, lapathin; oxalic acid, tannins, mucilage, mineral salts and vitamin C.	tonic, analgesic, hepatic diseases, poor digestion, spleen disorders, asthma, alcoholism	n.a.	East Africa, North America
R. woondii	seeds	n.a.	Dysentery	n.a.	South Africa
Where n.a. = not available					

Species of the *Rumex* genus are widespread throughout the world, mainly in Asia and Central Europe. Such plants are frequently grown for dietary purposes, and used as fresh food or cooked. The common ways of consumption are in salads, soups, snacks, but can also be prepared as decoction, infusion, juice, or powder (dehydrated plants). As medicinal plants, *Rumex* species can be used in for the treatment of gastrointestinal disorders, skin diseases, improving eyesight and stabilizing blood pressure, as well as preventing diseases such as scurvy (Figure 1).

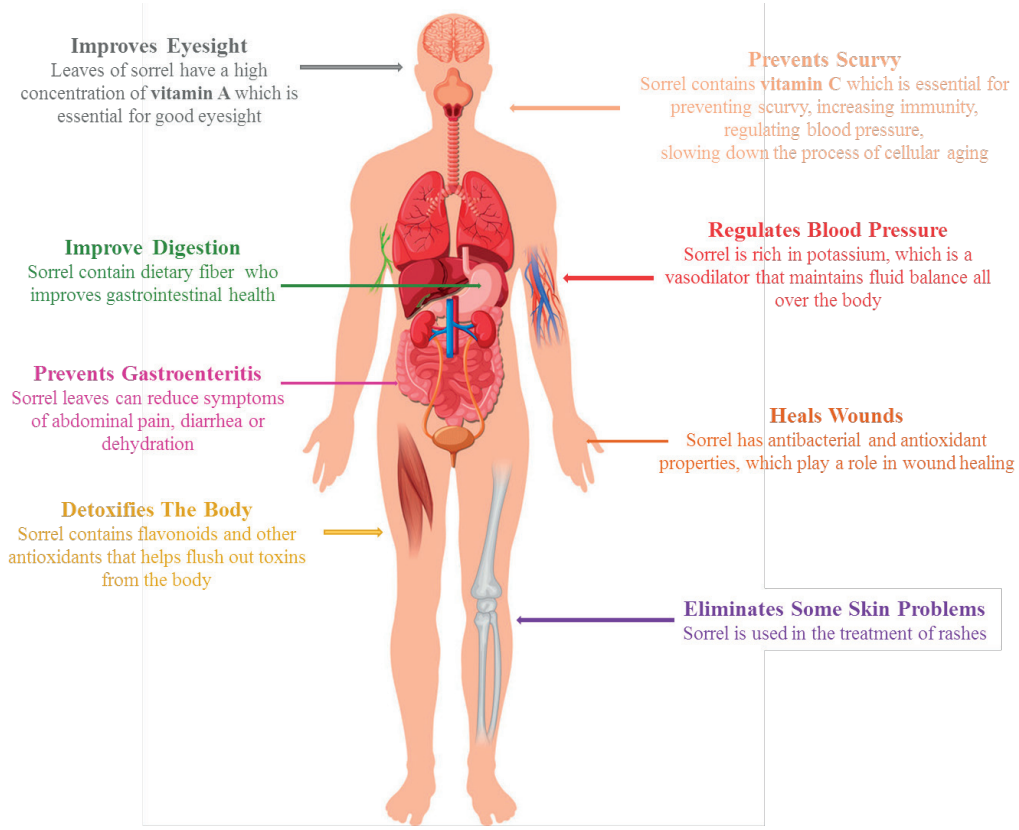

Figure 1. Utilization of *Rumex* spp. in traditional medicine

Note: This figure was created by authors usig various sources: https://niosrcranchi.org.in. and https://www.vectorstock.com

Nowadays, around 130 bioactive molecules have been identified from *Rumex* plants (Vasas et al., 2015). These include anthraquinones, naphthalenes, flavonoids, stilbenoids, tannins, carotenoids, polysaccharides, and other compounds.

CONCLUSIONS

The present review provides the current stage of knowledge regarding biologic active compounds, biochemical profile and safety use of *Rumex* species.

Among edible plants of *Polygonaceae* family, *Rumex* species are more and more cultivated, and consumed as innovative food.

Rumex spp. can also be considered an important source of bioactive substances with medicinal purposes. Therefore, the interest for this plant extracts are taken into consideration for the pharmaceutical industry.

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SOME FACTORS INFLUECING *TRICHODERMA* LACCASE PRODUCTION IN SUBMERGED CULTURE

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Abstract

The main goal of this study was to optimise the laccase production on laboratory scale, under submerged cultivation, in synthetic media. For this purpose, two Trichoderma spp. strains were isolated from different soil and compost samples. These isolates were then inoculated onto culture media under different pH conditions (4, 5, 6, 7 and 8). To induce laccase production, the culture media were supplemented with two different inductors such as guaiacol 0.03% and 2 - 2.5mM CuSO4. In the first 48 hours of submerged fungal culture, the media with initial pH values in the neutral-basic range (7 and 8), recorded drastically low of pH (between 4 and 5), which suggests an acidification of the culture medium. After 14 days from the experiment initiation, Trichoderma TdMI2 isolate grown on media supplemented with guaiacol, at an initial pH value of 8, had a maximum production of fungal biomass and recorded a slight increase in enzymatic activity (from 0.5 to 0 , 6 U / ml). Between the two isolates of Trichoderma spp - TdCP and TdMI2 - cultured on media supplemented with 2.5 mM CuSO4 as inducer for laccase, only TdMI2 had an enzymatic activity of 0.62 U / ml after 3 days of culture.

Key words*: Trichoderma spp., laccase activity, acidification, guaiacol, copper sulphate inducer.*

INTRODUCTION

The laccase enzyme (benzenediol: oxidoreductase) is an oxidoreductase capable of oxidizing phenolic and non-phenolic compounds that have been considered an essential tool in the fields currently known as white biotechnology and green chemistry. Laccase is one of the most robust biocatalysts due to its wide applications in different environmental processes such as detecting and treating chemical pollutants and dyes, pharmaceutical removal or lignin degradation for biofuel production (Popa et al., 2018; Burlacu et al., 2018; Alvarado-Ramirez et al., 2021). Laccases are produced by a wide range of organisms, including fungi, plants, bacteria, or insects (Albu et al., 2019).

Most of the studied laccases are of fungal origin, especially from white rot fungi, *Tramates versicolor, Pycnoporus sanguineus*, and *Trichoderma* spp. (Ranimol et al., 2018). Among *Trichoderma,* different species and isolates of diverse origin, were tested for their laccase production, purification and biotechnological application (Kalra et al., 2013; Ahmed et al., 2015). Most of the application relates to textile dye decolorisation and make use of *T. harzainum* (Bagewadi et al., 2017; Ranimol et al., 2018). Other studied species are *Trichoderma atroviride* and *Trichoderma longibrachiatum* (Bagewadi et al., 2017), while very recent publications refers to the use of *Trichoderma asperellum* (Shanmugam et al., 2020). Basically, this fungus may produce lacasse without any inductor; however, some authors reported that the addition of guaiacol or copper may increase the laccase activity under different pH and temperature conditions (Singh et al., 2014; Bagewadi et al, 2017; Ranimol et al., 2018).

Previously, our team has performed a screening for laccase production among different macro an micromycetes. As results, two strains of *Trichoderma* spp., one variety of *Pleurotus ostreatus* and two of *Agaricus bisporus* originating from supermarket wastes, were detected, by on-plate assay, as important

laccase producers, in the presence of different guaiacol concentrations (Albu et al., 2020).
The present work deals with the optimisation of laccase production under submerged cultivation conditions, in synthetic media, targeting the two isolates of *Trichoderma* spp. As fermentation factors, were chosen the enzyme inductors (guaiacol and copper sulphate) and the pH variation.

MATERIALS AND METHODS

Microbial strains. Two isolates of *Trichoderma* spp., TdCP (composting source) and TdMI2 (soil source) were used for the experiments. The fungal isolates were selected as a result of the agar-plate screening where they proved to be good producers of laccase. Both species were identified by sequencing as being *Trichoderma asperellum* (data not published).

Media. The fungal strains were cultivated 10 days at 28ºC for the spore/inoculum production on PDA (Potato Dextrose Agar) from VWR Chemical, UK. The laccase production was performed on PDB (Potato Dextrose Broth; VWR Chemicals, UK).

Experimental variants for laccase production. Two different inductors (guaiacol and cooper sulphate) were employed in the experiments conducted at different pH values (4, 5, 6, 7, and 8).
The guaiacol test was performed only for the TdMI2 isolate. After autoclaving, the PDB culture medium was supplemented with 30 µL guaiacol. To initiate the experimental variants, 100 ml of PDM was used, distributed in 250 ml Erlenmeyer flasks. The inoculation was done with a suspension of 10^6 spores/mL and the cultures were shanked at 120 rpm during 15 days at 28ºC. During incubation, variations in the pH of the culture medium were periodically monitored.
In the second test, for inducing laccase production by fungal cultures was added as inducer CuSO4 at different concentrations (2-2.5 mM). For this test were used both *Trichoderma* isolates (TdCP and TdMI2).
For each test, one flask containing 100 ml medium was taken as a sample periodically every two days, and filtrated using filter paper No. 1 to determine the growth dry weight (g/100 ml) and laccase enzyme activity in filtrate.

Assay for laccase activity
The laccase activity was determined by using guaiacol as substrate (Kalra et al., 2013). Oxidation of guaiacol by laccase is used to measure enzyme activity. The reaction mixture contained: 1 ml guaiacol (2 mM), 3 ml sodium acetate buffer (10 mM) and 1 ml enzyme source (fungal supernatant). The mixture was incubated at 30°C for 15 min and the absorbance was read at 450 nm using UV spectrophotometer. One unit of laccase activity (U) was defined as the amount of the enzyme required to oxidize 1 µM of guaiacol per min. The laccase activity in U/ml is calculated by this formula:
E.A. (U/mL) = A x V/ t x e x v,
where:
E.A = Enzyme activity; A = Absorbance;
V = Total mixture volume (ml); v = enzyme volume (ml); t = incubation time; e = extinction coefficient for guaiacol (0.6740 µM/ cm).

RESULTS AND DISCUSSIONS

Two isolates of *Trichoderma* spp., TdCP (composting source) and TdMI2 (soil source) were used to optimize laccase production under submerged culture with the participation of two inducers such as guaiacol and copper sulphate (CuSO4). Also, different levels of initial pH values ranged from 4 and 8 were applied.

The pH evolution during *Trichoderma* cultivation
The influence of guaiacol addition in PDB medium (0.3%) on the laccase production was initially tested on the isolate TdMI2, originating from soil. The tests were performed under different initial pH conditions (4, 5, 6, 7 and 8) and it was measured constantly (each two days) during the process. It was noticed that, in the absence of guaiacol, even from the second day of incubation, the medium with an initial pH of 8, has drastically decreased at 5, while the medium with an initial pH of 5 showed a less significant decrease, reaching

levels around 4.5. No changes were noticed in the case of the initial pH of 4 (Figure 1).

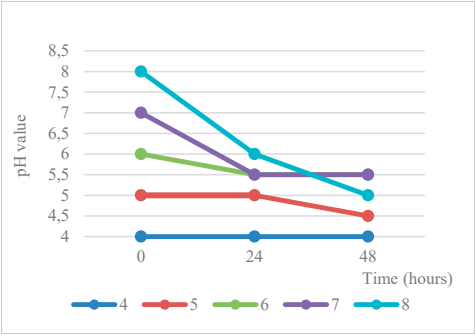

Figure 1. Evolution of pH during incubation of TdMI2 isolate on PDB medium; w/o guaiacol at different pH values

Similarly, in the presence of 0.3% guaiacol, the initial pH 8 has drastically decreased after 48 hours of cultivation, to even lower level, respectively 4; initial value of pH 7 also decreased to lower levels (pH 5) (Figure 2). The acidic pH values have kept their initial range (4 and 5).

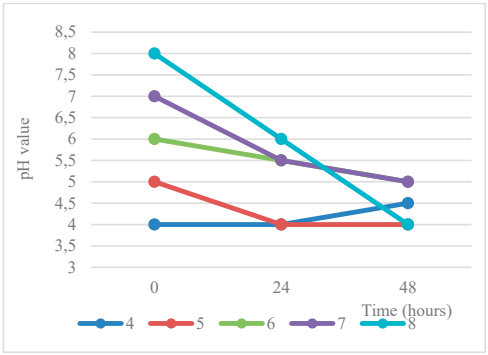

Figure 2. The evolution of pH during incubation of TdMI2 isolate in PDB medium with 0.3% guaiacol at different pH levels

As described by Pelagio-Flores et al. (2017), *Trichoderma* species isolated from soils have the property to induced the acidification of the environment. A few other fungi, mainly pathogenic, acidify their culture media such as: *Penicillium* spp., *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, and *Phomopsis mangiferae* (Prusky et al., 2016). *Trichoderma* spp. grows better in acidic conditions with an optimal growth at pH ranging from 4 to 6, and they can modify the pH of the environment (Singh et al., 2014), but the consequences of fungal-mediated pH changes for plant root growth and development have not yet been deepen analysed (Pelagio-Flores et al., 2017). They produce different secondary metabolites such as non-ribosomal peptides, terpenoids, pyrones and indolic-derived compounds (Contreras-Cornejo et al. 2016) which may induce such acidification, but further studies should be conducted in this regard.

The influence of pH and guaiacol on laccase production

The laccase activity was measured in evolution, every two days, for all the pH values (4, 5, 6, 7 and 8). As described above, the initial pH has decreased in the case of neutral and basic pH (7 and 8), and, after 24-48 hours, actually, all media had a pH on the range 4-5, which was reported as optimal for the *Trichoderma* development. The production of laccase was estimated based on the incubation time.

However, in our case, with or in the absence of guaiacol, the higher laccase activity was noticed in the case of an initial pH of 8, after 14 cultivation days, reaching 0.55-0.6 U/mL as enzymatic activity (Figure 3 versus Figure 4).

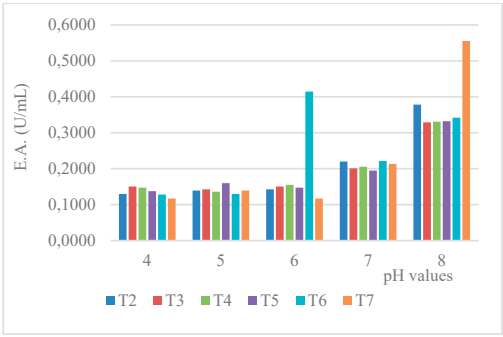

Figure 3. The laccase activity evolution in the presence of 0.3% guaiacol measured every 2 days in the culture of TdMI2 isolate

The guaiacol presence has not significant influence in the laccase production on an initial pH of 8, while for initial acidic pH (4 and 5) its presence influenced positively, but in a lower degree, the enzyme production. The activity measured for the acidic pH was more than 50% lower than in the case of an initial basic pH.

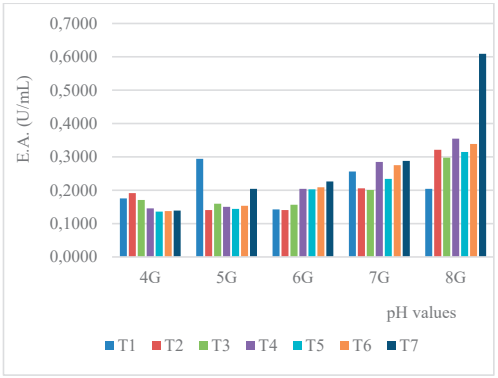

Figure 4. The laccase activity evolution in guaiacol absence for TdMI2 isolate (measured each 2 days)

In this attempt, we were trying to correlate also the biomass production during the cultivation. A correlation was established between the growth of the fungus (biomass weight) and pH with respect to time (Figure 5). The isolate showed maximum growth on 14th day at pH 8 with biomass to be maximum (1270 g). These results clearly suggest the ability of the fungus to grow on an initial pH of 8 which actually decreases after 48 hours in acidic range (4-5). This is close to the values reported by Ranimol et al. (2018) on *Trichoderma harzianum* on pH of 7-7.5.

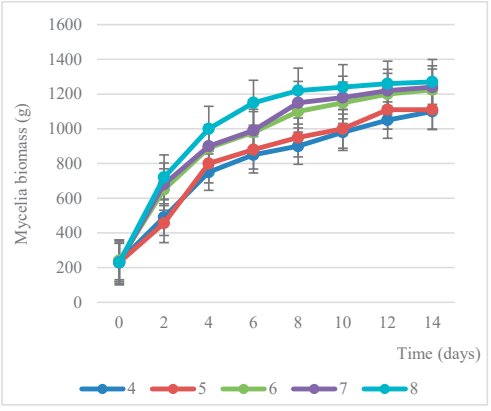

Figure 5. The mycelial biomass evolution of TdMI2 isolate in PDB with 0.03% guaiacol at different pH

The influence of CuSO4 presence on laccase activity

On visual level it was noticed that samples w/o copper sulphate (the control) kept a light yellow color constantly, during all 10 cultivation days, while in the presence of the CuSO4 as inducer, the media color gained different brown overtones, even from the 3rd cultivation day (Figure 6).

The samples with higher concentration of copper sulfate (2.5 mM) have a darker color; among the two isolates, the most intense color was noticed in the case of TdMI2. This results indicate also that both isolates are tolerant to the copper presence, which is an important property when using *Trichoderma* in application like heavy metals (copper) bioaccumulation in soils. For instance, Jovicic-Petrovic et al. (2014) have tested different *Trichoderma* soils isolates for their resistance to Cu (II) concentrations ranging from 0.25 to 10 mmol/L.

Regarding the enzymatic activity, the evolution is quite different. In the first 3 days, the copper inductor has a positive and equal influence on both isolates, being registered laccase activity increases of 0.15-0.2 U/mL against the control.

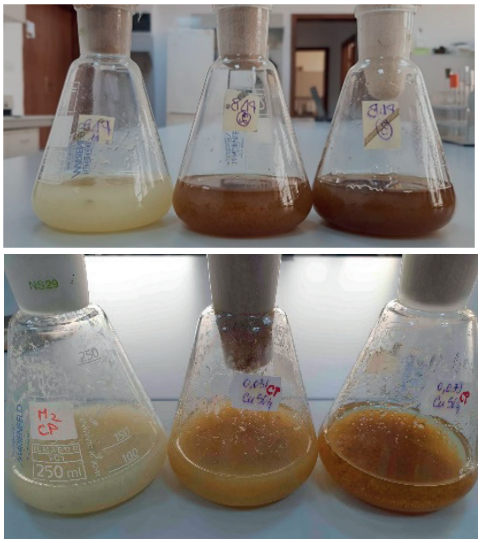

Figure 6. Cultural aspects of *Trichoderma* isolates in PDB additionated with CuSO4 at different concentrations; (up) TdMI2; (down) TdCP

A change was registered after 7 and 10 cultivation days, when, in the case of the isolate TdCP the enzymatic activity has decreased with 0.25 U/mL, while for the isolate TdMI2 the laccase activity increases, reaching double levels against the 3rd cultivation day when adding 2.5mM CuSO4 (Figure 7).

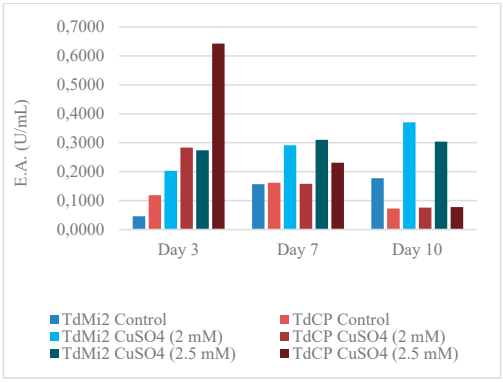

Figure 7. The influence of CuSO4 on laccase activity for two *Trichoderma* isolates

Khambhaty et al. (2015) showed that *Trichoderma atroviridae* could efficient produce laccase without addition of any supplement. However, the addition of CuSO4 increased the activity by almost 25% proving that $Cu2^{+}$ catalytically enhances the action of laccases. Copper is known as a constituent in the laccase's catalytic site; still, its presence greatly influences the enzyme action. Other studies have also shown that copper not only induces laccase by the expression of laccase genes, but it also positively affects the activity and stability of the enzyme (Baldrian et al., 2002) in other fungi (*Pleurotus ostreatus*). Even more, very recently a novel method for simultaneous enhancement of catalytic activity and reusability of laccase was carried out to overcome the limitations on industrial application of laccase, like lignin removal. The immobilization of laccase onto copper ferrite magnetic nanoparticles (CuMNPs); the increased catalytic activity of laccase was observed at 15 mM CuSO4 and the laccase immobilized CuMNPs exhibited 18% higher enzymatic activity when compared to that of laccase in free and immobilized MNPs form (Muthuvelu et al. 2020).

CONCLUSIONS

Different trials were performed on two *Trichoderma* isolates to test the influence of the pH and the inducers presence (guaiacol and CuSO4) on the laccase production in submerged culture, in synthetic media.

The maximum fungal biomass was obtained after 14 cultivation days at an initial pH of 8.

The results reveal the fact that, probably due to secondary metabolites production, the initial pH from neutral-basic range (7 and 8) are drastically decreasing in the first 48 hours reaching pH level of 4-5. Such situation was registered both, in the presence or absence of guaiacol (0.03%). The highest laccase activity was registered after 14 cultivation days at an initial pH of 8; the addition guaiacol has slightly increased the enzymatic activity (from 0.5 to 0.6 U/mL).

In the presence of copper sulphate as inducer, the two isolates behaviour in terms of laccase activity was quite different. While the isolate from the arable soil (TdCP) had a maximum activity after 3 cultivation days with 2.5 mM copper (0.62 U/mL) the isolate from the compost (TdMI2) reached the maximum activity only after 10 cultivation days (0.38 U/mL).

Further, it is expected to use the strain TdMI2 (identified as *Trichoderma asperellum*) for the laccase production on bioreactor scale, to purify and characterise the enzyme and to tests its potential biotechnological application, like lignin degradation or tumours inhibition. Considering the copper positive influence on its laccase production, potential immobilisation in copper based nanoparticle may be considered for the investigations.

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HEALTH BENEFITS OF FERMENTED COLOSTRUM - A REVIEW

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Abstract

Colostrum is the secretion produced by the mammary gland following parturition and transfers the passive immunity gained by the mother to the baby. Colostrum is a mixture of carbohydrates, proteins, growth factors, blood cells and immunoglobulins. Recent studies suggest that in order to improve the biological function of colostrum it is fermented with kefir grains enhanced with selected yeasts, for the development of new nutraceutical and cosmeceutical products. The symbiotic consortia of microorganism produces bioactive peptides with effects against microbial pathogens, cholesterol-lowering capacity and blood pressure-lowering effects, mainly due to inhibition of angiotensin converting enzyme (ACE), antithrombotic and antioxidant activities, opioid, cyto- and immuno-modulatory effects. The present article is a review of the current understanding of the colostrum composition and how it can be improved to an exceptionally safe and useful nutraceutical product.

Key words*: colostrum, milk, kefir, composition, immnunoglobulin.*

INTRODUCTION

In recent years, functional foods have an important role in the development of the food industry (Conte & Scarantino, 2013). The list of functional foods is constantly growing, and colostrum is also gaining popularity due to its benefits (Dzik et al., 2017).

Colostrum is breast milk secreted in the first 48 hours after birth and has a different composition than mature milk.

During breastfeeding, the composition of the breast secretion changes continuously (Ahmadi et al., 2016). Colostrum has the consistency of a dense, viscous, sticky liquid and usually has a color that can vary from yellow to orange (Shrinivas et al., 2010). It is extremely rich in nutrients, such as proteins, fat, lactose, vitamins and minerals and, in addition, it plays a fundamental protection role having an antimicrobial effect, due to immunoglobulins, lactoferrin, lacto- peroxidase, lysozyme and cytokines (Korhonen et al., 2012; Ayar et al., 2016).

Colostrum proteins offer a much lower lactose intolerance than milk, being considered safe and good for consumers healths (Bagwe et al., 2015). Newborns have a very small and immature gastrointestinal system and colostrum provides naturally produced nutrients in a highly concentrated low-volume form. Immunoglobulin A (Ig A) or antibodies present in colostrum not only provide protection to newborns against infectious diseases, but also provide passive immunity and good gastrointestinal development (Shrinivas et al., 2010; Gaspar-Pintiliescu et al., 2019).

Colostrum should not be processed at high temperatures, as its biological activity decreases. It is preferred to dose colostrum in a highly concentrated solid form. It has a short shelf life so the addition of preservatives is a necessity for its storage. (Bagwe et al., 2015)

Many studies have showed the value of colostrum as an agent that provides protection to the newborn against the new environment (Shrinivas et al., 2010).

In the present study we intend to review the main benefits of the colostrum and also, to support with data reports that the biological functions of bovine colostrum can be improved by fermentation and that the functional qualities of the resulted fermented products are important for developing new nutraceutical and cosmeceutical products.

COLOSTRUM COMPOSITION

Recent studies show that colostrum contains over 90 useful components, grouped in three main classes: immune factors, nutritional factors and growth factors: some of such factors are presented in Figure 1 (Shrinivas et al., 2010; Godhia et al., 2013).

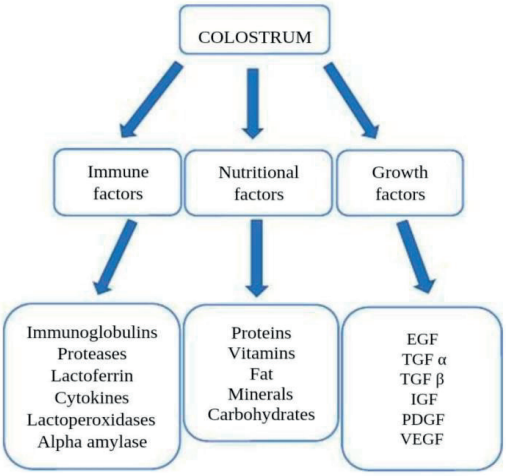

Figure 1. Main immune, nutritional and growth factors in colostrum (EGF: Epidermal growth factor, TGF α: Transforming growth factor, TGF β: Transforming growth factor, IGF: Insulin like growth factor, PDGF: Platelet derived growth factor, VEGF: Vascular endothelial growth factor – according to L.E. Hernandez- (Castellano et al., 2018)

Immune factors

Immunoglobulins are responsible for building the immunity in animals and humans (Bagwe et al., 2015). The immunoglobulins present in bovine colostrum are IgG1, IgG2, IgA, IgM and lactoferrin (Table 1).

Table 1. Immunoglobulins present in bovine colostrum (Bagwe et al., 2015)

Immunoglobulin	mg/mL
IgG1	35.0
IgG2	16.0
IgA	1.7
IgM	4.3
Lactoferrin	0.8

Colostrum is produced in the mammary gland primarily during the last three weeks of pregnancy.

IgG1 immunoglobulins are passively transferred to the mammary gland, and IgG2 immunoglobulins are selectively transferred, resulting in a much higher concentration in colostrum than in serum. The highest concentration of IgG1 in colostrum occurs a few days before calving and then becomes lower, mainly due to an increasing amount of colostrum in the mammary gland (Korhonen et al., 2000).

There is an important group of proteins in terms of the bacteriostatic properties of colostrum and which consist of non-specific antibiotic agents: lysozyme and lactoferrin.

Lysozyme is a cationic protein. Its activity increases in the presence of immunoglobulins (Ganz, 2006).

Lactoferrin it is a glycoprotein present at a concentration of 77g/L in human colostrum. Lactoferrin binds iron, makes it unavailable of *E. coli* in the gut and inhibits bacterial growth (Puppel et al., 2019).

Growth factors

Colostrum contains many hormones like prolactin, oxytocin, somatostatin luteinizing hormone, thyroid stimulating hormone, thyroxine, calcitonin, releasing hormone, progesterone and estrogen. These growth factors are able to stimulate the growth of the intestine and the repair process of the gastrointestinal tract (Godhia et al., 2013).

Epidermal growth factor (EGF) is a 53–amino acid peptide which stimulates the proliferation of different types of cells, fibroblasts and epithelial cells. EGF is present in human colostrum (200 mg/L) and milk (30-50 mg/L) and in many other species but is not found in significant amounts in bovine secretions (Playford et al., 2000).

Transforming growth factor (TGF-α) is present in human colostrum and milk at much lower concentrations than EGF. Instead, TGF-α is produced in the mucosa throughout the gastrointestinal tract.

Therefore, TGF-α may play an additional role to that of TGF-β in controlling intestinal epithelial proliferation and balance (Hoeflich et al., 2017)

Insulin-like growth factors (IGF) IGF-I and IGF-II are polypeptides with a molecular mass of 7.5 kDa (Bączyk et al., 2019).
Platelet-derived growth factor (PDGF) is a suitable mitogen for fibroblasts and arterial smooth muscle cells and administration of exogenous (Hoeflich et al., 2017).
Vascular endothelial growth factor (VEGF) glycoprotein that, induces vascular permeability and promotes monocyte migration (Vuorela et al., 2000).

Nutritional factors

Colostrum contains vitamins that are fat-soluble (retinol, tocopherol, beta-carotene, cholecalciferon, phylloquinone) and water-soluble (niacin, thiamin, riboflavin, vitamin B12, pyridoxal, pyridoxamine, pyridoxine) as it can be seen in Table 2 (McGrath et al., 2016).
The concentrations of riboflavin and retinol higher in colostrum (Calderón et al., 2007).

Table 2. Constituents of water-soluble and fat-soluble vitamins in bovine colostrum (McGrath et al., 2016)

Constituents	Bovine colostrum µg/mL
Water-soluble vitamins	
Niacin	0.34
Thiamine	0.90
Riboflavin	4.55
Vitamin B_{12}	0.60
Pyridoxal	0.15
Pyridoxamine	0.21
Pyridoxine	0.04
Fat-soluble vitamins	
Retinol (Vitamin A)	4.9
Tocopherol (Vitamin E)	2.9
Beta-carotene	0.7
Cholecalciferon (Vitamin D)	0.0305
Phylloquinone (Vitamin K_1)	4.9(µg/L)

Vitamins E and C have an antioxidant effect and help stabilize membranes (macrophages, granulocytes and lymphocytes).
Vitamins A, D and E have a significant role, and their deficiency can have an effect on lowering immunity (McGrath et al., 2016).

Minerals

Colostrum contains 10 times more minerals (except potassium) than milk. The bitter taste of colostrum is given by the mineral content. The amount of minerals in colostrum is shown in Table 3 (Tsioulpas et al., 2007).

Table 3. The content of minerals (%) in colostrum (Tsioulpas et al., 2007)

Minerals	Testing time	
	Calving time	After 11 days
Calcium	0.256	0.130
Magnesium	0.037	0.011
Potassium	0.137	0.153
Sodium Chloride	0.074	0.036
Phosphorus	0.235	0.113
Chloride	0.118	-

The biological and nutritional value of colostrum decreases over the time (Table 4). Colostrum loses its ability to absorb immunoglobulins after the first 6 hours after birth, and after two days an intestinal barrier will appear (Puppel et al., 2019).

Table 4. The composition of colostrum and milk at different hours after calving (Puppel et al., 2019)

Colostrum (h)	Protein %	Casein %	Fat %	Lactose %
0	16.8	4.1	6.7	2.9
6	11.7	3.5	6.1	3.5
12	6.3	3.1	4.4	3.9
24	5.5	2.9	4.1	4.1
48	4.8	2.8	3.9	4.2
Milk	3.2	2.6	3.8	4.6

ADVANTAGES OF COLOSTRUM INTAKE

Colostrum has many therapeutic applications in general health status of consumers. Also, it stimulates the properties of the immune system and contains hormones, growth factors and other bioactive components.
Due to its complex composition, it can be used in combating various diseases (Shrinivas et al., 2010).

Nutritional benefits

As colostrum contains high concentration of carbohydrates, proteins and fats, it delivers its nutrients in very concentrated low volume form (Shrinivas et al., 2010).

Near about twenty times more protein is present in colostrum as compared to the milk produced later (Starton, 2005).

Bioactive compounds in colostrum improve the balance of the intestinal flora, the immune system and tissue regeneration. Autoimmune and cardiovascular diseases are also diseases that can be ameliorated by colostrum (Bagwe et al., 2015).

Colostrum is also rich in polypeptides containing proline, which combat inflammatory response mechanisms. These peptides influence the production of cytokines, the main components of inflammation and inhibit the production of reactive oxygen species, which also cause inflammation (Janusz et al., 2010).

Immune systems benefits

Breastfeeding lays the foundations for good health for all children. One of the greatest significance of colostrum is the fight of the autoimmune diseases and the development of the digestive system. The property of increasing immunity is attributed to molecules called transfer factors.

Some studies (Davison, 2012) showed that colostrum intake can activates the immune system and antimicrobial factors, stimulating the immune functions.

As immune modulator, colostrum can be an important factor in autoimmune diseases (like rheumatoid arthritis) (Godhia et al., 2013).

Wong et al. (2014) demonstrated that a diet supplemented with colostrum improve the immune response to influenza virus. They concluded that bovine colostrum is an important immunomodulator that promotes a good immune response to some infections and that can be used as a therapeutic factor for enhancing immunity, especially in at-risk people.

MICROORGANISMS USED IN THE COLOSTRUM FERMENTATION

Colostrum is an under-used food in the dairy industry (Cotârleț et al., 2020). In order to enhance the benefits of colostrum, it has been fermented with artisanal kefir grains and selected yeasts. The cultures of bacteria and yeasts that act in symbiosis have a greater impact on the health of the consumer (Windayani et al., 2019; Cotârleț et al., 2019)

Kefir it is an old fermented milk product, with a sour taste and refreshing aroma. In Europe and the area of Caucasus Mountains of Russia, is still consumed for its nutritive and functional properties (Cotârleț et al., 2019; Shi et al., 2018).

The kefir grains used for obtaining fermented milk products (known as artisanal cultures grains) represent a complex of proteins, polysaccharides and beneficial microorganisms (lactic acid bacteria, acetic bacteria and yeasts) associated in a natural consortium (Dallas et al., 2016).

The fermentation of colostrum with kefir grains represents a suitable approach to improve the functionality of the obtained product in accordance with the metabolic activity of multiple cultures (bacteria and yeasts) which act in symbiosis. Fermentation also improves the preservation of colostrum, due to the fact that it is very sensitive to microbial contamination and also to heating lability due to the high protein content. (Windayani et al., 2019). During the colostrum fermentation with various microorganism consortia, some bio-peptides (with molecular mass between 1 and 50 KDa) are formed. Bioactive peptides are fragments of protein that bring health to the human body (Shimizu et al., 2007).

These peptides were separated (by ultrafiltration or ultracentrifugation) and tested for their activity *in vitro* (Gaspar-Pintiliescu et al., 2019). Another microorganisms consortia used for colostrum fermentation (in order to enlarge the assortment of dairy fermented products) is a symbiotic association of yeasts and acetic acid bacteria known as “kombucha” (Oancea et al., 2019). It was demonstrated that a symbiosis between microorganisms from kombucha and kefir enhances kombucha fermentation potential, which represents a valuable source of peptides with improved biological activities (Cotârleț et al., 2019; Malbaša et al., 2009; Yang et al., 2008).

HEALTH BENEFITS OF FERMENTED COLOSTRUM AND DERIVED BIOPEPTIDES

A pilot reproducible technology has been developed for bovine colostrum fermentation with symbiotic consortia of microorganism (kefir grains and *Candida lipolytica* selected strain) (Figure 2) (Cotârleț et al., 2019; Cotârleț et al., 2020). By this technology it was obtained a multifunctional, tribiotic product, which includes pre-biotic (bio-peptides with antioxidant effect and polysaccharides from kefiran), pro-biotic (lactobacilli and yeast from kefir grains) and post-biotic (short-chain fatty acids produced by microbial strains and bioactive peptides) (Ricci-Cabello et al., 2012; Cotâleț et al., 2020).

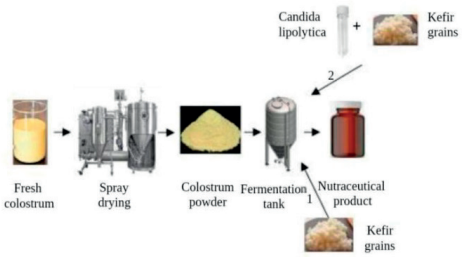

Figure 2. Nutraceutical product obtained by colostrum fermentation technology (Oancea et al., 2019)

Dairy bioactive peptides demonstrated microbicide effects against microbial pathogens, cholesterol lowering ability, and blood pressure lowering effects, mainly due to angiotensin conversion enzyme (ACE) inhibition, antithrombotic and antioxidant activities, opioid, cyto- and immuno-modulatory effects (Mohanty et al., 2016), which recommend them as potential nutraceuticals or therapeutic products for health supporting.

There are reported different benefits of colostrum derived – bioactive peptides on functional or metabolic sides (Figure 3).

Korhonen (2009) studied the effect of various dietary peptides on human health by reducing the risk of chronic diseases, but also by immunizing the immune system. This effect is based on the composition of the peptides and the amino acid sequence. The length of bioactive peptides is generally 2-20 amino acid residues and some peptides may have multifunctional properties (Hartmann et al., 2007).

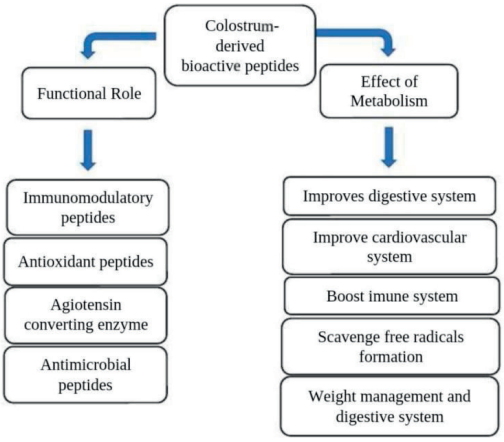

Figure 3. Benefits of colostrum - derived bioactive peptides (Punia et al., 2020)

Colostrum - derived bioactive peptides with antihypertensive effects

Angiotensin converting enzyme (ACE) is a enzyme that controls the arterial blood pressure and electrolyte balance by converting angiotensin I to the vasoconstrictor angiotensin II (Vardanyan el al., 2016). The use of the synthetic drugs for prevention of hypertension may cause serious side effects, such as cough, skin rashes, nausea, vomiting and dizziness (Lin et al., 2012). This has led researchers to search for other, more secure, innovative and cheaper ACE inhibitors (Wijesekara et al., 2011). Food-derived peptides with antihypertensive properties targeting ACE could reduce the risk associated with cardiovascular diseases (Gaspar-Pintiliescu et al., 2019). An important therapeutic strategy is the synthesis and isolation of these biopeptides from various natural sources (Shori et al., 2015). For example, some studies reported a significant variation in the ACE inhibitory activity of peptides derived from kefir-fermented +milk from human, bovine, ovine, buffalo or rat species. A randomized clinical trial recently reported a decrease in blood pressure in prehypertensive adults treated with fermented goat's milk for 8 weeks (Lu et al. 2018).

CONCLUSIONS AND PERSPECTIVES

Colostrum is a safe and useful nutraceutical product for use in a wide range of applications. Colostrum appears to have great potential on human health and this should inspire

researchers to conduct further studies on colostrum fermentation with different consortia in order to analyse the hidden therapeutic and functional properties that have not been revealed to date.

All the data presented in the present work demonstrated that fermented colostrum represents a valuable source of peptides with improved biological activities, compared to those obtained from unfermented (control) colostrum.

Specific bioactivity and functionality of isolated peptides make them suitable as ingredients in functional foods and nutraceuticals and will be further studied.

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MICROBIOLOGICAL CHARACTERISTICS OF LONG-TERM CONTAMINATED SOIL WITH ORGANOCHLORINE PESTICIDES

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Abstract

The aim of this study was to characterize the soil microbiome, involved in nitrogen transformation processes, long-term exposed to obsolete pesticides (HCH, DDT and their metabolites). The working technique was based on the usual bacteriological methods of analysis; nutrient media, suitable for the comparative study of these groups of microorganisms were used. Under the long-term impact of toxicants, the restructuring of soil microbial community in the direction of reducing microbial diversity took place. In the soil polluted with POPs, compared to the reference soil, the number of nitrogen transforming bacteria and micromycetes was diminished and the representatives of the actinomycetes group were absent. The lack or small number of Azotobacter genus was the characteristic feature for the polluted soil. The research allowed the detection of both the inhibition of soil microflora and the development of natural attenuation of pesticides in soil.

Key words*: soil microorganisms, nitrogen transformation, long-term pollution, organochlorine pesticides*

INTRODUCTION

Soil pollution with different persistent contaminants is a global problem. There are approximately 342,000 contaminated sites in Europe alone, which will increase to more than 500,000 by 2025 (Liedekerke et al., 2014; Storey, 2018).

The Republic of Moldova is no exception. Until the 1990s, significant amounts of pesticides were used in agriculture, which later contributed to the contamination of soils and crops in agricultural fields. Due to the ban on the use of persistent organic pollutants (POPs), storehouses, which contained significant amounts of pesticides, were abandoned. Currently, around 1,600 sites in the country - the former territories of pesticide deposits, reached a deplorable state (Focșa et al., 2020). These deposits, as well as the adjacent territories, are a continuous source of environmental contamination, due to the persistence of organic pollutants (Juc et al., 2006).

Synthetic organic compounds often include atoms (such as chlorine, fluorine, bromine or sulphur) inserted into their structures in positions not normally found in nature, and this complicates their natural degradation processes. As well as persisting in the environment, this also means that they can affect the structural and functional properties of soil microbial communities (Bohme et al., 2005) and at the same time create nutrient imbalances in agricultural soils. Soil microbiome including bacteria, fungi, protozoa, algae and viruses forms a vital component of agro-ecosystem and is responsible for many critical and fundamental soil functions such as nutrient-cycling, soil fertility, improving plant productivity through enhanced availability of limited nutrients and decomposition of organic as well as inorganic matter. At the same time, the soil microbial community is essential in the detoxification (bioremediation) of soils contaminated with toxins and unwanted components resulting from human activities.

In many cases, after the application of pesticides, an increase in total microbial biomass has been reported, while a corresponding reduction in functional diversity has been observed (Wang et al., 2008; Lupwayi et al., 2009). Under the long-term influence of chemical pesticides, only a few functional

groups of microorganisms tend to dominate in the soil, which affects the overall community structure and therefore different biological processes of the soil (Hussain et al., 2009; Lo, 2010; Tan et al., 2012; Ivantsova et al., 2015; Prashar & Shah, 2016).

In order to reduce the environmental impact caused by stocks of obsolete pesticides and stocks contaminated with POPs, a series of measures have been taken to implement the commitments made by the Republic of Moldova following the ratification of international environmental treaties regulating chemicals, stocks and their waste (National Implementation Plan, 2004). In the Environmental Strategy for 2014-2023 and the Action Plan for its implementation, approved by Government Decision no. 301 of 24.04.2014, activities are planned for the evacuation and destruction of unusable pesticides, including the POPs category (Focșa et al., 2020).

However, the information about the actual condition of soil after the repacking on former storages is not sufficient at present. The remains of the storages, the foundations can be a permanent source of soil contamination. Along with this, natural detoxification processes can take place in soil long-time contaminated with pesticides. In the framework of State Program Project 20.80009.7007.20 "The study and management of pollution sources for the elaboration of recommendations on the implementation of measures to reduce the negative impact on the environment and public health" (2020-2023) the investigation of pesticide pollution level and microbiological characteristics of soil around the former pesticide storage CR-Slobozia Duşca 01 was initiated.

The aim of this study was to characterize the soil microbiome, involved in nitrogen transformation processes, long-term exposed to obsolete pesticides (HCH, DDT and their metabolites) at former storage CR-Slobozia Duşca 01.

MATERIALS AND METHODS

The object of study was the polluted soil collected from the territory of the former pesticide storage, located near the village Slobozia-Duşca, Criuleni district, Republic of Moldova. Geographical coordinates of the warehouse: X = 29.087525404, Y = 47.174280 0600001; the approximate area of the site: 7600 m².

Primary sampling of soil samples from the pesticide-contaminated site was performed according to the protocol (GOST 17.4.4.02-2017). Three complex soil samples were collected from different zones of the site: sample 1 - near the basement of demolished storage, up the slope; sample 2 and 3 - down the slope. The sample of the reference soil (control soil) was taken at a distance of 200 m from the deposit on the rising slope.

After air-drying at 22-23°C and the removal of vegetal parts and other impurities, the samples were ground and sieved (mesh No. 2).

Soil pH and soil moisture content were determined using standard methods (GOST 17.5.4.01-84; Kozlova, 2009).

The concentrations of organochlorine pesticides were determined by gas chromatography GC/MS (Agilent 6890 equipped with a μECD detector) following the USEPA Method 8081A. The calibration interval was from 0.02 to 0.5 μg/ml. The following pesticides or pesticide metabolites were analyzed in the samples: α-, β, and γ-HCH isomers, hexachlorbenzene (HCB), heptachlor, aldrin, dieldrin, endrin, chlordane, DDE, DDD, and DDT.

Isolation of soil microorganisms was performed by spread plate method, on nutrient media considered the most informative for the comparative study of microorganisms involved in nitrogen transformation (Gerhardt, 1981; Zvyagintsev, 1991). Thus, the presence of ammonifying bacteria was determined by inoculation on Nutrient agar medium (Oxoid, England); bacteria which assimilate the mineral forms of nitrogen and actinomycetes – on Inorganic Salt Starch agar (ISP No.4) (in grams per liter: 10.0 Starch soluble, 1.0 K_2HPO_4, 1.0 $MgSO_4 \cdot 7H_2O$, 1.0 NaCl, 2.0 $(NH_4)_2SO_4$, 2.0 $CaCO_3$, 0.001 $FeSO_4 \cdot 7H_2O$, 0.001 $MnCl_2 \cdot 7H_2O$, 0.001 $ZnSO_4 \cdot 7H_2O$, 20.0 Agar-agar, final pH 7.2 ± 0.2); micromycetes - on Czapek-Dox agar (in grams per liter: 2.0 $NaNO_3$, 1.0 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.5 KCl, 0.01 $FeSO_4$, 30.0 Glucose, 30.0 Agar-agar, final pH 5.0 ± 0.2); and oligonitrophilic

bacteria and *Azotobacter* spp. - on Ashby's Mannitol Agar (Sigma-Aldrich).
Statistical analysis was performed using MS Excel. All results were expressed as mean of three individual replicates ± CI (confidence intervals). All differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSIONS

The former pesticide storage CR-Slobozia Dușca 01 is located extremely close to pastures and arable land. In a radius of 300 meters from the investigated land, the following risk receptors were identified: arable land/annual crops - distance 5 m, pastures - distance 5 m. In the sector up to 1000 m down on the relief from the investigated land, the following risk receptors were identified: river - distance 450 m, water basin - distance 690 m.
The results of the laboratory analyzes regarding the soil pollution in the perimeter of the investigated land showed a high level of soil contamination with POPs (Table 1).
In the present survey, 10 POPs substances were determined in concentrations corresponding to the high level of contamination. In addition to HCH, DDT and their metabolites, traces of other pollutants have been recorded, such as Heptachlor, gamma-Chlordane, alfa-Chlordane, Endosulfan Sulfate, Toxaphene, Trifluralin and Atrazine.

Table 1. The degree of soil pollution with POPs in the territory of the pesticide storage

Chemical compound	Concentration of POPs, mg/kg soil
alfa-HCH	74.077
beta-HCH	17.922
gamma-HCH	8.983
delta-HCH	5.742
o-p-DDE	1.452
p-p-DDE	7.908
o-p-DDD	0675
p-p-DDD	6.277
o-p-DDT	17.475
p-p-DDT	74.384
Total POPs	**214.895**

Note: Maximum permissible concentration of pollutant in soil - 0.1 mg/kg soil.

The pH value of the soil in the control sample, samples 1 and 3 was neutral, while in sample 2 it had increased acidity of 3.6-3.8 (Table 2).

Table 2. pH value of the reference and contaminated soil

Soil sample	pH
Reference soil	7.6-7.7
Sample 1	7.6
Sample 2	3.6-3.8
Sample 3	7.6

The microbiological analysis of the soil samples, and in particular, of the microbiome involved in the nitrogen transformation, showed a big gap between the soil samples (Figure 1).
Thus, the most abundant population of microorganisms was found in contaminated soil samples 1 (59.21 x 10^6CFU/1 g dry soil) and 3 (54.45 x 10^6CFU/1 g dry soil), where the population density exceeded even the control sample (28.35 x 10^6CFU/1 g dry soil) by 2 times. An analysis of microbial population revealed a strong decrease in the total number of microorganisms in the soil sample 2 (1.03 x 10^6CFU/1 g dry soil), it was by 27.5 times lower than in the control sample. This indicates that high doses of pesticides and high acidity of the soil inhibit the vital functions of soil microorganisms, and lead to a reduction of their biodiversity in comparison with the biodiversity in uncontaminated soil.
Such changes in the density and diversity of the polluted soil microflora have been observed in other research (Mohn et al., 2006; Nicol et al., 2008; Manickam et al., 2010; Doolotkeldieva et al., 2018; Lu et al., 2020).
The composition of the indigenous population of microorganisms that assimilate nitrogen and survived in the hard conditions of a long-term toxic stress is presented in Table 3.
It has been observed that, under the long-term influence of the toxicants, the restructuring of the soil microbial cenosis took place in the direction of the decrease of the microbial diversity, but with the appearance of more resistant species. The soil sample 1 is the richest one, both in terms of density and diversity of microorganisms. In this sample, all groups of tested microorganisms were detected, and the density of bacteria that assimilate mineral nitrogen, ammonifiers and oligonitrophilic exceeds by 2 times the control sample. Predominant in the soil sample 1 were bacteria that assimilate mineral nitrogen.
In the soil sample, collected from location 3 of the site, although the population density is

higher than in the reference soil, actinomycetes are missing, and the number of micromycetes and representatives of g. *Azotobacter* is small. Oligonitrophilic bacteria predominated in this soil sample. The increase in number of microorganisms, the predominance of one or other functional groups in samples 1 and 3, speaks about the adaptation of the microflora to the conditions of long-term pollution with POPs and the development of natural bioremediation processes in the soil.

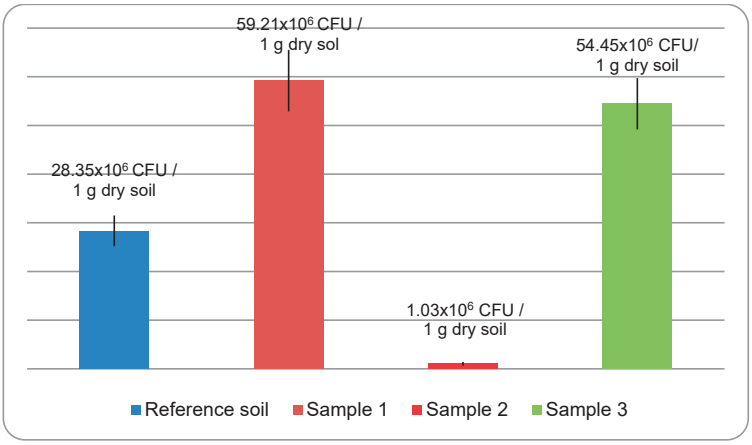

Figure 1. The total number of microorganisms in the reference and contaminated soil samples. The sample of the reference soil was taken at a distance of 200 m from the deposit on the rising slope. Soil sample 1 was collected near the basement of demolished storage, up the slope; soil sample 2 and 3 were collected down the slope

Table 3. Diversity of microorganisms involved in nitrogen transformation processes in polluted soil

Groups of microorganisms	CFU/ 1 g dry sol	Reference soil	Polluted soil		
			Sample 1	Sample 2	Sample 3
Bacteria that assimilate mineral nitrogen	$\times 10^6$	11.71 ± 1.12	26.13 ± 2.92	0.17 ± 0.18	14.20 ± 0.79
Actinomycetes	$\times 10^6$	3.99 ± 0.31	5.18 ± 0.28	0.00	0.00
Oligonitrophilic bacteria	$\times 10^6$	6.23 ± 1.55	13.14 ± 1.80	0.26 ± 0.01	27.04 ± 3.44
Ammonifying bacteria	$\times 10^6$	10.29 ± 0.47	19.89 ± 1.59	0.60 ± 0.18	13.24 ± 1.04
Micromycetes	$\times 10^3$	126.28 ± 8.80	51.58 ± 3.19	4.34 ± 0.87	5.05 ± 2.46
Azotobacter spp.	Cells	952.68 ± 39.27	61.94 ± 7.28	0.00	12.03 ± 0.10

The poor diversity of microorganisms was established for soil sample 2. In this soil no actinomycetes and *Azotobacter* spp. was detected. Representatives of the other groups were observed as single colonies.

The predominance of different groups of microorganisms in the studied soil samples can be explained by the complex pollution and the presence of a large number of POPs metabolites. There is a lot of research which shows that depending on the degradation phase and metabolic pathways involved in the decomposition of persistent pollutants, the groups of microorganisms with the best adapted set of enzymes predominate (Mohn et al., 2006; Manickam et al., 2010; Jeffries et al., 2018; Doolotkeldieva et al., 2018; Regar et al., 2019).

CONCLUSIONS

Thus, our results show that long-term contamination of soils with obsolete organochlorine pesticides affect representatives of soil microbiome differently.

In response to the conditions of toxic stress the reduction of the microbial population biodiversity, especially of fungi, actinomycetes and representatives of g. *Azotobacter* was established.

At the same time, compared to unpolluted soil, the predominance of some groups of microorganisms in polluted soil (bacteria that assimilate mineral nitrogen and oligonitrophilic bacteria) was observed. This is an indication that the microflora is adapting to long-term pollution with POPs and the natural bioremediation processes in the soil is developing.

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NEW INSIGHTS ON LIGNIN DEGRADATION

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Abstract

Lignin is one of the primary components of lignocellulosic biomass, that hinders the depolymerization of the carbohydrate polymers. Lignin is an aromatic heteropolymer which has a complex chemical structure and a linkage heterogeneity which can lead to various aromatic compounds, that are converted into central carbon metabolism, through various microbial catabolic pathways and mechanisms. Lignin degradation can be obtained through different methods: chemical, enzymatic and microbial. Due to negative impact on the environment of the chemical approach, scientists suggest that the biotechnological pathways should be preferred. Multiple aromatic catabolic microorganisms actively secrete ligninolytic enzymes: peroxidases and laccases. The main lignin-degrading peroxidases include manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP). Laccases, are usually found in plants, fungi, and bacteria and belong to the multicopper oxidase superfamily. In this review are described the most efficient approaches to depolymerize lignin in order to obtain value-added compounds.

Key words*: degradation; depolymerization; laccase; lignin; peroxidase.*

INTRODUCTION

Lignocellulosic materials consist of three major components: cellulose, hemicellulose and lignin. Lignin's matrix is closely linked to cellulose filaments and covalently connected with hemicellulose. Lignin can be found in plants, up to 30% of their cell walls. The heterogeneity of lignin determines in plants mechanical strength, defence against pathogens and water transportation to their tissues (Linger et al., 2014). Lignin is probably the most complex and least characterized molecular group among the wood components (Douglas, 1996). There is not one unique, well-defined lignin molecule with certain characteristic properties and functionalities. The structure of lignin depends on its origin, environmental factors during growth, the used extraction method and the applied pretreatment technology (Bruijnincx et al., 2016). Lignin is an amorphous biomacromolecule with a variable three-dimensional composition of p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers (Figure 1). These three monomers differ in the methoxylation pattern of the aromatic ring (Douglas, 1996). The percentage of guayacil, syringyl and p-hydroxyphenyl units varies from species to species. For example: softwoods contain mainly G type lignins, so that means that are mainly G units; hardwoods contain mainly GS-type lignins, so we can find mixtures of G and S units and grass lignins contains a higher proportion of H units (Bugg et al., 2011).

Lignin biosynthesis occurs via oxidative coupling reactions starting from aromatic alcohols that differ in the degree of methoxylation: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Beckham et al., 2016). The chemical composition of lignin consists of phenylpropanoid aryl-C_3 units linked through C-C and ether bonds.

ß-aryl ether is the most common connection, formed by an ether linkage to another aryl unit at C-2. The main sources of ß-aryl ether are softwoods, in which can be found up to 45-50% of units and 60% in hardwoods. Also, in softwoods a percentage of 20-25% units of biphenyl linkages can be found (Bugg et al., 2011). The diaryl propane in softwoods contains only a C–C bond to the second aryl ring, while the diaryl ether founded in hardwoods contains an ether linkage between both aryl rings (Bugg et al., 2011). These

bounds are formed through radical dimerization or polymerisation reactions from cinnamyl alcohol precursors (Freudenberg, 1965; Higuchi, 1971).

In Figure 1 are presented the major types of linkages found in lignin.

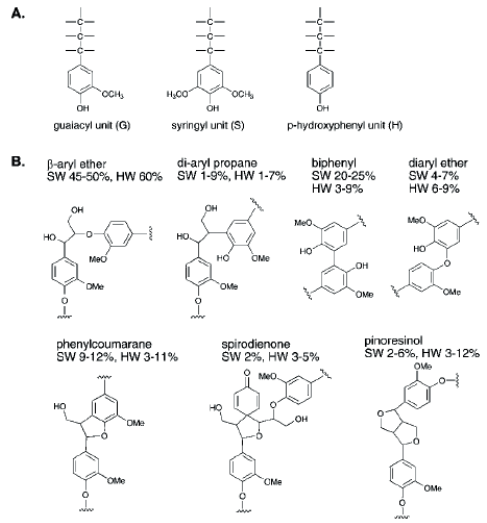

Figure 1. Structural units found in lignin,
A. Structures of G, S and H monomeric units.
B. Structures of chemical linkages found in lignin (shown as G units). SW-Softwood, HW-Hardwood (Bugg et al., 2011)

Linkages are generated by the hence reactions of the phenoxy radical which has stringent character on the "next to" aromatic carbon from the phenolic oxygen. The phenoxy radical is obtained through an oxidation by plant peroxidase and laccase enzymes, of the cinnamyl alcohol precursor (Bugg et al., 2011). Due to lignin heterogenic structure, most of the degradation processes lead to a mixture of aromatic compounds that usually are difficult to valorise (Vardon et al., 2015).

One of the main challenges in lignocellulose valorisation is considered to be lignin separation from lignocellulose components. Lignin removal is a necessary pretreatment, that will in return enhance hydrolysis of the complex polysaccharides left. This process is the most expensive step in the conversion of biomass into fermentable sugars (Karp et al., 2013). An advanced study on lignin structure and its degradation is imperative as it can lead to different value-added products, that can justify the importance of valorisation of all the components of lignocellulose.

Lignin degradation can be achieved through various types of methods: chemical, enzymatic and microbial.

CHEMICAL DEGRADATION OF LIGNIN

There are many methods used for lignin removal mainly being through thermo-chemical processes such as: organosolv process, alkaline hydrolysis, steam explosion, acid hydrolysis, wet oxidation, liquid hot water etc.

Acid hydrolysis is a method of lignin separation from biomass using dilute acid, most commonly sulfuric acid (Bruijnincx et al., 2016). In other experiments, several acids were used such as nitric, acetic, hydrochloric or phosphoric acid. Sulfuric acid process is considered to be the most efficient and low-cost method (Karp et al., 2013). An inconvenience in using these acid treatments is the formation of furfural or 5-hydroxy-methyl-furfural, that can act as inhibitors for microorganisms which are part of the degradation processes of cellulose and hemicellulose. Some organic acids such as fumaric and maleic acid were proposed as alternative treatments that will avoid the formation of 5-hydroxy-methyl-furfural (Karp et al., 2013), the efficiency being comparable with sulfuric acid process.

Alkaline hydrolysis is a pretreatment process often used to remove lignin from the biomass through saponification of ester bonds. It also removes acetyl groups from lignin and uronic acid units from hemicellulose (Karp et al., 2013).

This method uses either the Kraft process or the soda process. Lignin is dissolved in the pulping liquor (black liquor) and is present as lignin phenolate. This alkaline treatment usually performed with NaOH leads to a decrease of the degree of polymerization, crystallinity and a good degradation of the linkages between lignin and carbohydrates (Karp et al., 2013). This pre-treatment leads to extensive degradation of the native lignin structure. Kraft lignin, in contrast to soda lignin, has sulphur species incorporated in its structure.

The **Lignoboost technology** aims at extracting high quality lignin from a Kraft pulp mill. It works in conjunction with evaporation, and the

process begins by precipitating lignin from black liquor by lowering its pH with the help of CO_2. The precipitate is then dehydrated using a filter press, similar to those used in the mining and energy sectors. Conventional filtering and sodium separation problems are overcome by re-dissolving the lignin in spent wash water and acid. The resulting slurry is dehydrated again and washed with acidified water, to produce cakes of purified lignin. After acidification all phenols and carboxylic acids become protonated. The lignin obtained is highly pure with only a minor contamination of carbohydrates and ash, and 2-3% sulphur, half of which is being chemically linked to the lignin (Bruijnincx et al., 2016).

Acidic pulping of lignocellulose with excess aqueous (bi) sulphite together with either sodium, magnesium, calcium or ammonium hydroxide. The lignin ends up dissolved in the pulping liquor (black liquor) as lignosulfonate together with some degraded carbohydrates and some extractives. Lignosulfonates cannot be precipitated through by pH changing and are instead isolated through complete water evaporation (Bruijnincx et al., 2016).

Organosolv process is a treatment that uses an organic solvent mixture with or without an acid or alkaline catalyst (HCl, H_2SO_4, NaOH, NH_4OH etc.), that breaks the hemicellulose glycosidic bonds and degrade the internal structure of lignin (Sun & Cheng, 2002; Raita et al., 2017). Lignin is separated from the biomass through solubilization. The homogeneity of the organosolv processed lignin is higher than that of kraft lignins and lignosulfonates (Norgren & Edlund, 2014). Ethanol, methanol, acetone, cyclic ethers or organic acids, such as acetic acid and formic acid or combinations thereof, are often used for organosolv processing. The process is acid-catalysed, with the acid being formed in-situ from the hemicellulose fraction, or added deliberately (Bruijnincx et al., 2016).

The Bergius-Rheinau process, using concentrated hydrochloric acid at low temperature, produces a lignin fraction that is solid, water insoluble, high molecular weight and with less functional groups. Furthermore, the hydrolysed sugars and the lignin end up together with part of the hydrochloric acid, which must also be recovered (Bruijnincx et al., 2016).

Steam explosion is a most common used process for lignin degradation. The method can be performed with or without an acid or alkaline catalyst. The biomass is first grinded and then subjected to a high-pressure steam (0.69-4.83 Mpa) at 160°-260°C, followed by a rapid decompression. Besides lignin degradation, this process also helps with hemicellulose depolymerization (Karp et al., 2013). Also, fermentation to ethanol can be first performed followed by removal of the ethanol by distillation, leaving behind the lignin in the fermentation broth (Bruijnincx et al., 2016). This method is often used to depolymerize lignin after its isolation through some of the protocols described above.

Ammonia fibre expansion is another method used to degrade lignin, by suspending the lignocellulosic biomass in to a liquid ammonia at moderate pressure from 100 to 400 psi and temperature 70°-200°C. This process helps also with hemicellulose hydrolysis and decrystallizing of cellulose (Harmsen et al., 2010).

Other treatments such as **wet oxidation** performed at temperatures from 170°C to 200°C and at pressures from 10 to 12 bar O_2, for 10 to 15 minutes, are recognized as an efficient method for partial solubilization of lignin (McGinnis et al., 1983). Alkaline wet oxidation seemed to have a poor outcome of degradation compared with acid wet oxidation, where a significant part of the polysaccharides was lost (Karp et al., 2013).

Many new (catalytic) fractionation methods have been reported and might make use of **ionic liquids (Ils), liquid salts** such as zinc chloride hydrate or **deep eutectic solvents (DES)** to (selectively) dissolve lignin or cellulose from lignocellulosic biomass (Bruijnincx et al., 2016). Comparing all the main methods used for lignin degradation through chemical pathways, the major depolymerization of lignin was achieved with: alkaline treatments, organosolv process, wet oxidation and ammonia fibre expansion (Harmsen et al., 2010). Although these processes are considered effective in lignin depolymerization, they present several disadvantages, mainly high negative impact on

the environment due to the use of toxic compounds and harsh conditions or sometimes they are turning out to be expensive processes.

ENZYMATIC DEGRADATION OF LIGNIN

The most important enzymes known for their ability to oxidize lignin are: lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase (Lac). Amongst them, laccase is considered to be the most suitable because it doesn't need a heme cofactor or a supply of hydrogen peroxide and the oxygen serves as the terminal electron acceptor for this enzyme (Woolridge, 2014). According to Thurston (1994) Laccase is a trinuclear blue copper enzyme involved in the catalysation of the oxidation of anilines, phenols and aromatic thiols, accompanied by four electron reduction of O_2 to H_2O, as seen in Table 2. Laccase's cluster is composed of four active site copper atoms; (Solomon et al., 1996). The copper atoms located at T2/T3 site conduct to oxygen reduction, while the copper atom located at T1 site determines the electrochemical potential (Morozova et al., 2007; Yaropolov et al., 1994). Laccases are known for their relative low redox potentials (≤ 0.8 V) in comparison with ligninolytic peroxidases that have moderate redox potentials (>1 V) (Cañas & Camarero, 2010). According to Yanmis et al. (2016), it was observed that when laccase is used in combination with ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) leads to degradation of nonphenolic lignin model compounds and delignification of kraft pulp. When laccase is used in combination with a mediator (HBT – 1-hydroxybenzotriazole), veratryl alcohol is oxidized to veratraldehyde (Woolridge, 2014). Laccase coupled with a mediator is known as laccase mediator system (LMS) and usually the mediator has a low molecular weight and a low redox potential.

There are several mediators used in LMS such as NHA (N-hydroxyacetanilide), HPI (N-hydroxyphthalimide), ABTS, TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy), HBT and violuric acid. HBT and NHA combined with laccase seemed to lead to the best results (Woolridge, 2014). The mediators based on *N*-OH variety (NHA, HPI, HBT) and violuric acid were found to be the best for degradation of recalcitrant nonphenolic lignin. The nitroxyl radicals obtained oxidize their specific substrates by a mechanism that involves hydrogen atom transfer (Cañas & Camarero, 2010; Barreca et al., 2003; Cantarella et al., 2003). High redox potential laccases can oxidize the nitroxyl radical TEMPO to the oxoammonium ion through a non-radical, ionic mechanism (>N=O+) (Cañas & Camarero, 2010; Fabbrini et al., 2002). In laccase-HBT lignin was observed a better functionality of carboxylic acid than in laccase-NHA lignin (Chakar & Rgauskas, 2000). The LMS needs a low optimum oxygen pressure that can be 4tilized in used in lignin removal in the kraft pulp process. Depending on the treatments number of LMS using HBT as mediator and an alkaline extraction, the percentage of delignification can vary from 70% up to 80%. This is a strong proof that LMS with HBT as mediator is capable of degrading the most unreactive lignin (Bajpai et al., 2006). One thing observed during this degradation process was that the mediator has to be recycled constantly. However, the HBT mediator has proved to be less effective on converting to benzotriazole (Woolridge, 2014). Some concerns regarding the limitations of LMS using some mediators, and the toxicity and cost of HBT, were raised. One thing noticed was that the HBT oxidant derived from laccase is reactive, therefore supporting oxidation and inactivation of the latter, either due to adsorption to fibre either to adsorption to pulp, as seen in the case of bleaching flax pulp with *Pycnoporus cinnabarinus* laccase (Sigoillot et al., 2005).

A far better option as a mediator seems to be the synthetic NHA. This mediator is cheaper, biodegradable and sustains a better laccase retention of about 80% of LMS activity (Amann, 1997). Similar properties to NHA have been discovered in other two synthetic acids mediators: 7-cyano-4-hydroxy-2*H*-1,4-benzoxazine-3-one and *N*-(4-cyanophenyl)-acetohydroxamic acid, when utilised in the bleaching kraft process of softwood pulp (Geng et al., 2004). A 25% lignin degradation was observed when using natural phenolic mediators such as acetosyrignone and syringaldehyde (Camarero et al., 2007). A

higher rate of oxidation can be achieved with optimal pH activity (4-7) resulted from using laccases with higher redox potential together with N-OH compounds with lower redox potential.

Manganese Peroxidase (EC 1.11.1.13) catalyses the oxidation of Mn^{2+} to Mn^{3+} using H_2O_2 as an oxidizing substrate (Table 2). The reaction leads to a chelation of the manganese ion to an organic acid, such as oxalic or malonic acid. Being a strong oxidant, Mn^{3+} (1.54 V) can act on several phenolic structures found in lignin (Feijo et al., 2008). The MnP removes lignin and oxidizes residual lignin to a more accessible form to bleaching through alkaline process (Paice et al., 1997). High concentrations of Mn have been observed to lower the efficiency of the peroxide stage (Ni et al., 2000). These high concentrations, at high temperature and pH values, will generate darker pulps (Leary & Giampaolo, 1999). Manganese ion can be chelated by gluconic acid, formed when glucose oxidase and glucose produce H_2O_2 for MnP (Feijo et al., 2008). A significant impact on the fungal degradation of lignin could be represented by brightness amplification generated by MnP systems when unsaturated fatty acids (linoleic and linolenic acids) are added (Gruber et al., 1998). A reduction of a hydrogen atom from the benzyl position (Bao et al., 1994) or one electron oxidation of the aromatic ring (Srebotnik et al., 1997) are the result of the degradation of nonphenolic structures in lignin. Unsaturated lipids are oxidized to peroxyl radicals by Mn^{3+}-oxalate (Kapich et al., 1999). MnP-lipid system can oxidize compounds of nonphenolic β-*O*-4 lignin type (Jensen et al., 1996) compared to chelated Mn^{3+}, which cannot (Enoki et al., 1999).

Lignin peroxidase (EC 1.11.1.14) is a heme containing glycoprotein able to degrade non-specific lignin polymers, attacking non-phenolic compounds with β-O-4 linkages (Table 1) with redox potential up to 1.4 V (Dashtban et al., 2010). LiP can display its activity with the help of a redox mediators such as veratryl alcohol, oxidizing non-phenolic compounds that account for up to 90% of lignin, but its presence is not always need it. LiP can oxidize its substrates in multi-step electron transfers, forming unstable radical cations that will lead to non-enzymatic pathways such as rearrangements, removal of methyl groups, and cleavage of side chains (Falade et al., 2017).

Versatile peroxidase (EC 1.11.1.16) is a hybrid enzyme, because it combines the catalytic activities of LiP and MnP and therefore it is capable of degrading both phenolics and non-phenolics compounds in lignin (Table 2), including those with low and high redox potential (Dashtban et al., 2010). In comparison with MnP and LiP, VP is considered superior because it can display its activity without the help of veratryl alcohol (LiP) and Mn^{2+} (MnP).

According to Dashtban (2010), VP like other heme peroxidases (MnP, LiP) has the heme group deep inside the interior of the protein and has access to other medium through two channels, one that acts similarly to LiP (being hidden) and the other that acts like the one in MnP, where the oxidation of Mn^{2+} to Mn^{3+} happens. Lac does not need H_2O_2 to start the catalysis, compared to MnP, VP and LiP, but it does require the help of several mediators to be able to oxidize aromatic compounds in lignin.

Table 1. The main enzymes involved in lignin degradation

Enzyme	Oxidizing substrate	Cofactor	Lignin degradation
Lac	O_2	N/A	Phenolics, non-phenolics
LiP	H_2O_2	Heme	Non-phenolics
MnP	H_2O_2	Heme	Phenolics
VP	H_2O_2	Heme	Phenolics. Non-phenolics

In addition, new enzymes that act on specific linkages in lignin (ß-O-4 type), are described: aryl-alcohol oxidase (AAO), glyoxal oxidase (GOX), or aryl-alcohol dehydrogenase (AAD), as mentioned in Figure 2. One example is the ß-O-4 linkages (Beckham et al., 2016). Although enzymes can be sometimes expensive, compared with chemical approaches to lignin degradation, enzymatic depolymerization has a low cost for utilities, requires mild conditions and do not cause corrosion problems.

MICROBIAL DEGRADATION OF LIGNIN

In nature, it was observed that several microorganisms have developed some mechanisms to overpass lignin's heterogeneity. This mecha-

nism is using the so-called biological "funneling" pathways. The process is based on the microorganism's capacity to use the aromatic molecules formed as a carbon and energy source (Vardon et al., 2015). As a result of this abundance of aromatic carbon, microbes have evolved various catabolic pathways and mechanisms to utilize aromatic species. Aerobic bacteria employ 'upper pathways' for converting broad slates of aromatic compounds into a few central intermediates, such as catechol and protocatechuate (Fuchs et al., 2011). Dearomatization proceeds using reductive CoA thioesters that destabilize the aromatic ring structure and lead to common intermediates such as benzoyl-CoA (Fuchs et al., 2011; Boll et al., 2014). These intermediates can be further reduced by ATP-dependent (facultative anaerobic) or ATP-independent (obligate anaerobic) reductases to facilitate ring-opening and subsequent b-oxidation-like reactions to form central intermediates (Beckham et al., 2016). For biological funnelling to work, it will need to use a microbe being able to conduct efficient transport and catabolism of a wide spectrum of aromatic compounds simultaneously. Michalska et al. demonstrated that some bacterial ATP-binding cassette (ABC) transporters are able to transport a significant number of benzoate derivatives including vanillic acid and 4-hydroxybenzoic acid (Michalska et al., 2012). Scientists observed that microorganisms degrade lignin both through enzymatic pathways but also non-enzymatic ones. The enzymatic mechanisms involve either enzymes capable of oxidizing lignin, either enzymes that degrade cellulose and hemicellulose for a better access to lignin structure, as seen in Figure 2. The non-enzymatic mechanisms are usually assisted by oxidation through the formation of hydroxyl radicals. Therefore, many microorganisms were identified as lignin degraders (Dicu et al., 2020), mainly from fungal origin such as white and brown rot fungi. They produce H_2O_2 used either as a starter for MnP and LiP, either as a component of the Fenton reaction, in the end resulting in the formation of hydroxyl radicals, that will attack lignin and other lignocellulose components in a non-specific manner. It was also noted that these fungi produce low molecular weight compounds such as quinones, that are first converted into hidroquinones and then later subjected to Fenton reactions (Figure 2). Both white and brown rot fungi are associated with the quinone redox cycling (*Coniophora puteana, Phanerochaete. Chrysosporium).* Auxiliary enzymatic pathways for lignin degradation include: cellobiose dehydrogenase (CDH) and glycopeptides (Figure 2). CDH has been found to degrade cellulose, lignin and hemicellulose, providing hydroxyl radical through Fenton reaction. CDH is mainly synthesized by white rot fungi (*Ganoderma lucidum, Dichomitus squalens*) (Țețu, 2019). Brown and white rot fungi also produce glycopeptides (*P. chrysosporium, Gloeophyllum trabeum)*. These rot fungi are capable of catalysing redox reactions in the end also producing free hydroxyl radicals.

Several laccase isoenzymes with various optimum enzymatic activities and pH stability are synthesized by microorganisms such as *Trametes versicolor* or *Ceriporiopsis subvermispora*, that can oxidize with the lowest redox potential methoxybenzene congener (Woolridge, 2014). *C. subvermispora* could be utilized on both types of woods: hardwoods and softwoods, due to its selective properties towards lignin and extractives removal. One important aspect noted during several studies was that *C. subvermispora* can be cultivated on both *Eucalyptus grandis* and *Pinus taeda* wood chips, but only when *E. grandis* was used laccase production was detected, peroxidases being identified on both cultivation substrates (Ferraz et al., 2003). The results of different bleaching experimental studies with *C. subvermispora* and *T. versicolor* were that lignin degradation and MnP activity do not seem to be correlated (Addleman et al., 1995).

Several other microorganisms were considered as prominent solutions for microbial degradation of lignin: *Pseudomonas fluorescens, P. putida, Rhodococcus opacus, R. jostii*, *Acinetobacter baylyi*, *Amycolatopsis* sp*., Sphingomonas* SYK-6*, Aspergillus nidulans* (Beckham et al., 2016; Albu et al., 2019). Studies showed that *P. putida* KT2440 offers significant potential, including reduced lag periods, increased biomass yield, higher growth rates, improved heterologous protein expression, higher tolerance to oxidative stress and improved cell survival in stationary phase.

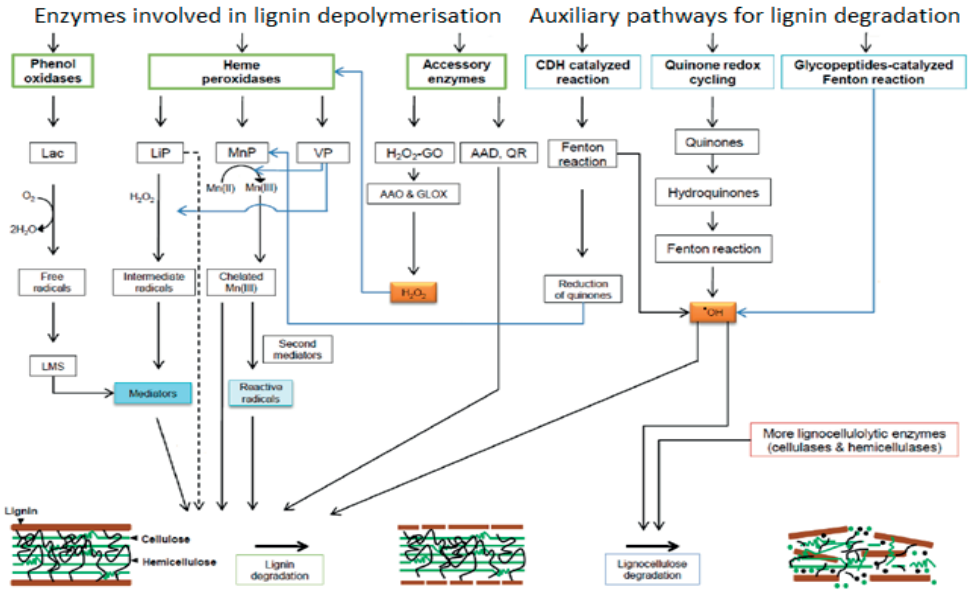

Figure 2. Microbial lignin degradation (adapted from Dashtban, 2010)

Several species have been described to accomplish aromatic catabolism via anaerobic mechanisms, including *Azoarcus* sp. CIB (Fernández et al., 2014), *Thauera aromatica* (Heider et al., 1998), *Aromatoleum aromaticum* (Trautwein et al., 2012), *Rhodopseudomonas palustris* (Fuchs et al., 2011), and others (Beckham et al., 2016). Microorganisms as *R. jostii* RHA1, *Amycolatopsis* sp., *Bacillus subtilis*, and *P. putida* MET94 have been useful in the discovery and description of lignin degrading enzymes. Salvachúa et al. (2015) recently demonstrated that multiple aromatic catabolic microorganisms actively synthesize ligninolytic enzymes, namely laccases and peroxidases, which leads to a significant extent of depolymerization of soluble, high molecular weight (HMW) lignin. According to Beckham, the resulting lower molecular weight compounds were taken up and converted to carbon storage products such as TAGs or PHAs, depending on the microbe. Subsequently, Yuan *et* al. demonstrated the addition of exogenous, commercial laccase from *Trametes versicolor* to *R. opacus* growth on commercial Kraft lignin, demonstrating a significant increase in TAG yield from 8 mg/L to 145 mg/L (Beckham et al., 2016). Delignification was realized over a wide range of acidic pH when *T. versicolor* laccase was utilized as part of a LMS (Bourbonnais & Paice, 1996). Biopulping process is fundamentally based on some fungi properties that makes them able to adsorb to wood chips and to synthesize extracellular enzymes, that will degrade lignin selectively, but will leave mostly intact the cellulose (Ferraz et al., 2008; de Souza-Cruz et al., 2004). Figure 3 presents the way guaiacol (2-methoxyphenol) is demethylated to catechol via a cytochrome P450 enzyme. Microorganisms capable of metabolizing aromatic monomers derived from depolymerized lignin, such as ferulate, phenol, 4-hydroxybenzoate, p-coumarate, vanillate or guaiacol, convert these molecules through 'upper pathways' to two central intermediates, protocatechuate and catechol. Protocatechuate can be decarboxylated by AroY to form catechol, which is then subjected to ring-opening to form cis–cis-muconate by CatA, a catechol 1,2-dioxygenase. Subsequently, muconic acid can be chemo-catalytically converted to (a) adipic acid via hydrogenation, or (b) terephthalic acid via isomerization, Diels–Alder reaction with ethylene, and dehydrogenation (Beckham et al., 2016). Vardon et al. demonstrated that cis, cis-muconate can be produced in an engineered

strain of P. putida KT2440 from aromatic components of lignin such as: ferulate and p-coumarate, and also the common model aromatic compound benzoate (Vardon D.R., 2015).

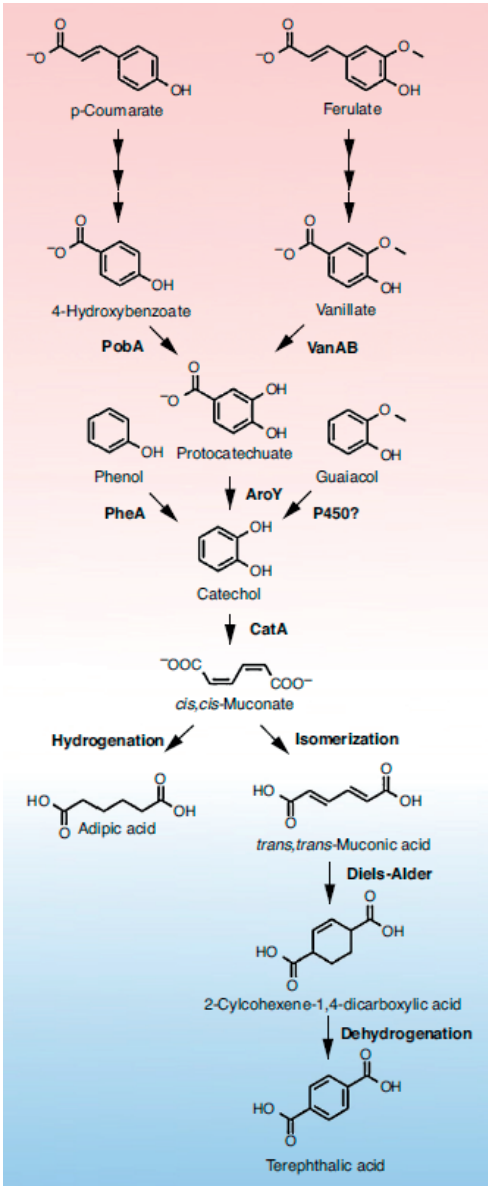

Figure 3. Metabolization of aromatic monomers derived from degraded lignin (Beckham et al., 2016)

T. versicolor and *Pycnoporus coccineus*, were proved to be some of the most prolific synthesizers of oxidative enzymes, especially laccase (Woolridge, 2014).

CONCLUSIONS

Lignin is one of the main components of lignocellulose, and has a distinctly different molecular structure and very different structural and chemical properties, that hinders the depolymerization of the carbohydrate polymers. The possibility of using native lignin for biotechnological applications is limited and therefore several steps are required in order to degrade lignin and provide value-added compounds.

In this paper, were presented the main pathways available for lignin degradation: chemical, enzymatic and microbial.

Although each one of them has both advantages and disadvantages, biotechnological approaches (enzymatic and/or microbial) are preferred due to low cost of utilities, mild conditions, lack of corrosive problems and overall less negative impact on the environment.

Enzymatic degradation of lignin involves a synergic action of four enzymes: laccase, lignin peroxidase, managanese peroxidase and versatile peroxidase, each of them with their own mechanism of action and very specific target components from lignin structure.

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BIODEGRADABLE POLYMERIC SYSTEMS FOR MEAT PACKAGING - A REVIEW

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Abstract

Nowadays, plastic materials have become one of the most widely used materials in all aspects of life, such as in automobiles, household appliances, computers and packaging materials. Because of the environmental issues caused by its disposal and incineration, biodegradable polymeric materials (or biopolymers) were recommended as an alternative to synthetic ones. Most of the disposed packaging come from food industry, especially from fresh and ready-to-eat food products, which are also characterized by high perishability. Meat and derived products represent such perishable food items with quick deterioration under improper storage. Many advances have occurred in the field of smart meat packaging; thus, the aim of this study was to review current advances in the development of novel packaging materials for fresh and processed meat, which are also in support of environmental sustainability.

Key words*: meat packaging, biopolymers, biodegradable.*

INTRODUCTION

The development of active and biodegradable materials for replacing conventional ones, having properties for enhancing the shelf-life and safety of packaged food is nowadays one of the most challenging research activities (Van Long et al., 2016; Xu et al., 2021). Antimicrobial packaging has been proposed as an alternative to post-packaging operations to improve the safety of food products. Main natural antimicrobials occur in nature or they are isolated from microbial (nisin, natamycin), plant (e.g., essential oil of basil, thyme, oregano, cinnamon, clove, and rosemary) or animal sources (e.g., lysozyme, lactoferrin), organic acids (e.g., sorbic, propionic, citric acid) and naturally occurring polymers (chitosan) (Perinelli et al., 2018; Tas et al., 2019; Bhavaniramya et al., 2019).

Development of biodegradable, active, edible and nontoxic films represent a new trend in packaging development (Sanches et al., 2021), mainly based on natural and renewable resources such as polysaccharides and proteins (Xu et al., 2021). These films have combined various concepts, such as food, preservation and packaging into a biomaterial that is edible, biodegradable, prevents loss of moisture, color, lipid oxidation, off-odors, enhancing shelf-life, and transmitting functionality on meat, fish and derived food products (Umaraw et al., 2020).

Spoilage of fresh meat during processing, distribution and commerce have a great negative impact on meat industry, in terms of safety and also from an economical point of view. As an alternative for conventional meat packaging, active biodegradable materials with antioxidant properties were proposed in recent years (Domínguez et al., 2018), given the fact that meat is highly perishable due to its high content in nutrients and polyunsaturated fatty acids, being susceptible to oxidation and microbial spoilage (Xiong et al., 2020).

The purpose of the present study was to review the current state of research regarding novel biomaterials for meat packaging, with great properties in terms of preservation and shelf life.

RESULTS AND DISCUSSIONS

Applications on poultry meat

Quail meat was packed using a composite film based on pectin from apple peel and potato starch, modified using ZrO_2 nanoparticles and

microencapsulated whey protein in *Zataria multiflora* essential oil. The packaging film showed stability for chemical, pH and microbial characteristics of quail meat (Sani et al., 2021).

Moreno et al. (2018) incorporated N-α-lauroyl-l-arginine ethyl ester monohydrochloride in starch - gelatin films and used them as food contact active layers in chicken breast fillets which were vacuum packed in pouches (polyamide/polyethylene). The results of this study showed an increased shelf life of the packed chicken samples without affecting meat oxidation process when compared to control samples.

Gelatin nanofibers containing thyme essential oil/βcyclodextrin ε-polylysine nanoparticles were developed by Lin et al. (2018), to control *Campylobacter jejuni* propagation. These nanofibers were subsequently incorporated into gelatin matrix using electrospinning method and applied as packaging for chicken meat. The packed samples presented lower pH, thiobarbituric acid (TBA) and total volatile basic nitrogen (TVB-N) values and also lower aerobic bacterial count. No impact was observed on texture, color and sensory properties, showing that the developed nanofibers represent promising food packaging.

Two double layer materials based on polybutylene succinate and polybutylene succinate-co-adipate obtained from renewable materials were used for vacuum packaging of raw chicken and turkey meat and also smoked turkey meat. For the packed products were found minor changes in pH, microbiological parameters, water activity, color and volatile compounds profiles during their shelf life storage, making these materials suitable for replacing conventional packaging materials (Vytejckova et al., 2017).

Kamkar et al. (2021) developed films based on chitosan and nano-liposomal garlic essential oil, which were applied on chicken fillet in order to study their preservation capacity. The results of the study showed lower values of pH, thiobarbituric acid reactive substances, peroxide, total volatile nitrogen and microbial count for the packed samples compared with control ones, making the developed films suitable as potential active packaging for chicken shelf life extension.

Biodegradable PVA-montmorillonite K10 clay nanocomposite films with *in situ* generated ginger extract mediated silver nanoparticles were developed by Shiji et al. (2019). These films have proven to be highly effective for reducing microbial load in chicken sausages compared to control films, being taken into consideration for shelf life extension of chicken meat products.

Pirsa & Shamusi (2019) developed films based on bacterial cellulose modified by polypyrrole-Zinc oxide nanocomposite, that were applied in chicken thighs for preservation purposes. The developed film controlled the pH increasing of the tested samples and also decreased the microbial load compared to the control samples, being suitable as antioxidant and antimicrobial packaging.

High pressure treatment combined with active packaging based on polylactide, polyethylene glycol and cinnamon oil was tested on the inactivation of *Salmonella typhimurium* and *Listeria monocytogenes* in chicken samples by Ahmed et al. (2017). The most efficient treatment was the combination of a 300 MPa treatment pressure on chicken packed in films containing 17% cinnamon oil, reducing the pathogens load to a safe level during a period of 21 days of refrigerated storage.

Fresh poultry meat was packed in active biocomposites based on chitosan reinforced with montmorillonite incorporated with rosemary and ginger essential oil. Films proved to be efficient for shelf life extension of the tested samples, reducing lipid oxidation by half and microbiological contamination by 6 to 16% (Pires et al., 2018).

Active edible films composed of semi-refined κ-carrageenan incorporated with a water extract of germinated fenugreek seeds were developed by Farhan & Hani (2020) for fresh chicken breast preservation. The results showed that the developed films efficiently controlled microbial growth on the surface of the samples, in comparison with the control samples.

Polyvinyl alcohol based materials containing *Laurus nobilis* and *Rosmarinus officinalis* essential oils were applied on chicken breast fillets by Goksen et al. (2021). The developed materials inhibited lipid oxidation process and microbial development of the chicken meat,

having a good effect in terms of pH values and color parameters during storage period.

Films based on watermelon rind pectin containing kiwifruit peel extract were developed and applied on chicken tights for preservation purposes. After 9 days of storage the thiobarbituric acid reactive substances were lower for the samples packed in the developed films, compared to control ones (Han & Song, 2021).

Applications on beef meat

Langroodi et al. (2018) studied the antibacterial and antioxidant properties of edible coating based on chitosan containing *Zataria multiflora* essential oil and hydroalcoholic extract of sumac on beef stakes packed in modified atmosphere (MAP) and refrigerated. The results showed that the tested coatings combined with MAP presented high antimicrobial effect, increasing the shelf life and presenting acceptable sensorial properties, while inhibitory effects on lipid oxidation of meat were obtained when using only the coating alone.

Films based on starch, sweet whey and red cabbage extract were used for beef packaging by Sanches et al. (2021). The most effective film to best preserve the quality of ground beef with minor changes was composed of starch, 64.18% red cabbage extract and 4.36% sweet whey, demonstrating also the role of anthocyanins in ground meat preservation.

Xavier et al. (2021) developed an active nanocomposite film based on chitosan and *Cinnamodendron dinisii* Schwanke essential oil nanoencapsulated in zein, which was applied as packaging material on ground beef. This packaging material stabilized the deterioration reactions in ground beef, preserving the color and increasing the shelf life under storage in refrigeration conditions.

Lin et al. (2019) incorporated *chrysanthemum* essential oil in chitosan nanofibers in order to obtain a novel antibacterial packaging. The obtained material was applied on beef and it was proved to be a great inhibitor for *L. monocytogenes* with an inhibition rate over 99.9% at temperatures of 4°C, 12°C and 25°C on a storage period of 7 days. Also, the packaging material presented lower antioxidant activity and pH, when compared to the control sample.

Red meat was packed in a PLA based system (tray and film) using modified atmosphere in comparison with a conventional system (polyethylene terephthalate/polyethylene tray and polyvinyl chloride film) (Panseri et al., 2018). Using the PLA system for beef packaging maintained the redness of the samples and a reduced content of volatile compounds associated with oxidation phenomena.

Alirezalu et al. (2021) packed beef fillets using active chitosan films containing ε-polylysine. These films protected the packed samples, for which lower values for microbial load and also higher sensory properties were obtained when compared to control samples.

Gelatin/palm wax/lemongrass essential oil coated Kraft paper was used for beef packaging by Syahida et al. (2021). The results of the study showed that the developed packaging material showed a reduction in pH, color and moisture changes, delaying also lipid oxidation and microbial spoilage of the tested samples.

Applications on pork meat

Blended films based on curdlan/nanocellulose were used as packaging material for chilled pork meat by Qian et al. (2021), which led to 12 days increased shelf life for the tested samples.

Zhang et al. (2020) developed curdlan/polyvinyl alcohol/thyme essential oil biodegradable films, which were applied for chilled pork meat preservation. The results showed great antibacterial and antioxidant activity of the developed materials, extending the shelf life of pork meat with 10 days.

Yang et al. (2016) developed films based on distiller dried grains with solubles rich in protein, with the addition of green tea, oolong tea and black tea extract as antioxidants. The films were further used as packaging for pork meat and during the storage period they led to lower lipid oxidation compared to control samples.

Edible coatings based on pectin loaded with a nanoemulsion from oregano essential oil and resveratrol were used for fresh pork loin preservation under high oxygen modified atmosphere packaging. The results of the study

showed that the tested materials prolonged the shelf life of fresh pork loin, leading to lower pH values and color changes, delaying lipid and protein oxidation and inhibiting microbial growth (Xiong et al., 2020).
Song et al. (2020) used two antioxidant packaging based on polyethylene terephthalate containing rosemary oleoresin and green tea extract for minced pork meat preservation. The results showed that both tested materials have antioxidant activity, inhibit the oxidation of lipids and proteins and thus extend the shelf life of minced pork samples.

Applications on fish preservation

A novel bacteriocin from *Weissella hellenica* BCC 7293 (Bac7293) was used in order to obtain biodegradable and antimicrobial food packaging, by diffusion coating onto PLA (polylactic acid)/sawdust particle biocomposite film. The resulted film inhibited the growth of both Gram-negative and Gram-positive bacteria in pangasius fish fillets during storage in chilled conditions, demonstrating good antimicrobial properties (Woraprayote et al., 2018).
Ehsani et al. (2020) developed biodegradable films based on chitosan, gelatin or alginate, containing lactoperoxidase system or sage essential oil as active ingredients for common carp fish burger packaging. The packed samples were maintained at refrigeration temperatures for up to 20 days. The results showed that the most effective film to inhibit the total viable count, psychrotrophic bacterial count, *Pseudomonas* spp., *Shewanella* spp. and TBARS was the film based on chitosan incorporated with lactoperoxidase system, extending the shelf life of the tested fish samples.
Socaciu et al. (2021) developed films based on heat-denatured whey protein isolate incorporated with tarragon essential oil, which were applied as packaging for brook trout. The film enhanced the quality preservation of samples, showing lower values for physical-chemical parameters, for microbial load and higher sensory characteristics compared to control samples.
Edible coating based on RA and ε-PL were prepared by Li et al. (2018) and were applied in combination with MAP for preservation of Half-smooth tongue sole. The results of the study showed great characteristics of the film and an extension of shelf life with 8-12 days.
Vizzini et al. (2020) developed an active packaging material with antibacterial properties, being represented by an alginate film reinforced with zinc magnesium oxide nanoparticles for cold-smoked salmon preservation. The salmon samples were inoculated with *L. monocytogenes*. The results showed the tested film provided good antibacterial properties, with no bacterial proliferation at refrigeration temperature for 4 days.
A whey protein concentrate film incorporated with green tea extract was used for fresh salmon packaging (Castro et al., 2019). The packaging proved to be efficient delaying lipid oxidation of salmon samples.

CONCLUSIONS

Active packaging represents a great alternative to conventional packaging, both from an economical point of view and great characteristics such as biodegradability, nontoxicity and availability of raw materials. This study showed that edible films and coatings applied to meat as packaging materials demonstrated great antimicrobial and antioxidant properties, capacity to reduce lipid oxidation and preservation of physical-chemical properties, thus prolonging the shelf life of the tested samples.

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METHODS FOR OBTAINING COLLAGEN FROM VARIOUS FISH SOURCES

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Abstract

Collagen is a natural biopolymer, widely used in various fields, including medical, pharmaceutical, cosmetic or food industry. It is obtained from terrestrial and aquatic sources. Waste from fish meat processing industry is an important source of collagen. The purpose of this paper was to show the possibility to obtain high quality collagen from the residues of the fish processing industry, the main methods of getting and characterizing it,as well as the possibilities of using it. The main methods of collagen obtaining are chemical methods using acetic acid, sodium chloride and sodium hydroxide and enzymatic methods using pepsin, trypsin or pronase. A more recent method, such as ultrasound improves the yield of collagen and its quality. In the case of hard tissues (bones, scales) it is necessary a decalcification often using EDTA or hydrochloric acid. The soft (meat, skin) and hard tissues are mainly treated with sodium hydroxide, butyl alcohol, acetone, etc. to remove non-collagenous proteins and fats. The obtained collagen is analyzed in terms of yield, hydroxyproline content, amino acids analysis, infrared spectra, molecular weight and denaturation temperature determination.

Key words*: collagen, hydroxyproline content, methods, chemical, enzymatic.*

INTRODUCTION

The agri-food industry generates a huge amount of biodegradable organic wastes, in solid and liquid form, with high humidity and biological instability, which favor microbial activity. They can disrupt the ecosystem around the place of storage, if improperly deposited (Nayaka & Bushand, 2019). The residues from fish processing industry represent 0.4% of the total waste amount. Total solid residues yielded by fish processing industry consist of the head, tail, skin, bones, scales, fins and viscera, which represent up to 75% of the fish weight, while fish skin and bones represent 30% from the fish residues (Sylvipriya et al., 2016). A series of valuable products, such as gelatin, collagen, bioactive peptides, proteins, amino acids, enzymes or biogas can be obtained by their processing and are useful for the pharmaceutical, food, cosmetic or medical industries.

Collagen is the main protein present in the extracellular matrix of connective tissues, such as skin, bones, ligaments, tendons, cartilage, internal organs and eyes. It is found in significant amounts in the vertebrates body, but also in invertebrates. In vertebrates body, it represents 30% of total protein content. Collagen molecule has a molecular weight around 300 kDa, a diameter of 14-15Å and a length of about 2800Å (Subhana et al., 2020). The polymeric chain contains repetitive sequence domains of Gly-X-Y or Gly-Y-X, in which Y is proline or hydroxyproline, the latter being the collagen-specific amino acid. Glycine is present in 30-40% and hydroxyproline/proline residues represent 25-35% of total amino acid content.

Collagen molecule consists of 3 helical polypeptide chains, twisted to the right, which have different amino acids composition. The resulting triple helix structure turns to the left (Figure 1). The triple helical structure is unique to collagen and confers its mechanical stability, elasticity and strength. The structure is stabilized by the presence of hydrogen bonds formed between constituent chains, involving the participation of amino and carboxyl groups,

water molecules and hydroxyl groups of hydroxyproline.

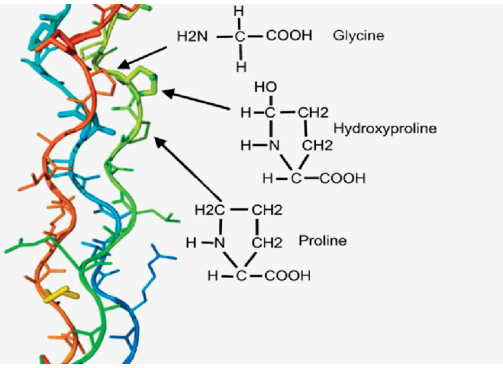

Figure 1 Collagen triple helix formation (Berillis, 2015)

Collagen present on the market has animal origin, being isolated from pigs or cattle. It is known that collagen protein of porcine origin is not accepted by population of Muslim or Jewish religion, while bovine collagen possess a certain risk of transmitting diseases to humans, for example bovine spongiform encephalopathy (Yuswana et al., 2021). Collagen of fish origin eliminates the religious restrictions and zoonosis risks (Sylvipriya et al., 2016).

Collagen can be extracted from any type of solid residue resulting from fish processing, such as bones, skin, scales and cartilage. These tissues represent a quite accessible raw material. The technological flow used for collagen extraction is presented in Figure 3. In order to obtain collagen with high purity and yield, a pretreatment of the raw material it is necessary. Depending on the used raw material, the preliminary processing stage has certain particularities. The second stage represents the effective extraction of collagen and it is performed by chemical methods (using dilute acids), enzymatic methods (using pepsin, trypsin, etc.) and methods that use ultrasound to improve the extraction yield

The aim of this work was to review recent approaches used for collagen isolation, purification and characterization from different fish tissues, in order to valorize wastes, improve environmental conditions and to establish its possible use as main ingredient of medical, food or cosmetic formulations.

COLLAGEN SOURCES AND THEIR PRETREATMENT

The raw material pretreatment is necessary to improve the collagen extraction efficiency and the final product quality. At this stage, the tissue is washed of any impurities and noncollagenous proteins are extracted (Arumugan et al., 2018; Ahmed et al., 2018; Tan et al., 2018). In some cases, the tissue is degreased, e.g. the skin, while in others tissue decalcification is required, for example in the case of bone tissue (Skierka et al., 2007).

Skin

Fish skin is rich in type I collagen, representing up to 70% of total protein content, depending on the species and the season (Chin et al., 2019). Collagen extracted from fish skin has an excellent ability to retain water, about 6% of its weight, and has no irritant potential, thus being suitable for medical applications (Nayaka & Bushand, 2019).

The pretreatment process consists of the mechanical cutting into small pieces of the skin, after removal of the adherent tissues (fat, blood, etc.), followed by extensively washing with tap water and then, with distilled water. To remove noncollagenous proteins, the skin is treated with basic solutions, generally with NaOH in concentrations ranging between 0.1-0.3 M or salts like NaCl for different time periods. Arumugan et al. (2018) treated the skin tissue with 0.3 M NaOH solution, for 4 h, while Ahmed et al. (2019) treated the skin with 0.1 M NaOH solution, for 24 h. In both cases, the ratio between the tissue and the basic solution was 1:10 (w/w) and NaOH solution was changed 3 times during the pretreatment. After NaOH treatment, the skin is washed with distilled water until reaching neutral pH. The next stage is degreasing, which is done with cold acetone, hexane or butanol. Arumugan et al. (2018) used 20% butanol solution, for 10 h, to remove fat. The ratio between the tissue and the degreasing solution is generally 1:10 (w/w). Wang et al. (2008) treated the deep-sea redfish skin with 1M NaCl solution, for 24 h to remove noncollagenous proteins and degreasing was performed with hexane.

Bones

A fairly high percentage of the fish processing industry residue is represented by bone tissue. Bones, including fish heads and backbones, are mineralized tissues with an extremely complex structure. They are rich in type I collagen, in a proportion of at least 30% (Jafari et al., 2020).

Preparation of bone tissues for collagen extraction begins with mincing and washing in common water. In order to obtain as much collagen as possible, it is necessary to decalcify the bones. This process can be done with EDTA or HCl solutions of different concentrations, in the ratio of 1:10 (w/w) between the bone tissue and the decalcification solution. The duration of the decalcification stage varies from 2 h to several days, depending on the type of solution used and the hardness of the bone tissue. Ahmed et al. (2019) decalcified the fish bones in 0.5 M EDTA solution, pH 7.5, for 3 days. Other studies have used a treatment with 0.1 M and 0.5 M EDTA solutions, pH 5, followed by 0.1 M, 0.5 M and 1 M HCl solutions treatment, at intervals of 1-4 days, using a ratio of 1:5 (w/w) between the bone tissue and the decalcification solution, in order to obtain bone decalcification (Skierka et al., 2007). It has been shown that decalcification with EDTA is less efficient, reaching up to 65%, compared with the method that uses HCl, which can reach up to 100% when using a concentration of 1 M HCl. The percentage of tissue decalcification is proportional with the concentration of the used solution and the time of incubation. Thus, incubation of bone tissue with 0.5 M EDTA solution for 96 h determined a slightly higher demineralization percentage, compared to that achieved after 72 h of treatment (Skierka E. et al., 2007). Bone tissue softened by decalcification pretreatment is then subjected to basic solution treatment to remove noncollagenous proteins and degreasing treatment, identical to that used for the skin tissue.

Scales

Fish scales contain inorganic components, such as hydroxyapatite (40-45%) (Zainol et al., 2012) and a percentage of 40-55% organic material, which mainly contains collagen (Chin et al., 2019). The decalcification of fish scales is done using a similar treatment as that performed on bone tissues. First of all, the scales are washed and then, they are treated with strong organic acids, HCl or EDTA, at pH 7.4-7.5. Chin et al. (2019) have obtained collagen from carp scales after demineralization with HCl in concentrations of 1 M and 1.25 M, and H_3PO_4 in concentrations of 0.5 M, 1 M and 5 M, or H_2SO_4 in concentrations of 0.5 M and 1 M. The highest amount of collagen was obtained after decalcification with a solution containing a mixture of 0.2 M HCl and 0.5 M H_2SO_4 (Chin et al., 2019). Ahmed et al. (2019) decalcified the scales using an identical pretreatment to that of bones decalcification using 0.5 M EDTA solution, pH 7.5. After decalcification, the process of noncollagenous proteins and fat removal takes place as described for the skin tissue.

Cartilages

Cartilages are rich in type II collagen, but also contain small amounts of types IX and XI collagen, which represent about 1-6% of the total collagen amount. The pretreatment stage consists of tissue washing and then, the minced tissue is treated with 4 M hydrochloric guanidine, in order to remove the proteoglycan components from its structure (Cumming et al., 2019).

METHODS FOR COLLAGEN EXTRACTION

Collagen fibers are found in the triple helix form with stable inter- and intra-molecular hydrogen bonds, which gives them water insolubility. Therefore, it is necessary to use specific techniques to increase the solubilization of collagen and the extraction efficiency (Jafari et al., 2020). The main methods used for collagen extraction are: chemical methods, which use dilute acids, enzymatic methods using different enzymes and methods that use ultrasound to improve the extraction yield (Kim et al., 2012).

Chemical methods

Acid extraction of collagen uses organic acids, such as acetic acid, or inorganic acids, such as HCl. Regardless of the used acid type, a low concentration is preferred. The ratio between the extraction solution and the tissue is of high importance, as well as the extraction period. The extraction is done at low temperature with

continuous stirring, necessary to increase the contact surface. The most used reagent for collagen extraction is acetic acid in a concentration ranging between 0.2 M and 1 M. Previous research has shown that the collagen extraction yield obtained after 0.5 M acetic acid treatment of tuna by-products, for 36 h has reached 13.5% (Ahmed et al., 2019). Chin et al. (2019) extracted collagen from fish scales using 0.5 M acetic acid, for 24 h, obtaining a yield of 15.33%. Wang et al. (2008) extracted collagen from deep-sea redfish skin, scales and bones using 0.5 M acetic acid, in a ratio of 100:1 (w/w) relative to wet tissue. A treatment of 24 h led to obtaining a yield of 47.5% in case of skin extraction, 6.8% in case of scales extraction and 10.3% for bone collagen extraction (Wang et al., 2008).

Collagen was extracted from the channel catfish skin using 4 different acids: acetic acid, hydrochloric acid, citric acid and lactic acid, in a ratio of 50:1 (w/w) relative to the tissue, with stirring, for 48 h. The used solutions had different pH values of 1.8, 2.1, 2.4, 2.7 and 3. The highest amount of collagen was obtained when using HCl extraction at pH 2.4 (42.36%), followed by extraction with acetic acid at pH 2.7 (39.45 %). The lowest yield results were obtained in the case of extractions with lactic acid and citric acid (Tan & Chang, 2010). In order to increase the extraction yield, the tissue residue obtained after collagen extraction is subjected to another extraction by repeated acid extraction or applying an enzymatic extraction.

Enzymatic methods

In order to extract collagen from fish tissues, a series of proteolytic enzymes, such as pepsin, pronase, trypsin, etc. are usually used. To obtain the best possible results, it is necessary to work at the optimal pH of the enzyme. The most widely used enzyme is pepsin, which is frequently dissolved in acetic acid at pH 2-3 (Jafari et al., 2020). A series of working parameters are of great importance for collagen extraction, such as pepsin concentration, the time of hydrolysis, the ratio between enzyme and substrate. Extraction of collagen from catfish skin was performed by digestion with varying pepsin concentration (0.118-23.6 KU/g) dissolved in hydrocloric acid solution pH-2.4, obtaining the highest protein recovery rate (64.19%) at a pepsin concentration of 23.6 KU/g (Tan et al., 2018). Ali et al. (2018) extracted type I collagen from golden carp skin using 1% pepsin solution in 0.5 M acetic acid, in a ratio of 1:15 (tissue: enzyme solution) with continuous stirring, for 24 h, to obtain an extract yield of 49.8%. Enzymatic extraction with pepsin is often repeated to extract collagen, if a large amount of tissue residue is obtained after the first acid extraction. Thus, Ahmed et al. (2019) subjected the remaining residue after acidic extraction to a digestion with 0.5% pepsin solution dissolved in 0.5 M acetic acid, using a ratio of 0.2 g pepsin/mg residual tissue. To inactivate pepsin in the obtained collagen solution, a dialysis stage was performed against Na_2HPO_4 at pH 7.2 (Ahmed et al., 2019). Tan & Chang (2010) extracted collagen from the channel catfish skin using different concentrations of pepsin ranging between 0.118-23.6 units (KU)/g skin, dissolved in HCl at pH 2.4, at a ratio between the tissue and the extraction solution varying between 1:5 and 1:20. It was observed that the highest protein yield was obtained at the pepsin concentration of 23.6 units (KU)/g tissue.

Previous research has evaluated the effect of bacterial collagenolytic enzymes extracted from *Bacillus cereus* FORC005 and *Bacillus cereus* FRCY9-2 on the extraction of collagen from skin of bigeye tuna (Ahmed et al., 2018). Thus, the undissolved residue obtained after acetic acid extraction was treated with bacterial collagenolytic enzyme in Tris HCl buffer containing $CaCl_2$, at 40°C, for 48 h. The yield of collagen extraction using this method reached values up to 18.8%.

Ultrasound methods

Ultrasound is a sound wave with frequencies higher than the upper limit of human hearing. Ultrasound devices operate at frequencies from 20 kHz to a few GHz. They are used in different types of extractions using various frequencies. Ultrasonic treatment generates a large amount of vibration-induced energy that increases the kinetic energy of the particles, providing energy for a reaction that can promote extraction (Kim et al., 2012).

Kim et al. (2012) have used ultrasound to isolate collagen from sea bass skin using an ultrasonic processor. After pretreatment, the

skin was incubated in 0.5 M acetic acid solution, in a ratio of 1:200 and subjected to ultrasound treatment with a frequency of 20 kHz with amplitudes of 20%, 40%, 60% and 80%, the exposure time being between 0 and 24 h. The temperature was maintained at 4°C by a circular cooling system in the water bath. In parallel, a control extraction was performed, under the same conditions, but without ultrasound. At 80% amplitude, 10.3 times more collagen was extracted than in the case of control extraction (Kim et al., 2012). Ali et al. (2018) have used ultrasound to extract collagen from the skin of the golden carp. The tissue was incubated in 0.5 M acetic acid solution, in a ratio of 1:15 and it was introduced into an ultrasonic reactor with a flat tip probe of 20 kHz, at a temperature of 4°C. The treatment was performed at different degrees of amplitude: 20%, 50%, 80% for variable periods of time of 10, 20 and 30 min, respectively, and after that, the extraction continued with stirring, for 48 h. An ultrasound-free extraction method was performed under the same conditions. The collagen concentration was proportional to the used amplitude and the period of sonication time. The highest amount of collagen was obtained in the case of sonication exposure at 80% amplitude, for 30 min. The application of ultrasonic treatment increased the amount of collagen by 81.53%, compared to the treatment without ultrasound (Ali et al., 2018). Ultrasonic collagen extraction could also be used to improve the enzymatic extraction with pepsin. Thus, the tissue was incubated in 0.5 M acetic acid, in a ratio of 1:15 between the tissue and the enzyme solution, and different pepsin concentrations were added to reach 0.1%, 0.5% and 1%. Sonication was performed at 80% amplitude, for 30 min. A parallel extraction experiment was done in identical conditions, but without sonication. The amount of extracted collagen was proportional to the concentration of pepsin and the yield was 120% higher in the case of sonication, compared to the identical method, but without sonication (Ali et al., 2018).

Other extraction methods

Another method of obtaining collagen is based on the extrusion process, mainly used to prepare food. Thus, fish powder was mixed with a solution of 1.26% citric acid, pH 2, or 9.37% acetic acid, pH 2, in a ratio of 4.7:1 (w/v) and subjected to extrusion cooking at 135 °C, than grounded. The resulting product was dried, mixed with water in a ratio of 1:10 and incubated at 25°C and 50°C, for 1 h. The mixture was centrifuged at 10200 g and the resulting supernatant was dried by lyophilization. The yield of type I collagen extraction using this protocol was 10.9% (Huang et al., 2016).

Supercritical fluids technology using CO_2 acidified water has been used to extract collagen from the skin of Atlantic cod fish. Supercritical fluid is a gas at temperatures and pressures above its critical temperature and pressure, but a pressure below the critical value is needed to compress it into a solid. The physical properties of a supercritical fluid lie between those of a gas and a liquid, and can be controlled by pressure and temperature, allowing the ability to dissolve some materials better than some gases. It was reported that, after tissue pretreatment, the mixture of cod skin and water was placed in a high pressure vessel heated to 370°C and pressurized to 50 bar, for 3 h. After depressurizing the vessel, the extract was filtered and lyophilized, and the yield of collagen obtaining process reached a value of 13.8% (Sousa et al., 2020).

METHODS FOR COLLAGEN PURIFICATION

The solution of acid- or enzyme-solubilized collagen resulting from the collagen extraction process, regardless of the performed method, is subjected to centrifugation and then, collagen present in the supernatant is purified using precipitation with salts, such as NaCl or chromatography techniques (Yata et al., 2001; Chin et al., 2019).

Salt precipitation

NaCl precipitation is the most widely used method and is based on the insolubility of collagen in NaCl solutions at different concentrations, but it depends on the used extraction method. Thus, the solution containing extracted collagen is centrifuged and NaCl is added until reaching the necessary concentration for precipitation. Then, the

solution is centrifuged, in order to collect the precipitated collagen, which is dissolved and dialyzed against dilute solutions of acetic acid. This method of precipitating collagen is convenient and can be used in industry. Ahmed et al. (2019) added a concentration of 2 M NaCl to the resulting centrifuged supernatant containing collagen, followed by centrifugation. The resulting precipitate was dissolved in 0.5 M acetic acid solution and then, it was dialyzed against 0.1 M acetic acid, for 24 h and against water, for 48 h. The resulting collagen was conditioned by lyophilization (Ahmed et al., 2019). Other studies purified collagen by precipitation with NaCl at a concentration of 0.9 M NaCl, after which it was centrifuged and the obtained precipitate was dissolved in 0.5 M acetic acid and then, dialyzed against 0.1 M acetic acid and water (Wang et al., 2008). Tan & Chang (2010) also used a concentration of 0.9 M NaCl to precipitate the chemically extracted collagen in acetic acid, hydrochloric acid, lactic acid and citric acid solutions. The same concentration was used to precipitate the enzymatically extracted collagen in pepsin/hydrochloric acid solution. The resulting precipitate was dialyzed against distilled water to remove NaCl. The obtained collagen was then lyophilized.

Chromatography technique

Purification of collagen by chromatography is frequently performed after precipitation with a salt, the most used being NaCl. Chromatography technique is used for separating the components of a mixture based on the ionic charge differences by their binding to a stationary phase and elution with a mobile phase. Chromatography performed on ion exchange columns allowed separation of type V collagen from other types of collagen, in particular types I and II. Yata et al. (2001) have extracted and separated type V collagen and type I collagen from the skin of 3 types of fish (common horse mackerel, yellow sea bream and tiger puffer). The collagen was solubilized with pepsin and precipitated with ammonium sulfate, resulting in 2 fractions. Then, the collagen extracts was purified by ion exchange chromatography using a phosphocellulose column. The fractions were separately loaded on the column and then, eluted with different NaCl concentrations. Finally, they were dialyzed against water and a solution of 20 mM sodium phosphate (Yata et al., 2001).

Yield of purified collagen

Regardless of the used extraction method, the efficiency of collagen isolation from different tissues is finally determined by extraction yield calculation. The main method used to estimate the yield is the determination of hydroxyproline content (Wang et al., 2008; Ali et al., 2018), the total protein content (Arumugan et al., 2018) or the amount of lyophilized collagen, which are reported to the initial tissue weight (Tylingo et al., 2016).

METHODS OF COLLAGEN CHARACTERIZATION

Analysis of the primary structure

This analysis has an important role for the evaluation of protein properties. To determine the amino acid composition of collagen, the acid hydrolysis of a sample is performed, followed by ion exchange liquid chromatography analysis. The amount of amino acids within samples differs depending on the source from which the collagen is extracted. Glycine represents 30% of the total amino acids and hydroxyproline is the specific amino acid in collagen molecule. It was reported following amino acids analysis that glycine was found in a proportion of 222-227 residues/1000 amino acid residues in the collagen extract obtained from bigeye tuna skin (Ahmed et al., 2018), 328-341 residues/1000 amino acid residues in the collagen extract obtained from the deep-sea redfish skin, bones and scales (Wang et al., 2018), 378-390 residues/1000 amino acid residues in collagen extracted from fish scales and 325-332 residues/1000 amino acid residues in collagen from golden carp skin (Ali et al., 2018). The presence of hydroxyproline in bigeye tuna skin collagen was 80-82 residues/1000 amino acid residues (Ahmed et al., 2018), 61-65 residues/1000 amino acid residues in the case of collagen obtained from the skin, bones and scales of deep-sea redfish (Wang et al., 2018) and 77-81 residues/1000 amino acid residues in the case of collagen extracted from the skin of golden carp (Ali et al., 2018).

Analysis of the secondary and tertiary structure

Identification of the chemical bonds and tertiary structure of isolated collagen is usually performed by FT-IR technique, which is based on an infrared spectrum of absorption or emission. Previous studies have shown that the specific absorbance of the -NH group from the amide involved in a hydrogen bond occured at frequencies less than 3330 cm^{-1} and was present in the case of collagen extracted from bigeye tuna skin (Ahmed et al., 2018), hoki hyaline cartilage (Cumming et al., 2019) and in collagen extracted from the skin, scales and bones of tuna bigeye (Ahmed et al., 2019). In the same studies, the C=O-coupled amide group had frequencies between 1600-1700 cm^{-1}. The presence of these chemical bonds confirms the existence of the triple helix.

Electrophoresis technique

The separation of proteins in polyacrylamide gels, based on their mobility, depending on the molecular weight is performed by electrophoresis technique. Typical electrophoretic patterns of type I collagen extracted from different tissues is presented in Figure 2.

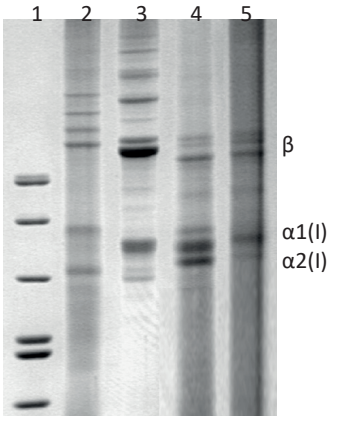

Figure 2. SDS-polyacrylamide gel electrophoresis pattern of collagen extracted from deep-sea redfish tissues: skin (3), scales (4), bones (5). A molecular weight marker (1) and type I collagen from bovine tendon (2) were migrated as controls (Wang L. et al., 2008)

Previous research has shown that the fish collagen is similar to bovine collagen, used as a control, observing well defined α1 and α2 chains, but also the presence of β dimers (Ahmed et al., 2019; Ali et al., 2018; Wang et al., 2018; Arumugan et al., 2018).

Analysis of denaturation temperature

The denaturation temperature represents the temperature at which the tertiary structure of collagen triple helix changes by the hydrogen bonds breaking, leading to variation of the viscosity degree of the collagen solution. Previous research has reported that collagen extracted from the skin, scales and bones of deep-sea redfish had denaturation temperatures of 16.1°C, 17.7°C, and 17.5°C, respectively (Wang et al., 2008), while those of collagen extracted from tuna bigeye skin, scales and bones were 33.7°C, 31.6°C and 32.3°C, respectively (Ahmed et al., 2019). The denaturation temperature is directly proportional to the amount of hydroxyproline. Thus, the redfish collagen extract presenting lower denaturation temperature had 6.1-6.5% hydroxyproline content (Wang et al., 2008), while the tuna collagen extract with higher denaturation temperature had 8.2-8.7% hydroxyproline content (Ahmed et al., 2019).

Collagen extracted from fish sources has biodegradability, low antigenicity and high biocompatibility. Its applicability in different fields, such as medical, pharmaceutical, food and cosmetics industry (Figure 4) is due to its resistant fibers, which can get additional stability by self-aggregation and cross-linking.

Collagen extract is easy to handle and it has the ability to combine with other natural or synthetic polymers to give valuable biomaterials (Moldovan et al., 2009). The main biomaterials obtained from collagen are conditioned in the form of sponges, membranes and films.

The collagen sponge (spongy foil) is obtained by freeze-drying (lyophilization) process (Figure 5), the membrane results after collagen gel drying in glass plates, in an oven, at a temperature below 35°C and the film is obtained by drying the viscous collagen solution in a thin layer, at a temperature close to room temperature.

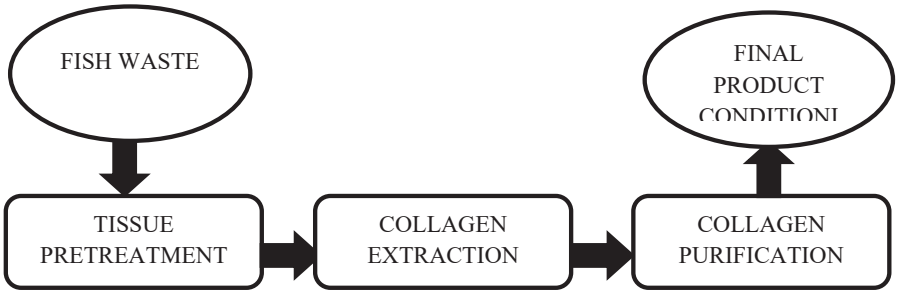

Figure 3. The technological flow chart of collagen extraction from fish residues

APPLICATIONS OF FISH ORIGIN COLLAGEN

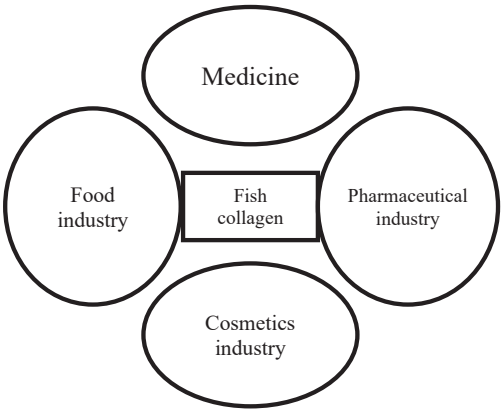

Figure 4. Applications of fish origin collagen

Figure 5. Macroscopic view of a collagen sponge (Cherim & Sarbu, 2009)

Collagen-based films can be used to replace the plastic packaging of fresh foods, such as meat (Jeevitah et al., 2013). Edible coatings developed using a thin film of collagen can prevent dehydration and oxidation of lipids in food products, thus prolonging their shelf life. In the case of fresh or frozen meat, the use of collagen layers or films helps to keep the texture, aroma, color and weight as long as possible. In combination with other compounds, such as fish bioactive peptides, the collagen films may have antioxidant and antimicrobial properties that prolong the life of products and improve their nutritional value.

In medical field collagen is widely used as a transporter of drugs to tissues or organs, eliminating the instability, solubility and poor absorption of drugs (Jafari, 2020). Collagen-based biomaterials play an essential role in repairing skin wounds, such as injuries and burns, due to the fact that this protein is the main component of the skin. Collagen membranes have high porosity, which allows the proliferation of cells at the site of application, the penetration of nutrients and oxygen, high biostability and low immunogenicity. The collagen sponge obtained from fish sources demonstrated a high capacity to stimulate cell proliferation of fibroblasts and keratinocytes, as main cells of the skin (Subhana et al., 2020).

In cosmetics, fish collagen is used to obtain skin care products with high moisturizing and softening effect. It alleviates the harmful effects of solar radiation and reduces the aging process of the skin (Jeevitah et al., 2013).

Collagen is an ideal material for tissue engineering applications due to its biological properties and the efficiency of extraction and purification processes. A disadvantage of fish collagen would be the low denaturation temperature, but after mixing with other synthetic or natural compounds, the value of the denaturation temperature could increase. Multifunctional matrices based on fish collagen have proven useful in the process of regeneration of bone tissue, dermal tissue or cartilage (Subhana et al., 2020).

CONCLUSIONS

The huge amount of residues resulted from fish industrial processing is a useful source for obtaining proteins of interest, such as collagen, a widely used biopolymer in medicine, cosmetics, the pharmaceutical industry and the food industry. Collagen can be extracted from fish tissues (skin, bone, cartilage, scale etc.) by chemical and enzymatical methods, mainly. Preliminary treatments of raw materials before obtaining collagen are of a major importance, such as washing, degrasing or decalcification, in the case of hard tissues. The evaluation of the protein properties is performed by determining the primary, secondary and tertiary structure, by determining the molecular mass, the denaturation temperature using spectrophotometry, electrophoresis, chromatography techniques.

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SIZE AND TYPE OF POLLEN GRAINS OF SOME MELLIFEROUS PLANTS IN ROMANIA

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Abstract

In Romania there are about 1000 melliferous plant species, from these about 200 are considered important for beekeeping. Based on the descriptions found in the literature, a classification according to the type and size of the hydrated pollen grains of these melliferous plant species was attempted in the present study. The melliferous species that were analysed belong to 57 plant families, and more than half of them have medium size pollen grains. Several pollen types were noted: inaperturate; monoporatae; triporate; tetraporate; pentaporate; periporate; tricolpate; hexacolpate; pericolpate; monosulcate; tricolporate; tetracolporate; pericolporate; heteroaperturate (pseudocolpus); spiraperturate; syncolpate; bisaccate; pollen grains in groups (tetrad, polyad). From these, tricolporate pollen is the most frequent (about half of the species), followed by tricolpate pollen. These two morphological characters of the pollen grain of some melliferous species present in Romania were used to determine the species that show similar traits for identification purposes when using the light microscope technique. Pollen shapes, symmetry, wall thickness and surface patterns can further help group the plant species and narrow the search, when pollen identification is carried out.

Key words*: botanical identification; light microscope; melliferous plants; pollen morphology.*

INTRODUCTION

The knowledge of the melliferous plants that are available in a given area is very important for apiculture. The microscopic analysis of the pollen of various plants visited by honeybees for their nectar and/or pollen is an established method that helps to determine the source of honey and the botanical origin of bee pollen.

Pollen is the male gametophyte in higher plants, a very unusual vegetative cell that carries the male gametes or their progenitor cells (Sarkar et al., 2017). The structure of the pollen grains is very complex, in order to protect the male genetic material during the transport from the anther to the stigma.

Pollen grains have different morphological characteristics and colour, depending on the plant species, therefore they have great significance in taxonomic and phylogenetic studies. The light microscope images of the hydrated pollen grains can provide a description of pollen size, polar and equatorial shapes, symmetry, type and number of apertures, aperture size and position, wall thickness and surface patterns, that allow a botanical identification at family, genus or species level.

Romania has a great diversity of melliferous plants (ICDA Bucureşti & Asociaţia Crescătorilor de Albine din România, 2007; Ion, 2012; Ion et al., 2018) and has very good potential for commercial beekeeping (Isopescu et al., 2017). Comprehensive studies regarding the pollen spectrum of honey and bee pollen in Romania can be found in the literature, for example Dobre et al. (2013) provided a palynological evaluation of selected honeys from different regions of Romania, while other studies include the description of the palynological spectrum of bee pollen samples (Mărgăoan et al., 2010; Bobiș, 2014; Spulber et al., 2017; Stanciu et al., 2016).

The current study presents a preliminary analysis of the pollen of some melliferous species in Romania according to the size of the pollen grain and aperture type (number, shape and position of the aperture/s). In addition, the appearance of the pollen-unit was also used (monad, tetrad or polyad). Such data can be enlarged by adding distinctive differences and giving a key for plant taxons with similar pollen, also by providing tailored lists of the floral sources for specific areas.

MATERIALS AND METHODS

In the present study 220 melliferous plant species that belong to 57 plant families were analysed (Table 1). The selection of the plant species was based on the descriptions of the melliferous flora of Romania by the ICDA Bucureşti & Asociaţia Crescătorilor de Albine din România (2007) and of Ion (2012), with only a few species added from other sources (Table 1). The morphological description of the pollen was also taken from different publications.

Table 1. Melliferous plants included in the present study and the references for the morphological descriptions of the pollen

Botanical family	Botanical name	Common name	References for the morphological descriptions of the pollen
Aceraceae	*Acer campestre*	Field maple	Hesse, Halbritter & Heigl (2020a)
	Acer platanoides	Norway maple	Sam, Auer & Halbritter (2020a)
	Acer pseudoplatanus	Sycamore	Sam, Auer & Halbritter (2020b)
	Acer tataricum	Tatar maple	Halbritter & Heigl (2020a)
Adoxaceae	*Sambucus nigra*	Elderberry	Halbritter, Diethart & Auer (2020)
	Viburnum opulus	Guelder rose	Halbritter & Heigl (2020jj)
Amaranthaceae	*Atriplex patula*	Spear saltbush	Sam & Heigl (2021)
	Beta vulgaris	Beet	Hecker (1988)
	Beta vulgaris var. altissima (*B. vulgaris saccharifera*)	Sugar beet	Angelini et al. (2014)
Amaryllidaceae	*Allium cepa*	Onion	Halbritter & Heigl (2020b)
	Allium ursinum	Wild garlic	Halbritter, Aktuna & Heigl (2020)
	Galanthus nivalis	Snowdrop	Halbritter, Heigl & Schneider (2020a)
Apiaceae	*Angelica archangelica* (Mârza şi Nicolaide, 1990)	Angelica	Halbritter (2016a)
	Carum carvi	Caraway	Heigl (2020b)
	Coriandrum sativum	Coriander	Auer (2020a)
	Daucus carota	Wild carrot	Halbritter & Heigl (2020k)
	Eryngium campestre	Field eryngo	Halbritter & Heigl (2020n)
	Foeniculum vulgare	Fennel	Heigl (2020e)
	Oenanthe aquatica	Fineleaf water dropwort	Auer (2020f)
	Pastinaca sativa (Mârza şi Nicolaide, 1990)	Parsnip	Heigl (2020o)
	Pimpinella anisum	Anise	Stebler (2021w)
	Pimpinella saxifrage	Burnet-saxifrage	Heigl (2020q)
	Sium latifolium	Great water-parsnip	Şerbănescu-Jitariu et al. (1994)
Apocynaceae	*Asclepias syriaca*	Milkweed	Tarnavschi et al. (1981)
	Periploca greaca	Periploca	Tarnavschi et al. (1981)
Araliaceae	*Hedera helix*	Ivy	Tarnavschi et al. (1981)
Asparagaceae	*Convallaria majalis*	Lily of the valley	Aktuna & Heigl (2020a)
	Scilla bifolia	Alpine squill	Aktuna & Heigl (2020b)
Asteraceae	*Bellis perennis*	Lawn daisy	Halbritter & Heigl (2020c)
	Carduus acanthoides	Plumeless thistle	Halbritter & Auer (2020a)
	Carduus nutans	Plumeless thistle	Halbritter & Heigl, 2020g
	Carthamus tinctorius	Safflower	Stebler (2021e)
	Centaurea cyanus	Cornflower	Halbritter & Bombosi (2016)
	Chrysanthemum leucanthemum (*Leucanthemum vulgare*)	Dog daisy	Halbritter & Heigl (2021b)
	Cichorium intybus	Common chicory	Halbritter & Heigl (2020h)
	Cirsium arvense	Field thistle	Halbritter (2016b)
	Cynara scolymus (C. cardunculus var. scolymus)	The globe artichoke	Heigl (2020d)
	Echinops sphaerocephalus	Great globe-thistle	Halbritter, Heigl & Svojtka (2020a)
	Helianthus annuus	Sunflower	Halbritter, Heigl & Svojtka (2020e)
	Helianthus tuberosus	Jerusalem artichoke	Halbritter, Heigl & Buchner (2020)
	Leontodon autumnalis (*Scorzoneroides autumnalis*)	Fall dandelion	Heigl (2020y)
	Senecio subalpinus (*Jacobaea subalpina*)	Mountain ragwort	Heigl (2021b)
	Solidago virgaurea	European goldenrod	Halbritter & Heigl (2020gg)
	Taraxacum officinale	Dandelion	Bombosi & Heigl (2021)
	Tragopogon pratensis	Meadow salsify	Heigl (2020bb)
	Tussilago farfara	Coltsfoot	Halbritter, Heigl & Schneider (2020e)
Berberidaceae	*Berberis vulgaris*	Barberry	Tarnavschi et al. (1981)
Betulaceae	*Alnus glutinosa*	Common alder	Tarnavschi et al. (1981); Halbritter, Sam, Weber & Auer (2020)
	Betula alba (Mârza şi Nicolaide, 1990)	Silver birch	Stebler (2021c)
	Corylus avellana	Common hazel	Halbritter, Diethart & Heigl (2020a)
Boraginaceae	*Borago officinalis* (*Echium amoenum*)	Starflower	Tarnavschi et al. (1981); Halbritter & Heigl (2020e)
	Echium vulgare	Blueweed	Tarnavschi et al. (1981)
	Phacelia tanacetifolia	Lacy phacelia	Halbritter & Kratschmer (2016a)

Botanical family	Botanical name	Common name	References for the morphological descriptions of the pollen
	Pulmonaria angustifolia	Narrow-leaved lungwort	Halbritter & Heigl (2021d)
	Pulmonaria officinalis	Lungwort	Halbritter, Bombosi, Weber & Heigl (2020)
	Symphytum officinale	Comfrey	Bombosi, Halbritter & Heigl (2020)
Brassicaceae	*Brassica napus*	Rapeseed	Diethart & Heigl (2020b)
	Brassica nigra	Black mustard	Bombosi (2016a)
	Brassica oleracea	Cabbage	Heigl (2020a)
	Brassica oleracea var. gongyloides	Kohlrabi	Stebler (2021d)
	Brassica rapa (*Brassica campestris*)	Oil seed rape	Tarnavschi et al. (1987)
	Raphanus sativus (*R. raphanistrum subsp. sativus*)	Radish	Stebler (2021y)
	Rorippa sylvestris	Creeping yellowcress	Auer (2020h)
	Sinapis alba (*Brassica alba*)		Bombosi & Heigl (2020c); Stebler (2021cc)
	Sinapis arvensis	Wild mustard	Heigl (2020z)
Campanulaceae	*Campanula carpatica*	Carpathian harebell	Tarnavschi et al. (1981)
	Phyteuma orbiculare	Round-headed rampion	Halbritter & Heigl (2020w)
Cannabaceae	*Humulus lupulus*		Halbritter & Heigl (2020q)
Cornaceae	*Cornus mas*	European cornel	Weber, Halbritter, Heigl & Schneider (2020); Stebler (2021g)
	Cornus sanguinea (Mârza și Nicolaide, 1990)	Common dogwood	Halbritter & Heigl (2020i)
Cucurbitaceae	*Citrullus colocynthis* (Mârza și Nicolaide, 1990)	Wild gourd	Stebler (2021f)
	Citrullus lanatus	Watermelon	Tarnavschi et al. (1987)
	Cucumis melo (Bura et al., 2005)	Melon	Halbritter (2016c)
	Cucumis sativus (Mârza și Nicolaide, 1990)	Cucumber	Halbritter & Heigl (2020j)
	Cucurbita maxima	Squash	Tarnavschi et al. (1981)
	Cucurbita pepo	Summer squash	Halbritter & Hesse (2016)
Elaeagnaceae	*Elaeagnus angustifolia*	Wild olive	Halbritter & Heigl (2020l)
	Hippophaë rhamnoides	Sea-buckthorn	Halbritter, Heigl & Svojtka (2020b)
Ericaceae	*Calluna vulgaris*	Common heather	Halbritter & Heigl (2020f)
	Vaccinium myrtillus (*V. oreophilum, Myrtillus niger*)	Bilberry	Halbritter & Heigl (2021e)
	Vaccinium vitis-idaea	Mountain cranberry	Halbritter, Heigl & Sonnleitner (2020)
Fabaceae	*Amorpha fruticosa*	False indigo-bush	Halbritter & Heigl (2021a); Stebler (2021b)
	Coronilla varia (*Securigera varia*)	Crownvetch	Halbritter, Heigl & Vladović (2021); Stebler (2021h)
	Galega officinalis	Galega	Heigl (2020h)
	Gleditsia triacanthos	Honey locust	Heigl (2020j); Stebler (2021k)
	Lotus corniculatus	Common bird's-foot trefoil	Halbritter & Heigl (2020s); Stebler (2021r)
	Medicago falcate	Yellow lucerne	Halbritter, Heigl & Svojtka (2021)
	Medicago lupulina	Hop clover	Heigl (2020k)
	Medicago sativa	Alfalfa, lucerne	Heigl (2020l)
	Melilotus albus	Honey clover, white melilot	Heigl (2020m)
	Melilotus officinalis	Yellow melilot	Halbritter, Auer & Svojtka (2020b)
	Onobrychis viciifolia (*O. sativa*)	Common sainfoin	Halbritter & Heigl (2020v)
	Robinia pseudacacia (*R. pseudoacacia*)	Black locust	Heigl (2020v); Stebler (2021aa)
	Sophora japonica (*Styphnolobium japonicum*)		Heigl (2020aa)
	Sophora jaubertii (*S. prodanii*)		Tarnavschi et al. (1990)
	Trifolium arvense	Hare's-foot clover	Tarnavschi et al. (1990)
	Trifolium campestre	Hop trefoil, field clover	Halbritter & Kratschmer (2016b)
	Trifolium fragiferum	The strawberry clover	Auer (2020i)
	Trifolium hybridum	Alsike clover	Heigl (2020cc)
	Trifolium pratense	Red clover	Halbritter & Auer (2020h); Stebler (2021ff)
	Trifolium repens	White clover	Halbritter, Heigl & Schneider (2020d)
	Vicia pannonica	Hungarian vetch	Halbritter & Auer (2021)
	Vicia sativa	Common vetch	Auer (2020k)
Fagaceae	*Castanea sativa*	Sweet chestnut	Halbritter, Sam, Heigl & Buchner (2020)
	Quercus robur (*Q. pedunculata*) (Dobre et al., 2013)	Common oak	Diethart, Bouchal & Heigl (2020)
Gentianaceae	*Gentiana punctata*	The spotted gentian	Halbritter & Svojtka (2016)
Geraniaceae	*Geranium sanguineum* (Stanciu et al. 2016)	Bloody cranesbill	Heigl (2020i)
Grossulariaceae	*Ribes aureum*	Golden currant	Halbritter (2016f)
	Ribes nigrum	Blackcurrant	Heigl (2020t)
	Ribes rubrum	Redcurrant	Heigl (2020u)
	Ribes uva-crispa (*R. grossularia*)	Gooseberry	Halbritter, Heigl & Schneider (2020c); Stebler (2021z)
Hypericaceae	*Hypericum perforatum*	St John's wort	Halbritter (2015)

Botanical family	Botanical name	Common name	References for the morphological descriptions of the pollen
Iridaceae	*Crocus olivieri*		Uzundzhalieva & Todorova (2012)
Lamiaceae	*Dracocephalum moldavica* (*D. moldavicum*)	Moldavian dragonhead	Heigl (2021a)
	Glechoma hederaceum	Ground-ivy, creeping charlie	Halbritter & Auer (2020b)
	Hyssopus officinalis	Hyssop	Halbritter & Ulrich (2016); Stebler (2021m)
	Lamium album	White dead-nettle	Bombosi & Auer (2020a); Stebler (2021p)
	Lamium purpureum	Red dead-nettle	Halbritter & Auer (2020d); Stebler (2021q)
	Lavandula angustifolia (*L. officinalis*)	Lavender	Halbritter, Weber & Heigl (2020a)
	Leonurus cardiaca	Motherwort	Auer (2020b)
	Lycopus europeus	Bugleweed	Halbritter, Ulrich & Auer (2020a)
	Marrubium vulgare	Common horehound	Auer (2020d)
	Melissa officinalis	Lemon balm	Ulrich & Auer (2020)
	Mentha aquatica (*M. hirsuta*)	Water mint	Ulrich (2016)
	Mentha longifolia (*M. spicata var. longifolia*, *M. sylvestris*, *M. tomentosa*, *M. incana*)	Horse mint	Halbritter, Ulrich & Heigl (2020a)
	Mentha pulegium	Squaw mint	Auer (2020e)
	Mentha × piperita	Peppermint	Stebler (2021v)
	Origanum marjorana (*Majorana hortensis, M. majorana*)	Marjoram	
	Prunella vulgaris		Halbritter & Heigl (2020bb)
	Salvia nemorosa	Wild sage	Ulrich, Heigl & Tweraser (2020a)
	Salvia officinalis	Sage	Halbritter & Heigl (2020ff)
	Salvia pratensis (*S. virgata*)	Meadow sage	Tweraser, Loos & Heigl (2020)
	Salvia verticillata	Lilac sage	Ulrich, Heigl & Tweraser (2020b)
	Satureja hortensis	Summer savory	Heigl (2020x)
	Scutellaria alpina	Alpine skullcap	Halbritter & Weis (2016)
	Stachys annua	Annual yellow woundwort	Halbritter, Ulrich & Auer (2020b)
	Stachys officinalis (*Betonica officinalis*) (Mârza și Nicolaide, 1990)	Common hedgenettle	Auer (2021b)
	Stachys palustris		Halbritter, Ulrich & Heigl (2020c)
	Teucrium montanum		Halbritter & Heigl (2020hh)
	Thymus serpyllum	Wild thyme	Stebler (2021ee)
Liliaceae	*Erythronium dens-canis*	Dogtooth violet	Halbritter (2016d)
	Gagea lutea	Yellow star-of-Bethlehem	Halbritter & Heigl (2020p)
Linaceae	*Linum usitatissimum*	Common flax	Auer (2021a)
Loranthaceae	*Loranthus europaeus* (Dobre et al., 2013)	European yellow mistletoe	
Lythraceae	*Lythrum salicaria*	Purple loosestrife	Halbritter, Weber & Heigl (2020b)
Malvaceae	*Gossypium hirsutum*	Mexican cotton	Stebler (2021l)
	Hibiscus trionum	Bladder hibiscus	Halbritter & Auer (2020c)
	Malva silvestris (Mârza și Nicolaide, 1990)	Common mallow	Halbritter & Heigl (2021c)
Oleaceae	*Fraxinus excelsior*	Ash	Diethart & Heigl (2020c)
	Fraxinus ornus	Manna ash	Halbritter, Diethart & Heigl (2020b)
	Ligustrum vulgare	Common privet	Halbritter & Heigl (2020r)
Onagraceae	*Chamaerion angustifolium* (*Chamaenerion angustifolium, Epilobium angustifolium*)	Fireweed	Halbritter & Heigl (2020m)
	Epilobium parviflorum	Smallflower hairy willowherb	Halbritter & Auer (2020g)
Papaveraceae	*Corydalis solida*		Heigl (2020c)
	Papaver rhoeas	Field poppy	Oberschneider & Heigl (2020c)
	Papaver somniferum	Breadseed poppy	Heigl (2020n)
Paulowniaceae	*Paulownia tomentosa*	Princess tree	Halbritter & Auer (2020e)
Phytolaccaceae	*Phytolacca americana* (*P. decandra*)	Dragonberries	Heigl (2020p)
Pinaceae	*Abies alba* (Antonie, 2020)	Silver fir	Wrońska-Pilarek et al. (2020)
	Picea abies (Antonie, 2020)	European spruce	Halbritter & Heigl (2020x)
	Pinus sylvestris (Mârza și Nicolaide, 1990)	Pine	Halbritter & Heigl (2020y)
Plantaginaceae	*Plantago lanceolata*	Narrowleaf plantain	Halbritter, Ulrich & Heigl (2020b)
	Veronica officinalis (Mârza și Nicolaide, 1990)	Common speedwell	Auer (2020j)
Poaceae	*Sorghum bicolor*	Sorghum, great millet	Stebler (2021dd)
	Zea mays	Corn	Diethart & Heigl (2021)
Polemoniaceae	*Polemonium caeruleum*	Jacob's-ladder	Svojtka, Heigl & Halbritter (2020)
Polygonaceae	*Fagopyrum esculentum* (*Polygonum fagopyrum*)	Common buckwheat	Halbritter, Heigl & Svojtka (2020d)
	Rumex acetosa	Common sorrel	Sam & Heigl (2020a)
Primulaceae	*Primula officinalis* (*P. veris*)	Cowslip primrose	Halbritter & Heigl (2020aa)
Ranunculaceae	*Clematis vitalba*	Traveller's joy	Oberschneider & Heigl (2020a)
	Delphinium consolida (*Consolida regalis*)	Field larkspur	Oberschneider & Heigl (2020b)
	Ranunculus sceleratus	Celery-leaved buttercup	Auer & Svojtka (2016)
Rhamnaceae	*Paliurus spina-christi*	Jerusalem thorn	Halbritter & Weber (2016)
	Rhamnus frangula (*Frangula alnus*)	Alder buckthorn	Heigl (2020g)
Rosaceae	*Alchemilla vulgaris* (*A. acutangula, Potentilla acutiloba*)	Lady's mantle	Șerbănescu-Jitariu et al. (1994); Reitsma (1966)
	Amygdalus communis (*Prunus dulcis, P. amygdalus*)	Almond	Halbritter (2016e)

Botanical family	Botanical name	Common name	References for the morphological descriptions of the pollen
	Cotoneaster horizontalis		Stebler (2021i)
	Crataegus monogyna	Common hawthorn	Bombosi & Heigl (2020a)
	Cydonia oblonga	Quince	Stebler (2021j)
	Filipendula ulmaria	Meadowsweet	Bombosi & Heigl (2020b)
	Fragaria × *ananassa*	Garden strawberry	Heigl (2020f)
	Fragaria moschata	Musk strawberry	Halbritter & Heigl (2020o)
	Malus domestica	Apple tree	Auer (2020c); Stebler (2021s)
	Malus sylvestris	European crab apple	Halbritter & Heigl (2020t); Stebler (2021t)
	Potentilla anserina (*Argentina anserina*)	Silverweed, silver cinquefoil	Auer (2020g); Stebler (2021x)
	Potentilla reptans	Creeping cinquefoil	Halbritter & Heigl (2020z)
	Prunus armeniaca (*Armeniaca vulgaris*)	Apricot	Heigl (2020r)
	Prunus avium	Sweet cherry	Halbritter & Heigl (2020cc)
	Prunus cerasus (*Cerasus vulgaris*)	Sour cherry	Şerbănescu-Jitariu et al. (1994)
	Prunus domestica	European plum	Halbritter & Heigl (2020dd)
	Prunus persica	The peach	Halbritter & Heigl (2020ee)
	Prunus spinosa	Blackthorn	Halbritter & Heigl (2020mm)
	Pyrus communis	Common pear	Heigl (2020s)
	Rosa canina	Dog rose	Bombosi & Auer (2020b)
	Rubus caesius (*R. caeruleus*)	European dewberry	Halbritter & Auer (2020f)
	Rubus hirtus (*R. fruticosus*)	An European blackberry species	Bombosi (2016b)
	Rubus idaeus	Raspberry	Heigl (2020w); Stebler (2021bb)
Salicaceae	*Populus alba*	Silver poplar	Diethart (2016)
	Populus nigra (Mârza și Nicolaide, 1990)	Black poplar	Halbritter, Sam & Heigl (2020)
	Salix alba	White willow	Diethart & Auer (2020)
	Salix caprea	Goat willow	Hesse, Halbritter & Heigl (2020b)
	Salix cinerea	Common sallow	Halbritter (2016g)
	Salix viminalis	Basket willow	Şerbănescu-Jitariu et al. (1994)
Santalaceae	*Viscum album*	Common mistletoe	Halbritter, Auer & Schneider (2020)
Sapindaceae	*Aesculus hippocastanum*	Horse chestnut	Stebler (2021a)
	Koelreuteria paniculata	Goldenrain tree	Stebler (2021o)
Scrophulariaceae	*Verbascum densiflorum*	Denseflower mullein	Halbritter (2016i)
Simaroubaceae	*Ailanthus altissima*	Ailanthus	Diethart & Heigl (2020a)
Solanaceae	*Lycium barbarum* (*L. halimifolium*)	Chinese wolfberry	Halbritter, Auer & Svojtka (2020a)
	Nicotiana tabacum	Cultivated tobacco	Halbritter, Heigl & Schneider (2020b)
Tamaricaceae	*Tamarix gallica* (Mârza și Nicolaide, 1990)	French tamarisk	Halbritter (2016h)
	Tamarix ramosissima (Mârza și Nicolaide, 1990)	Saltcedar	Şerbănescu-Jitariu et al. (1994)
Tiliaceae	*Tilia cordata*	Small-leaved linden	Halbritter & Heigl (2020ii)
	Tilia platyphyllos (*T. grandifolia*)	Large-leaved linden	Halbritter, Hesse & Heigl (2020)
	Tilia tomentosa (*T. argentea, T. alba*) (Vințan, 2015)	Silver linden	Sam & Heigl (2020b)
Ulmaceae	*Ulmus minor* (*U. campestre*, *U. glabra*)	Field elm	Halbritter, Diethart & Heigl (2020c)
Verbenaceae	*Verbena officinalis*	Common verbena	Schneider & Halbritter (2016); Stebler (2021gg)
Violaceae	*Viola odorata*	Sweet violet	Halbritter & Heigl (2020kk)
Vitaceae	*Vitis silvestris* (*V. sylvestris*)	Wild grape	Bucher & Kofler (2021)
	Vitis vinifera	Common grape vine	Halbritter & Heigl (2020ll)

RESULTS AND DISCUSSIONS

The analysis of pollen types, showed that inaperturate pollen have only the two species of poplar, from the list of studied species (Table 1).

Monoporate pollen have only the Poaceae species, all of them have the same pollen type and although there are some size differences (large size pollen have some cultivated Poaceae) the microscopic pollen identification does not allow an analysis of the species or genus (therefore this pollen is referred to Poaceae-type). Anemophilous Poaceae pollen is widespread, especially in summer in temperate climates.

In the triporate category there are 6 species: *Betula alba, Corylus avellana, Humulus lupulus, Cucumis melo, Cucumis sativus* and *Chamaerion angustifolium*. In this group, several species have distinctive characteristics: the two Betulaceae species have small (*Betula*) and medium (*Corylus*) pollen grains with protruding pores (onci), also the Onagraceae species *Chamaerion angustifolium* has a distinctive characteristic, the presence of viscin threads. The Cucurbitaceae species have large size pollen grains. *Humulus lupulus* has triporate, small size pollen grain.

Tetraporate pollen have the two Campanulaceae species - *Campanula carpatica*

and *Phyteuma orbiculare*, both have medium size pollen.

Pentaporate pollen have *Alnus glutinosa* and *Ulmus minor*. Although they have similar size, alder has distinctive protruding pores (onci), similar to other Betulaceae species.

Fourteen plants have periporate pollen, they are from 6 families: Amaranthaceae - *Atriplex patula, Beta vulgaris, B. vulgaris saccharifera*; Cucurbitaceae - *Cucurbita maxima, C. pepo*; Grossulariaceae - *Ribes aureum, R. nigrum, R. rubrum, R. grossularia*; Malvaceae - *Gossypium hirsutum, Hibiscus trionum, Malva silvestris*; Plantaginaceae - *Plantago lanceolata* and Polemoniaceae - *Polemonium caeruleum*. All these species have pantoporate pollen grains. The three Malvaceae species have very large pollen grains. Large and very large pollen grains have also the two *Cucurbita* species, while the rest of species have small or medium size pollen grains.

In the tricolpate category there are 41 species, only one of them has large size pollen grains (*Linum usitatissimum*), 27 species have medium size pollen grains and 12 species have small size pollen grains (Table 2).

Table 2. Melliferous species with tricolpate, small and medium size pollen grains

Small size	Medium size
Brassica oleracea var. gongyloides *Clematis vitalba* *Leonurus cardiaca* *Papaver rhoeas* *Raphanus sativus* *Rorippa sylvestris* *Salix alba, S. viminalis* *Scutellaria alpina* *Sophora jaubertii* *Tamarix gallica, T. ramosissima*	*Acer campestre, A. platanoides, A. pseudoplatanus* *Brassica napus, B. nigra, B. oleracea, B. rapa* *Delphinium consolida* *Fraxinus excelsior* *Lamium album, L. purpureum* *Loranthus europaeus* *Marrubium vulgare* *Onobrychis viciifolia* *Papaver somniferum* *Phytolacca americana* *Quercus robur* *Ranunculus sceleratus* *Sinapis alba, S. arvensis* *Stachys annua, S. officinalis, S. palustris* *Teucrium montanum* *Tilia platyphyllos* *Veronica officinalis* *Viscum album*

In the hexacolpate category there are 19 species, 18 of them have medium size pollen grains (Table 3), and only one species, *Mentha pulegium,* has small size pollen grains.

Pericolpate (>6 apertures) pollen has only one species - *Primula officinalis* - whose pollen is stephanocolpate and of medium size.

Table 3. Melliferous species with hexacolpate, medium size pollen grains

Botanical name
Corydalis solida
Dracocephalum moldavica
Glechoma hederaceum
Hyssopus officinalis
Lavandula angustifolia
Lycopus europeus
Melissa officinalis
Mentha aquatic, M. longifolia, M. x piperita
Origanum marjorana
Prunella vulgaris
Salvia nemorosa, S. officinalis, S. pratensis, S. verticillata
Satureja hortensis
Thymus serpyllum

Monosulcate pollen have some monocotiledonate plants from: Amaryllidaceae family - *Allium cepa*, *Allium ursinum*, *Galanthus nivalis*; Asparagaceae family - *Convallaria majalis*, *Scilla bifolia* and Liliaceae family - *Erythronium dens-canis*, *Gagea lutea*. These species have medium or large size pollen grains.

Based on the descriptions used in the current study, 108 species have tricolporate pollen grains of different size, ranging from very large - only one species - *Geranium sanguineum*, to small pollen grains (Table 4).

Tricolporate, heteroaperturate with pseudocolpus, pollen grains have *Phacelia tanacetifolia* and *Lythrum salicaria*. These two species have different pollen size*: Phacelia tanacetifolia* has small size pollen and *Lythrum salicaria* has medium size pollen grains.

Tetracolporate pollen grains of medium size have the two *Pulmonaria* species.

Two species have 8-10-colporate, stephanocolporate pollen grains that differ in size: *Borago officinalis* has medium size pollen grains and *Symphytum officinale* has small size pollen grain.

Spiraperturate pollen is found in only one species: *Berberis vulgaris* that has medium-large, spiraperturate pollen grain.

Similarly, only one species has syncolpate pollen. *Crocus olivieri* has large, syncolpate pollen.

Large or very large bisaccate pollen have the three Pinaceae species (*Abies alba, Picea abies, Pinus sylvestris*).

Although most pollen grains of the plants included in the present study occur as monad, pollen grains shed in tetrads are found in the three Ericaceae species (*Calluna vulgaris, Vaccinium myrtillus* and *Vaccinium vitis-idaea*)

and the Onagraceae specie *Epilobium parviflorum* (Table 5), while pollen grains shed in larger groups: polyads, with pollinarium made of two elongated pollinia have the two Apocynaceae species (*Asclepias syriaca* and *Periploca greaca*).

Table 5. Melliferous species with pollen grains shed in tetrads

Botanical name	Pollen sie and type
Calluna vulgaris	Medium size, triporate
Epilobium parviflorum	Large size, triporate
Vaccinium myrtillus	Medium size, tricolporate
Vaccinium vitis-idaea	Medium size, tricolporate

Table 4. Melliferous species with tricolporate pollen grains

Small size	Medium size		Large size
Aesculus hippocastanum	*Acer tataricum*	*Paliurus spina-christi*	*Carduus nutans*
Amorpha fruticosa	*Ailanthus altissima*	*Pastinaca sativa*	*Carthamus tinctorius*
Bellis perennis	*Alchemilla vulgaris*	*Pimpinella anisum, P. saxifraga*	*Citrullus colocynthis*
Carum carvi	*Amygdalus communis*	*Prunus armeniaca, P. avium, P.*	*Citrullus lanatus*
Castanea sativa	*Angelica archangelica*	*cerasus, P. domestica, P. persica,*	*Cornus sanguinea*
Cornus mas	*Carduus acanthoides*	*P. spinosa*	*Cynara scolymus*
Coronilla varia	*Centaurea cyanus*	*Pyrus communis*	*Echinops sphaerocephalus*
Echium vulgare	*Chrysanthemum leucanthemum*	*Robinia pseudacacia*	
Filipendula ulmaria	*Cichorium intybus*	*Rosa canina*	
Fragaria × ananassa	*Cirsium arvense*	*Rubus caesius, R. hirtus, R. idaeus*	
Fragaria moschata	*Coriandrum sativum*	*Sium latifolium*	
Fraxinus ornus	*Cotoneaster horizontalis*	*Tilia cordata, T. tomentosa*	
Galega officinalis	*Crataegus monogyna*	*Tragopogon pratensis*	
Hypericum perforatum	*Cydonia oblonga*	*Trifolium arvense, T. campestre, T.*	
Lotus corniculatus	*Daucus carota*	*fragiferum, T. pratense, T. repens*	
Medicago lupulina	*Elaeagnus angustifolia*	*Tussilago farfara*	
Melilotus albus	*Eryngium campestre*	*Verbascum densiflorum*	
Paulownia tomentosa	*Fagopyrum esculentum*	*Verbena officinalis*	
Potentilla anserina	*Foeniculum vulgare*	*Vicia pannonica*	
Potentilla reptans	*Gentiana punctata*	*Vicia sativa*	
Rhamnus frangula	*Gleditsia triacanthos*	*Viola odorata*	
Rumex acetosa	*Hedera helix*		
Salix caprea	*Helianthus annuus, H. tuberosus*		
Salix cinerea	*Hippophaë rhamnoides*		
Sambucus nigra	*Koelreuteria paniculata*		
Senecio subalpinus	*Leontodon autumnalis*		
Solidago virgaurea	*Ligustrum vulgare*		
Sophora japonica	*Lycium barbarum*		
Taraxacum officinale	*Malus domestica, M. sylvestris*		
Trifolium hybridum	*Medicago falcata, M. sativa*		
Viburnum opulus	*Melilotus officinalis*		
Vitis silvestris	*Nicotiana tabacum*		
Vitis vinifera	*Oenanthe aquatica*		

CONCLUSIONS

The aim of this study was to use two morphological characters in the pollen grain of some melliferous species present in Romania, to determine the species that show similar traits for identification purposes when using the light microscope technique.

Of the 218 species that have data regarding their size (no data was used for *Asclepias syriaca* and *Periploca greaca* that have pollen grains in polyads), 7 species have very large size pollen grains, 18 species have large size pollen grains, 138 species have medium size pollen grains and 55 have small size pollen grains.

The studied pollen grains differ in aperture type and the appearance of the pollen-unit. Few species in the list that was analysed are having pollen of the following types (the plant family they belong to is mentioned below in brackets): pericolpate (Primulaceae); spiraperturate (Berberidaceae); syncolpate (Iridaceae); inaperturate (Salicaceae); monoporatae (Poaceae); tetraporate (Campanulaceae); pentaporate (Betulaceae, Ulmaceae); tricolporate – heteroaperturate (Boraginaceae; Lythraceae); tetracolporate (Boraginaceae); pericolporate (Boraginaceae); bisaccate (Pinaceae); triporate (Betulaceae, Cannabaceae, Cucurbitaceae, Onagraceae); monosulcate (Amaryllidaceae, Asparagaceae, Liliaceae). Also, few species in this list have pollen grains in groups (the plant family they belong to is mentioned in brackets): tetrads (Ericaceae, Onagraceae); polyads (Apocynaceae).

Fourteen/nineteen species have periporate (Fam. Amaranthaceae, Fam. Cucurbitaceae, Fam. Grossulariaceae, Fam. Malvaceae, Fam. Plantaginaceae, Fam. Polemoniaceae),

hexacolpate pollen, respectively (Fam. Lamiaceae, Fam. Papaveraceae).

The most common pollen types are the tricolpate pollen, found in species from numerous plant families (Aceraceae, Brassicaceae, Fabaceae, Fagaceae, Lamiaceae, Linaceae, Loranthaceae, Oleaceae, Papaveraceae, Phytolaccaceae, Plantaginaceae, Ranunculaceae, Salicaceae, Santalaceae, Tamaricaceae, Tiliaceae) and the tricolporate pollen that is found in more than half of the species in this list, comprising a wide range of plant families (Aceraceae, Adoxaceae, Apiaceae, Araliaceae, Asteraceae, Boraginaceae, Cornaceae, Cucurbitaceae, Elaeagnaceae, Fabaceae, Fagaceae, Gentianaceae, Geraniaceae, Hypericaceae, Oleaceae, Paulowniaceae, Polygonaceae, Rhamnaceae, Rosaceae, Salicaceae, Sapindaceae, Scrophulariaceae, Simaroubaceae, Solanaceae, Tiliaceae, Verbenaceae, Violaceae, Vitaceae).

The development of a larger database that includes additional, distinctive morphological differences for the use of pollen analysis might prove useful as well as the knowledge of the distribution and ecology of the source plants.

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MICROBIAL LIPASES: OBTAINING, PURIFYING AND CHARACTERIZATION REVIEW

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Abstract

Microbial lipases present a real interest in the biotechnology field, due to their special properties, efficiency and easy obtaining. These enzymes are characterized by high rates of substrate conversion, versatility, resistance and wide applicability, being produced by many species of bacteria, fungi and yeasts. From a commercial point of view, due to their special abilities to efficiently hydrolyze fats or other esters, they are considered innovative, feasible technology because they can easily cover a complex and multi-branched spectrum of industrial applications.

Key words*: lipases, bacteria, yeasts, fungi.*

INTRODUCTION

Literature data report that a considerable range of over 3200 enzymes have been prepared isolated and purified, with varying degrees of purity. Such enzymes are present in all living organisms, in body fluids and secretions specific for different organs (stomach, pancreas, small intestine, kidneys), in plants and in different microorganisms, where the enzymatic content depends on the species and environmental conditions. The enzymes isolation methodologies are relatively easy due to the sources that have an abundance advantage, accessibility, with significantly reduced costs and time processing. Microorganisms as lipase sources, are grown rapidly, in large quantities, and the used culture media are economical (e.g. agricultural or food industry by-products or wastes).

According to the reported data, microbial enzymes account approximately 80% of the total enzymes produced today in the world. Some enzymes are of current interest, due to the ease of obtaining, their special properties, versatility (Singh and Mukhopadhyay, 2012), large-scale productivity (Chahinian et al., 2005), as microbial lipases case, presents a real industrial interest, with special implications in chemical, pharmaceutical, cosmetic or food applications (Vulfson, 1994).

By their origin, lipases can be classified into: microbial lipases; plant lipases; animal lipases (milk lipases; pancreatic lipases; lipoprotein lipase; hormone-sensitive lipases) (Pascoal et al., 2018). Rich sources of lipases are represented by bacteria, especially the genus *Chromobacterium, Pseudomonas* and *Staphylococcus*; by yeasts, *Candida lipolytica* (Ionita et al., 2001), fungi, especially *Aspergillus, Geotrichum, Rhizopus, Mucor, Penicillium* (Pascoal et al., 2018; Jurcoane et al., 2009).

An important criterion in the description of industrial applications is specificity, and from this point of view lipases can be classified as follows: (i) substrate-specific, (ii) regioselective and (iii) enantioselective (Sarmah et al., 2017).

Substrate-specific lipases can be used effectively in reactions, in which they act selectively on a specific substrate in a mixture of crude raw materials, facilitating the synthesis of the desired product, as demonstrated by the use of

lipases in biodiesel and diacylglycerol production of high purity. (Sarmah et al., 2017)

Regioselective lipases tend to lead the reaction in a favorable direction compared to other adverse reactions. Such a property of lipases is of extraordinary importance for the chemical and pharmaceutical industries, especially in the production of isomeric compounds which have an optimal function only in a specific configuration (Sarmah et al., 2017**)**.

Based on positional specificity (regiospecificity), lipases are divided into three groups: (i) nonspecific lipases, which have the ability to catalyze triglycerides to free fatty acids and glycerol (Kapoor and Gupta, 2012; Ribeiro et al., 2011); (ii) 1,3 specific lipases, capable of releasing fatty acids from positions 1 and 3 of triglycerides, but unable to hydrolyze the ester bonds in the secondary position; they can rapidly hydrolyze triglycerides into diglycerides (Kapoor and Gupta, 2012; Ribeiro et al., 2011); (iii) specific fat lipids with fatty acid selectivity, catalyzing the hydrolysis of long-chain fatty acid esters (Kapoor and Gupta, 2012; Ribeiro et al., 2011). Due to their diversity, wide range of actions and their independent production facilitated by genetic manipulation, microbial lipases are produced at industrial scale and represent the most common class of enzymes used in biotechnological applications (Dey et al., 2014; Lee et al., 2015; Priji et al., 2015; Ullah et al., 2015).

Some of the processes catalyzed by enatiospecific lipases include the transesterification of secondary alcohols in pharmaceuticals, the hydrolysis of menthol benzoate in cosmetics/food, and the hydrolysis of glycidic acid methyl ester in medical/health care products (Sarmah et al., 2017).

LIPASE-CATALYZED REACTIONS

Mainly used in bioprocess industries for the production of various significant products, such as biodiesel fertilizers, cosmetics, flavored foods. Although some applications are reported in ringopening polymerization reactions, generally lipase-catalyzed reactions are chiefly grouped in two categories, namely, hydrolysis and synthesis. Further synthesis reactions can be classified as esterification, aminolysis, interesterification, alcoholysis, and acidolysis. However, the interesterification, alcoholysis, and acidolysis reactions are together considered as transesterification reactions. (Sarmah et al., 2017). Triacylglycerol acyl hydrolases (E.C. 3.1.1.3) or lipases, belong to the family of serine hydrolases (Pascoal et al., 2018). These enzymes have multiple roles such as: fats and oils hydrolysis (Sarac et al., 2017); oils hydrolysis in the oil-water interface, with free fatty acids, diglycerides, monoglycerides and glycerol release (Jurcoane et al., 2009; Gopinath et al., 2013; Guldhe et al., 2015).

MICROBIAL LIPOLYTIC ENZYMES

Due to their diversity and versatility, lipases are of current industrial interest, ranking them on the 3rd place of the most used enzymes (Rios et al., 2018), after proteases and amylases (Javed et al., 2017). Of all lipase types, microbial lipases are preferred because of their high substrate specificity, higher stability and low production costs (Lee et al., 2015).

Microbial lipolytic enzymes differ in terms of pH range and optimum temperature activity, as well as in thermal stability duration (Table 1).

Table 1. Microbial lipases properties (Pascoal et al., 2018)

Producing microorganism	Optimum pH	pH stability	Optimal temp. (^{0}C)	Thermostability (^{0}C)
Candida sp.	6,0	5.0 - 7.5	40	45
Aspergillus niger	5.6	2.2 - 6.8	25	50
Rhizopus delemar	5.6	3.0 - 8.0	35	65
Geotrichum candidum	6.0	4.5 - 10.0	35	50
Penicillium roqueforte	8.0	-	37	50
Penicillium cyclopium	7.0	6.5 - 9.0	30	40
Achromobacter lipolyticum	7.0	-	37	99
Pseudomonas fragi	7.0 - 7.2	-	32	72

The microbial lipases are different and specific depending on the producing microorganism: type I lipases are obtained by *Pseudomonas aeruginosa, Candida lipolytica, Penicillium roqueforti*. Triglycerides have fast attack and the role of priority, namely the 1 or 3 position of fatty acids but have a low activity on mono- and diglycerides; type II lipases are produced by *Aspergillus oryzae, Rhizopus* sp. and hydrolyze a wide range of fats, natural oils and synthetic glycerides; type III lipases are produced by the fungus *Geotrichum candidum*, which possesses a unique but not absolute specificity to unsaturated fatty acids, regardless of the fatty acid position in glyceride structure (Fogarty and Kelly, 1990). The lipase action interface is environmentally dependent and manifests a "*closed*" or "*open*" conformation due to the presence of a "*lid*" type protection structure, with a hydrophilic and a hydrophobic area. The hydrophilic side realizes the contact with the aqueous environment, while the hydrophobic side is orientated within the core of the enzyme structure. In the absence of the specific substrate, represented by triglycerides, lipase has a "*closed*" conformation, and the hydrophobic side is exposed to the maximum. In the presence of the specific substrate, the lipases are receptive by making a signal to the active site, for hydrophobic side exposure, with "*open*" conformation enzyme form. This mechanism allows the enzyme to contact the preferred substrate, resulting in substrate catalysis (Lotti, 2007; Ugo et al., 2017). In the case of serine lipases, the active site is characterized by the triad serine-histidine-aspartic acid and it is essential for all reactions in which this enzyme is involved (Jaeger et al., 1999; Reetz, 2002). The mechanisms involved suppose a serine proton removal, a mechanism by which aspartate and histidine residues are required (Reetz, 2002; Brady et al., 1990). The hydroxyl group of serine reacts with the carbonyl carbon forming an intermediate substrate. The presence of an oxo-anionic spacer contributes to the load distribution stabilization and to reducing the minimum energy for intermediate substrate formation. The last step is deacylation, where the acyl group is transferred to enzyme and the catalytic center is regenerated (Brady et al., 1990; Pascoal et al., 2018).

OBTAINING MICROBIAL LIPASES OF INDUSTRIAL INTEREST

Microbial lipases are considered the most advantageous enzymes. They have wide applicability due to their special abilities, resistance and stability to the conditions imposed by the industrial environment. Bacterial strains with a high productivity degree which have been studied and evaluated so far, are part of Gram-positive bacteria (*Bacillus, Staphylococcus, Lactobacillus, Streptococcus, Micrococcus, Propionibacterium)*, Gram-negative bacteria (*Pseudomonas, Chromobacterium, Acinetobacter, Aeromonas*), fungi (*Rhizopus, Aspergillus, Penicillium, Mucor*), yeasts (*Candida, Rhodotorula, Pichia, Saccharomyces, Torulospora trichosporon)* or actinomycetes (*Streptomyces*) (Rohit et al., 2001).

SCREENING AND ISOLATION OF LIPASE-PRODUCING MICROORGANISMS

Lipase-producing microorganisms are present in various industrial habitats, in wastes, vegetable oil from processing companies, dairy factories, oil-contaminated soil, oilseeds, compost, coal quarters and thermal springs. A simple and safe method of detecting microbial lipase activity uses an agar culture medium with Tween 80 surfactant, following the opaque areas formation corresponding to lipases presence. Changes to this working technique include Tween surfactants, Cu^{2+} oils or salts (Cardenas et al., 2001), or Rhodamine B chromogenic substrates (Sharma et al., 2001) and are used in screening methodologies. Lipases are mainly produced by submerged culture (Ito et al., 2001), for which numerous studies have been carried out to optimize the culture media subjected to the influence of the reaction factors involved (substrate concentration, carbon and/or nitrogen sources, pH, temperature, dissolved oxygen concentration) (Elibol and Ozer, 2001). High yields of lipase production were also observed in vegetable oils, like olive, soybean, sunflower, sesame, cottonseed, corn and peanut oil, but the maximum production of lipase was recorded when using olive oil. For example, the

presence of 1% olive oil for *Bacillus* sp. culture, induces the production of lipolytic enzymes (Lee et al., 2015); for *Pseudomonas fluorescens S1K WI,* olive oil induced very good lipase biosynthesis yields, with 7395 U/mg specific activity at pH 8.5 (Lee et al., 1993); for *Penicillium expansum,* the presence of only 0.1% olive oil and a pH of 8.3 in the culture medium is favorable, and the yield of production and stability of the enzyme can be improved by the addition of Tween 20 and PX lubricant (Sharma et al., 2001). The thermophilic *Bacillus thermoleovorans ID-1,* isolated from Indonesia thermal springs, exhibited extracellular lipase activity with high growth rates on lipid substrates at high temperatures, using 1.5% olive oil as unique carbon source (Lee et al., 1999). Carbon sources such as fructose and palm oil are beneficial in lipase production, especially for thermostable lipases produced by *Rhodotorula glutinis. Bacillus Wai 28A 45*, in presence of tripalmitin, at 70°C, while *Bacillus A30-1* adds corn oil and olives (1%), or beef/palm oil for *Bacillus stearothermophilus L1* (Sharma et al., 2001). On the other hand, the addition of different oils (eg. rapeseed or maize) to the culture medium, leads to lipase yields stimulation of (e.g. for *Rhizopus oryzae*). An alkaline lipase with excellent ability to remove greasy stains in alkaline solution, is produced by *Pseudomonas alcaligenes M-1*, when culturing on citric acid and soybean oil substrate (Sharma et al., 2001). Fermentation with 2% castor oil is beneficial for *Pseudomonas aeruginosa KKA-5*, which produces lipases capable of hydrolyzing 90% of castor oil and is stable under alkaline conditions (pH 7-10) (Sharma et al., 2001).

The nitrogen source effect can be decisive in microbial lipases production. In the presence of 2-5% peptone at 7.2 pH *Penicillium citrinum, Pseudomonas* sp. *KW1-56* (Izumi et al., 1990, Sharma et al., 2001), lipase synthesis had high yields in culture medium. Similar effects were observed with corn extract (7% for *Rhizopus oryzae),* soybean meal (35% for *Acremonium structum*, 1% for *Pseudomonas alcaligenes F-111*). Thermostable lipases produced by thermophilic fungi as *Emericella rugulosa, Humicola* sp*., Thermomyces lanuginosus, Penicillium purpurogen* and *Chrysosporium sulfureum*, need yeast extract, and thermostable alkaline lipases produced by *Bacillus A 30-1* are obtained in good yields in the presence of 0.1% yeast extract and 1% ammonium chloride, at 60°C, optimum pH of 9.5. The presence of metal ions in the culture medium can beneficially influence the microbial lipases production: magnesium ions enhance biosynthesis in *Aspergillus niger, Pseudomonas pseudoalcaligenes F-111, Bacillus* sp*. A 30-1, Pseudomonas pseudoalcaligenes KKA-5,* those of iron and calcium in *Bacillus* sp*.,* those of magnesium, calcium, copper, cobalt for *Acinetobacter calcoaceticus, Bacillus* sp*. A 30-1.*

MICROBIAL LIPASES PURIFICATION

Most enzymes isolated from plant or animal tissues or microbial cultures, require purification steps, for removing the contaminants that can catalyze unwanted side reactions. Extracellular enzymes, like most microbial lipases, are secreted into the culture medium and the biomass is separated by centrifugation or filtration techniques. The resulting solution can be concentrated using ultrafiltration membranes, and the separation of different proteins is achieved by fractional precipitation. The solvation capacity of the different proteins by water can be modified by water-miscible organic solvents, in other to dissolve substances or by combining these methods, together with the change of temperature (Jurcoane et al., 2009). The main used techniques in lipase purification processes consist of: selective precipitation techniques using neutral salts; fractional precipitation techniques using water-miscible organic solvents such as ethanol or acetone; gel filtration; hydrophobic interaction and affinity chromatography (Jurcoane et al., 2009; Javed et al., 2018). Purification methodologies purpose is not only to isolate the enzymes from contaminants, but also to improve the activity, stability and shelf life of the enzymes (Javed et al, 2018). On the other hand, after the purification stages, structural and conformational studies can be carried out (Nadeem et al., 2015) following the kinetic and thermodynamic mechanism for substrate hydrolysis, the transesterification reaction and

the structure-function relationship (Javed et al., 2018). The lipase purification step is very important and necessary for product formulations with industrial or medicinal applications and uses. Purification is a key step, which is performed for enzyme specific function assessing. The cell-free culture medium is subjected to salts precipitation, up to column chromategraphy, depending on the proteins nature and the purification level desired. The variety of chromatographic techniques includes anion exchange, cations or exclusion chromategraphy. Data from the literature indicate that lipases have been purified with high yields (Javed et al., 2018).

Often ultrafiltration technique is applied for concentration and then ion exchange chromatography and gel filtration are performed.

Purification stages may vary depending on bacterial strains used in order to obtain lipases: generally, the first step is ammonium sulphate precipitation or solvent precipitation, followed by different chromatographic techniques using on different chromatographic materials (e.g. DEAE-Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-60), Sephadex G-75, G-100 gel filtration, DEAE-Sephadex A-50 column, Sephacryl S-200 gel filtration chromatography and isoelectric focusing (Sharma et al., 2001). For example, anion exchange chromatography (for *Bacillus* sp*., Aneurinibailus thermoaerophilus HZ*), Sephadex G-100 gel filtration (for *Pseudomonas aeruginosa BUP2* and *NB-1*), DEAE-cellulose purification (for *Geobacillus stearothermophilus AH22, Idiomarina* sp., *Bacillus* sp.), ultrafiltration (for *Bacillus* sp*., NS5, Pseudomonas aeruginosa BN-1 and CS-2*), affinity chromatography (for *Acinetobacter* sp. *XMZ-26, Geobacillus* sp.), hydrophobic interaction fast performance liquid chromatography (FPLC) (for *Acinetobacter calcoaceticus)* (Sharma et al., 2001).

MICROBIAL LIPASES CHARACTERIZATION

The molecular mass has been determined by electrophoretic techniques. Also, the optimal pH range, as well as temperatures, at which the enzymatic activity is expressed at its maximum level were determined. The molecular mass was determined in 19-92 kDa range, the lowest being recorded for *Bacillus stratosphericus* lipase, and the largest one for *Pseudomonas gessardii* lipases. The optimum temperature range at which the lipase activity was maximal was determined to be between 15-80°C, this specific temperature leads to a decrease in lipase activity. Thus, the lipases produced by *Acinetobacter* have an optimum temperature of only 15°C, while the vast majority of microbial lipases have an optimum enzyme activity at temperatures above 35°C, reaching even 60 or 80°C (*Cohnella* sp*., Idiomarina* sp*., Janibacter* sp. lipases) (Javed et al., 2018).

MICROBIAL LIPASES APPLICATIONS

From a commercial point of view, microbial lipases are of great interest, because they have a good fat hydrolyzing ability being considered a feasible technology, as they can cover a wide range of applications (pharmaceutical, food, cosmetics, biofuels) (Israel- Roming, 2014).

The detergent industry needs high activity lipases capable of acting on different type of fats, in harsh washing conditions (alkaline pH, high temperature, surfactants) (Guerrand, 2017).

In food industry, there are many possibilities of using lipases in edible oil production, bread making, diary products obtaining and egg processing (Guerrand, 2017; Panyachanakul et al., 2019).

In the biofuel industry, lipases are able to synthesize biodiesel by catalysing the trans-esterification reaction in media based on oily plant material or waste oils or fats (Melani et al., 2019).

Bioremediation of wastes, but also grease contaminated soils and waters, represents an opportunity for enzymes to show their capacity of acting for environmental preservation in a sustainable way. Another application of great interest in the field of environmental protection is the use of microbial lipases in accelerating the degradation of polyurethane and polyesters derived from renewable resources (Rohit et al., 2001).

A new and successful approach is represented by the use of immobilized microbial lipases on different substrates, due to the many advantages, such as the continuous, repeated and easy use of biocatalyst,, as well as the

increased stability of the enzyme (Yunus, 1995; Ivanov and Schneider, 1997; Pascoal et al., 2018).

CONCLUSIONS

Due to the large field of applications that address lipases, a continues work is done for obtaining these enzymes from novel sources or by optimised processes that lead to higher activity, more stability against environmental factors and lower production costs.
In the meantime, because of the involvement in different reactions and due to the relative substrate specificity, new potential uses are investigated.

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