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CORRESPONDENCE TO: Ms. Joanna Molga
Polish Journal of Food and Nutrition Sciences
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of Polish Academy of Sciences
ul. Tuwima 10, 10-747 Olsztyn, Poland
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REVIEW

ORIGINAL PAPERS

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W latach 2019–2020 kwartalnik naukowy ***Polish Journal of Food and Nutrition Sciences*** realizuje zadania z zakresu digitalizacji i upowszechniania publikacji naukowych, które ukazują się na jego łamach oraz utrzymania procedur zapewniających ich oryginalność, w ramach zadania nr 1 pt. ***Digitalizacja artykułów publikowanych w kwartalniku PJFNS w celu zapewnienia otwartego dostępu do nich przez sieć Internet*** oraz zadania nr 2 pt. ***Utrzymanie procedur zabezpieczających oryginalność artykułów naukowych publikowanych w kwartalniku PJFNS***. Oba zadania finansowane są na podstawie umowy nr 607/P-DUN/2019 ze środków Ministra Nauki i Szkolnictwa Wyższego przeznaczonych na działalność upowszechniającą naukę.

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Review article
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Lemongrass (*Cymbopogon citratus*) Essential Oil: Extraction, Composition, Bioactivity and Uses for Food Preservation – a Review

Ewa Majewska, Mariola Kozłowska, Eliza Gruczyńska-Sękowska, Dorota Kowalska, Katarzyna Tarnowska*

Department of Chemistry, Faculty of Food Sciences,
Warsaw University of Life Sciences, 159C Nowoursynowska str., 02–776 Warsaw, Poland

Key words: lemongrass, *Cymbopogon citratus*, essential oils, citral, antimicrobial activity, food preservation

Lemongrass essential oil comes from the lemongrass plant (*Cymbopogon citratus*), which grows mainly in tropical and subtropical parts of the world. The prefix 'lemon' indicates its typical lemon-like odour, which is caused mainly by the presence of citral. Citral is a combination of two stereoisomeric monoterpene aldehydes; the *trans* isomer geranial is in predominance to the *cis* isomer neral. Lemongrass essential oil has been used since ancient times in folk medicine as a remedy to improve circulation, stabilise menstrual cycles, promote digestion or increase immunity. It is also used to produce perfumes, flavours, detergents, and pharmaceuticals. The method found to be the most suitable for the extraction of lemongrass essential oil is steam distillation, since it allows obtaining the oil without altering product quality. The chemical composition of the essential oil of *C. citratus* varies according to the geographical origin, farming practices, plant age, photoperiod, harvest period, genetic differences, and extraction methods. The chemical constituents of the essential oil which have constantly been detected and determine its biological activity are aldehydes, hydrocarbon terpenes, alcohols, ketones, and esters. The lemongrass essential oil shows a wide spectrum of biological activities. High antibacterial and remarkable antifungal activities make the lemongrass oil a potential food preservative. This paper reviews recent information on extraction methods of lemongrass essential oil, its chemical composition depending on the origin of the plant, bioactivity of the oil constituents as well as potential application as a food preservative.

ABBREVIATIONS

ADI: acceptable daily intake; BHT: butylated hydroxytoluene; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: gallic acid equivalents; GC: gas chromatography; GC/MS: gas chromatography coupled with mass spectrometer; HD: hydrodistillation; MAHD: microwave-assisted hydrodistillation; MIC: minimum inhibitory concentration; and SFE: supercritical fluid extraction

INTRODUCTION

In recent years, the quality and safety of food has gained a great concern among consumers, who increasingly search for healthier, nutritious, and safer food products. This tendency is reflected in research on advanced technologies for better preservation of food during production, storage and transport. For this reason, new alternatives from natural sources have been investigated such as the use of essential oils. The essential oil from *Cymbopogon citratus* leaves is one of the essential oils extensively studied in terms of its application as a food preservative.

C. citratus – commonly known as lemongrass – belongs to the *Poaceae* family which counts more than 635 genera and 9000 species. This herb plant is widely distributed around the world. There are more than 140 cultivated species only for the *Cymbopogon*, 52 of them grow in Africa, 45 in India, 6 in Australia, 6 in South America, 4 in Europe (only in Montenegro), 2 in North America, and the others in South Asia. *Cymbopogon flexuosus* and *Cymbopogon citratus* represent the two major species vastly cultivated for their essential oils in different regions of the world. *C. citratus* is known by numerous international common names, such as West Indian lemon grass or lemon grass (English), citronelle or verveine des indes (French), hierba limon or zacate de limón (Spanish), xiang mao (Chinese), capim-cidrao or capim-santo (Portuguese), and locally there are identified more than 28 indigenous names from different countries of the world. The genus *Cymbopogon* has been reviewed comprehensively in several articles [Avoseh *et al.,* 2015; Haque *et al.,* 2018].

C. citratus is an aromatic, evergreen, clump-forming, perennial grass producing numerous stiff stems arising from a short rhizomatous rootstock, and growing around 1.5 m tall. It rarely produces flowers. The leaves are blue-green in colour, flat, erect, linear in shape and give off a characteristic lemon flavour when they are crushed. *C. citratus* is considered to have its origin in Malaysia, nowadays it is widely cultivated in the Central and South America and parts of Africa, Southeast Asia, and the Indian Ocean Islands, both on a commercial scale and in gardens – especially in the South-

* Corresponding Author: Tel.: +48 22 59 376 12; Fax: +48 22 59 37 635
E-mail: ewa_majewska@sggw.pl (E. Majewska)

east Asia [Skaria *et al.,* 2012]. Lemongrass is used as a food flavouring, and can be dried and powdered, or used fresh. It is commonly used in teas, soups, and curries, it may also be served with poultry, fish, beef, and seafood. Many studies have confirmed that infusion of lemongrass leaves and other parts is highly effective in combatting various stomach infections, prevents ulcers, stimulates digestion and excretion, and is beneficial for treating nausea, stomach aches, and constipation [Carbajal *et al.,* 1989; Leite *et al.,* 1986]. Therefore, in many countries lemongrass is used as a medicinal herb [Avoseh *et al.,* 2015]. In Egypt, a hot water extract of the dried leaves is taken orally as a diuretic and renal antispasmodic. In Indonesia and Malaysia, a hot water extract of the whole plant is administered orally to stimulate blood flow in the pelvic area and uterus. The Brazilians prepare a tea from the leaves of lemongrass and use it for its antispasmodic, anti-inflammatory, and analgesic effects. In Thailand, a decoction of the dried entire plant is taken orally as a remedy for stomach ache and a hot extract of dried roots is recommended for diabetes. Health benefits of lemongrass plant were reviewed comprehensively by Tapsell *et al*. [2006]. The biological activity of lemongrass is due to the presence of the essential oil and phenolic compounds including phenolic acids, flavonoids, and tannins [Olorunnisola *et al*., 2014; Roriz *et al.,* 2014; Tavares *et al*., 2015]. From the nutritional point of view, lemongrass is a rich source of essential vitamins, such as: vitamin A, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin C and folate, along with being a host of important minerals like calcium, potassium, phosphorous, magnesium, copper, iron, and zinc [USDA National Nutrient Data Base, 2019].

Commercially, *C. citratus* is cultivated mostly for its essential oil which is biosynthesised mainly in leaves of the plant [d'Avila *et al*., 2016]. Lemongrass essential oil is of a considerable commercial importance because it is used in the manufacture of fragrances, flavours, perfumery, cosmetics, detergents, and pharmaceuticals. According to the Food and Drug Administration [21 CFR § 182.20, 2018], lemongrass essential oil is generally recognized as safe (GRAS) and may be used as a food additive replacing the synthetic ones. As a culinary flavouring lemongrass essential oil is added to alcoholic and non-alcoholic beverages, frozen dairy desserts, candy baked foods, gelatins and puddings, meat and meat products as well as fats and oils. It improves the flavour of some fish, wines, and sauces. Biological research has shown that various chemical compounds of lemongrass essential oil exhibit antimicrobial [Bassolé *et al.,* 2011], antioxidant [Mansour *et al.,* 2015], antiparasitic [Kpoviesi *et al.,* 2014], insecticidal, and insect repellent activities [Brugger *et al*., 2019]. Owing to its antimicrobial and antioxidant properties, it can be exploited in food preservation as an alternative to synthetic compounds which are recently less acceptable by consumers [Ekpenyong & Akpan, 2017].

This review article provides information on recent advances in the extraction methods of lemongrass essential oil, its composition, biological activity as well as its potential application for food preservation. The literature was carefully viewed in search for *C. citratus* essential oil data only.

EXTRACTION OF LEMONGRASS ESSENTIAL OIL

Lemongrass essential oil may be extracted by many different methods like solvent extraction, steam distillation, hydrodistillation (HD), microwave-assisted hydrodistillation (MAHD), and supercritical fluid extraction (SFE) with CO_2. A number of studies have proved that the quality of essential oils depends mainly on its constituents, which is significantly influenced by the extraction techniques [Desai & Parikh, 2015; Schaneberg & Khan, 2002; Wu *et al.,* 2019]. Moreover, methods involving heating may induce thermal degradation or hydrolysis of fragile constituents.

In solvent extraction, a hydrocarbon solvent (usually *n*-hexane) is added to the plant material in order to dissolve the essential oil. After filtering the solution and concentrating by distillation, a substance containing resin (resinoid) or a combination of wax and essential oil remains. The method is quite efficient for lemongrass essential oil extraction [Schaneberg & Khan, 2002] and relatively simple but generally requires high volumes of the solvent and sometimes yields unsatisfactory reproducibility. After the extraction operation, the sample is concentrated by evaporation, during which volatiles may be reduced. Moreover, contamination of the essential oil with solvent residues may occur. The Soxhlet apparatus is sometimes used to solvent extraction of lemongrass essential oil [Alhassan e*t al.* 2018]. In Soxhlet extraction, plant material has a continuous contact with refluxing liquid phase, which results in increased extraction efficiency. When compared to the other conventional methods, the most significant drawback of Soxhlet extraction is long heating period at high temperature (usually close to the boiling point of the solvent) which may lead to thermal degradation of fragile compounds. Both solvent extraction by maceration and Soxhlet extraction require the correct choice of the solvent to obtain a good extraction yield as well as to prevent the loss of volatiles. In recent years, the lemongrass essential oil was successfully extracted from dry and fresh lemongrass leaves using solvent extraction by Suryawanshi *et al.* [2016] and Alhassan *et al*. [2018]. The oil yield obtained by Suryawanshi group was 1.85% and that achieved by Alhassan group was 4.5%. In both experiments, *n*-hexane was used as a solvent. In turn, Schaneberg & Khan [2002] reported that sonication-assisted *n*-hexane extraction allowed obtaining lemongrass essential oil with comparable contents of the main compounds to steam distillation.

Currently, the most popular method for the extraction of the lemongrass essential oil is steam distillation. It is mainly used for temperature-sensitive materials like oils, resins, hydrocarbons, *etc*. which are insoluble in water and may decompose at their boiling point. It is often used to extract other essential oils as well [Fernandes *et al.*, 2019]. In this method, steam passes through the dried or fresh plant material which softens the cells and enables the essential oil to escape in vapour form. The temperature of the steam should be high enough so that the oil could vapourize, but not so high that it damages the plant material or burns the essential oils. The released essential oil, together with steam molecules, is subsequently cooled in a condenser and collected. This process enables conducting extraction process below

the boiling point(s) of the individual component(s). Essential oils consist of chemical compounds which boiling points often exceed 200°C [Berk, 2013]. In the presence of steam, these substances may be extracted at a temperature close to 100°C, at atmospheric pressure. The basic advantage of steam distillation is simplicity, and low costs of the apparatus. The properties of the essential oil are not changed since its constituents never decompose. This technique can also be used under pressure. Lemongrass plant may be distilled fresh or after wilting. Wilting herbage before the distillation process decreases the moisture content and increases oil yield [Skaria *et al.,* 2012]. The yields of the essential oil obtained with this method reported in the literature differ substantially, ranging from 0.24% [Anggraeni *et al.,* 2018], 0.3% [Santin *et al.,* 2009], 0.6% [Boukhatem *et al.,* 2014a], to 0.71% [Kpoviessi *et al.,* 2014]. Steam distillation is still a leading preparative technique for the extraction of lemongrass oil. A newer methodology, such as subcritical carbon dioxide extraction, although promising but expensive, has not really threatened to surpass this simple type of distillation.

In the HD technique, the plant material is totally submerged in water, which is boiled by applying direct heat. Essential oil distills together with water molecules and then is separated after condensation. The method is relatively easy and does not involve high expenses associated with the setup. The process is very slow, which may result in the degradation of some compounds in hydrolysis or polymerisation reactions of sensitive constituents due to extended heat exposure. The yield depends on various parameters such as size and weight of raw material, the nature of the herb or volume of water. A typical HD of lemongrass is conducted in a Clevenger-type apparatus for 3 h, according to the method recommended by Guenther in 1950 [Guenther, 1950]. In recent reports, the yields of the essential oil obtained *via* HD range between 0.43% [Marongiu *et al*., 2006] and 1.80% [Desai & Parikh, 2015]. Ajayi *et al.* [2016] proved that essential oil extraction from lemongrass leaves by HD, using not only neutral but weakly acidic and basic medium, significantly affected essential oil composition. The total yield of the volatile fractions was 0.73% for HD, 0.64% for MAHD, 0.7% for acid-distillation, and 0.45% for base-distillation. The main component in the obtained essential oil was citral with the content of 72.6% for HD, 44.7% for MAHD, 30.07% for acid-distillation, and 78.61% for base-distillation. Significant differences in contents of major components (geranial, neral, myrcene) were observed as well. The low concentration of citral in the essential oil distilled in acid conditions was a result of chemical transformations of this compound that may take place in an acidic solution [Ajayi *et al.,* 2016]. Citral contains an aldehyde moiety which can react with hydroxyl group of other terpenoids in the presence of acid molecules forming acetals. Other reactions such as polymerisation, disproportionation, cyclisation, are also likely. Basic medium provides the best environment for the extraction of lemongrass essential oil with a high citral content.

MAHD works basically the same as traditional HD, but the heating of the solvent is achieved by using microwaves. The flask containing the solvent (generally water) and the parts of the plant are placed inside a microwave oven (usually operating at 2.45 GHz). The use of microwaves for the heating process accelerates the extraction of oil, which can be completed in a matter of minutes, which consequently shortens time needed to obtain similar quantities of extracts. This method is very attractive to use in laboratories as well as industry due to its effective heating, fast energy transfer, and being environmentally friendly. Its acceptance as a potential and powerful alternative to conventional extraction techniques of lemongrass oil has been verified [Desai & Parikh, 2015]. The chemical composition of the essential oils obtained with traditional HD and by MAHD does not differ substantially [Desai & Parikh, 2015]. Significant differences may, however, be observed in contents of major components (geranial, neral, myrcene) [Desai & Parikh, 2015]. The yields of MAHD and HD methods reported by Tran *et al*. [2019] were 0.35% and 0.2%, respectively, the MAHD method produced oil with 93.28% while HD 83.85% with citral content.

Over the last decades, the increasing demand for high quality natural products has contributed to the development of more environmentally friendly technique of essential oils isolation, namely supercritical fluid extraction (SFE). SFE can be regarded as an alternative to the solvent extraction of various compounds from natural solid matrices without any trace of the solvent [Haloui & Meniai, 2017]. The properties of the supercritical fluid can be modified by adjusting the critical temperature and pressure, which influences the density of the solvent allowing for selective extraction. This is particularly important for pharmaceutical and food systems. Carbon dioxide is used in most cases as the supercritical fluid due to its chemical and physical properties and to its relatively low critical temperature and pressure values (23–50°C and 8–12 MPa, respectively) [Carlson *et al.,* 2001]. As a natural constituent of many foods, it is a non-toxic, non-corrosive, non-flammable, and inexpensive gas. It has GRAS status [21 CFR § 184.1240, 2019]. Essential oils obtained by means of this technique are of a much better quality than those obtained using solvent extraction or steam distillation and HD [Al-Marzouqi *et al.,* 2007]. SFE with carbon dioxide can be performed at temperatures around 30°C, thereby preserving original oil composition and properties. However, the cost of the equipment is one of the main disadvantages of this method, which restrains its use for extremely sensitive industrial sectors where high quality and purity of the final products are of the outmost priority. When it comes to the extraction of lemongrass essential oil with supercritical carbon dioxide, only few reports can be found in literature. Usually, the experiments are aimed at the optimisation of temperature and pressure of the extraction process. Carlson *et al.* [2001] performed supercritical CO_2 extraction of fresh lemongrass leaves under different temperature and pressure conditions. They found that the composition of the essential oil significantly depended on changes in temperature and pressure parameters. The largest yields and extraction rates were determined under the conditions of 9 MPa and 23°C and 12 MPa and 40°C, *i.e.* 1.7% and 1.51%, respectively. The compounds recorded in larger quantities in the essential oil for all the pressure and temperature conditions applied were neral and geranial which contents ranged from 26.7% to 31.9% and from 44.6% to 53.0%, respectively. Marongiu *et al.*

[2006] also analysed the influence of pressure on the supercritical extraction of lemongrass oil. A series of experiments were carried out, for 360 min, at 50°C and at different pressures: 9, 10, 11 and 12 MPa. Extraction conditions were selected so as to maximise citral content in the extracted oil. The collected extracts composition was compared with that of the essential oil isolated with HD and steam distillation. They determined the highest yield (0.65%) and the highest citral content in the essential oil (68%) at 9 MPa, while HD allowed achieving process yield of 0.43% and citral content of 73%. The appearance of the extract was changing at higher solvent density, passing from a characteristic yellow oil to yellowish semi-solid products due to the extraction of high molecular mass compounds. Using the response surface methodology, Wu *et al.* [2019] predicted the optimum operational parameters of the supercritical CO_2 extraction of lemongrass (*C. citronella*) essential oil. Under these conditions (extraction time 120 min, pressure 25 MPa, temperature 35°C, CO_2 flow rate 18L/h), they conducted SFE extraction with the yield of 4.4% and compared their results with these obtained using HD. The main components of the essential oil extracted with SFE and HD were geranial (20.02% for SFE and 15.12% for HD), geraniol (10.22% for SFE and 25.45% for HD), and neral (15.11 for SFE and 11.15 for HD). Although, the experiment was carried out for different species of lemongrass, it was proved that the supercritical fluid did not alter the main effective components in lemongrass essential oil.

A greener approach to the extraction of lemongrass essential oil was proposed by Yen & Lin [2017] who utilised solar energy for the extraction process. The yield of the solar energy extraction (1.28%) was comparable with the yield of the essential oil obtained with HD (1.3 %) but the essential oil extracted by solar energy showed a higher antioxidant activity than that obtained with HD. This new technique may hold promise as a potent completely sustainable green extraction method in future.

COMPOSITION OF LEMONGRASS ESSENTIAL OIL

Lemongrass essential oil is mainly produced by leaves which contain about 1–2% of essential oil in a dry matter [Skaria *et al.,* 2012]. The essential oil is accumulated by plant in specific oil cells of the parenchyma tissues [Ganjewala & Luthra 2010]. The characteristic feature of lemongrass essential oil is a sherry colour, pungent taste, and lemon like odour. The chemical composition of the essential oil obtained from leaves of *C. citratus* has been extensively studied using GC and GC/MS methods. It varies according to the geographical origin, geobotanical conditions of the environment, farming practices, plant age, photoperiod, harvest period, genetic differences, and the extraction methods. Despite these differences, such compounds as hydrocarbon terpenes, alcohols, ketones, esters and mainly aldehydes have always been detected (Table 1).

The main component of lemongrass essential oil is citral. It is a mixture of two geometric isomers. The *E*-isomer is known as geranial or citral A and the *Z*-isomer is known as neral or citral B. Geranial (0.99–48.14%) dominates over neral (0–38.32%) as shown in Tables 1 and 2. The quality of lemongrass essential oil is generally evaluated by its citral content. According to literature data, lemongrass oil should contain at least 75% of citral, in order for *C. citratus* essential oil to be considered as a product of high quality [Barbosa *et al.*, 2008]. Quality and quantity of lemongrass essential oil are highly dependent on the time of plant harvest, because the composition and the content of the essential oil are strictly connected with the developmental stage of the whole plant, plant organs, and cells [Verma *et al.,* 2015]. The harvesting methods usually have little effect on the essential oil yield. The increase in citral content of lemongrass may be influenced by fertilizer application or rhizosphere fungi present in the soil [Shaikh *et al.,* 2019]. The proportion of young leaves to older leaves during harvesting determines the high citral content and subsequently the quality of the essential oil [Tajidin *et al.,* 2012].

Another chemical compound which is often present in the lemongrass essential oil is myrcene. Its percentage content greatly varies and ranges from 0.8% in the essential oil obtained in Egypt [Mansour *et al*., 2015] to 25% in oils obtained in Brazil [Farias *et al.,* 2019] and Nigeria [Kasali *et al.,* 2001]. Such great differences in myrcene content in the essential oil are strictly connected with the geographical region of the world (Table 2).

Besides citral and myrcene, geraniol, citronellal, and limonene are usually detected in amounts higher than 1% in some samples. The essential oil obtained with HD from Nigerian plants was found to be rich in limonene (7.90%), whereas the essential oil obtained with the same method from lemongrass from Kenya was lacking this terpene (Table 1). The amount of citronellal in the essential oil is very diverse: from 0.12% to 12.77% (Table 1). As for alcohols identified in the essential oils of *C. citratus*, geraniol was by far the most frequently found one (1.34% to 21.86%). Ester which was identified in significant amounts was geranyl acetate (0.24% to 3.42%). Terpenes which are most frequently detected, besides myrcene and limonene, are α-pinene (0.01–2.12%), β-pinene (0.3–6.00%), β-caryophyllene (0.1–2.46%), β-ocimene (0.15––0.3%), and α-copaene (1.13–1.29%). Other minor constituents found in the essential oil are: β-citronellol, globulol, linalool, hinesol, borneol, isopulegol, *trans*-verbenol, *cis*-verbenol, nerol, *trans*-farnesol, 3-methylcyclohexanol, γ-eucalyptol, carotol, α-cardinol, cubenol, terpinen-4-ol, citronellyl acetate, citronellyl isobutyrate, piperitone, (+)-carvotanacetone, lauraldehyde, 2-undecanone, α-cubebene, α-muurolene, γ-muurolene, and β-guaiene. Table 1 summarises the components found in the essential oil of *C. citratus* obtained with various extraction methods in different regions of the world.

The chemical composition of *C. citratus* essential oil is strictly correlated with the geographical origin. Analysis of the essential oils from *C. citratus* species from Brazil, Asia, West and Eastern Africa showed high contents of neral and geranial chemotypes (Table 2). In turn, the essential oil from *C. citratus* of African origin was found to contain a high amount of myrcene, whereas the essential oil from the Ethiopian lemongrass contained geraniol (40%) as its main compound, followed by citral (13%) and α-oxobisabolene (12%) [Ekpenyong *et al.,* 2014]. The major components identified in the Egyptian essential oil were geranial (20.90–40.72%),

neral (16.20–34.98%), geraniol (8.30%), and linalool (5.60%) [Hanaa *et al*., 2012; Mansour *et al.,* 2015], whereas considerable amounts of geranial (37.80%) and neral (33.60%) were found in Saudi Arabia sample [Mansour *et al*., 2015]. The essential oil obtained from the lemongrass cultivated in Nigeria was poor in citral (0.99%), but rich in geraniol (21.86%), limonene (7.90%), and camphene (7.89%) [Moutassem *et al.,* 2019]. Geranial and neral, limonene, citronellal, myrcene, and geraniol were identified as marker compounds in lemongrass essential oil [Schaneberg & Khan, 2002]. Marker compounds refer to chemical constituents within a medicinal plant that can be used to verify its potency or identity. As shown in Table 1, limonene, citronellal, and geraniol are not always found. Even geranial and neral are not detected in essential oils obtained from *C. citratus* of different chemotypes [Moutassem *et al.,* 2019].

According to the research conducted by Hanaa *et al.* [2012], the method of drying has a significant effect on the essential oil content of *C. citratus*. The highest essential oil content (2.45%) was determined by these authors in the oil extracted from lemongrass leaves dried in an oven at 45°C. Whereas, lemongrass leaves dried in sunshine and in shade yielded the oil at percentages of 2.10% and 2.12%, respectively, without significant difference between them. Geranial (31.53%, 37.24%, and 39.86%), neral (30.08%, 31.28%, and 34.52%), and myrcene (16.61%, 15.42%, and 14.49%) were the major components of the essential oils extracted from lemongrass leaves dried by sun, in an oven or in shade, respectively. Generally, drying methods of lemongrass leaves have no tangible effect on the composition of main essential oil components (neral and geranial) [Kumar *et al.,* 2015].

The essential oil composition of lemongrass differs significantly at various harvesting stages [Tajidin *et al.,* 2012]. When lemongrass was harvested 5.5 months after planting, 44 compounds representing 98.64% of the essential oil were found. In the essential oil obtained from lemongrass harvested 6.5 month after planting, there were only 15 chemical compounds, representing 98.62% of the essential oil. However, at 7.5 months after planting, 50 chemical compounds were analysed accounting for 97.2% of the essential oil. Only 13 compounds were always present at each maturity stage, out of which only 7 compounds had concentrations greater than 1% (neral, geranial, myrcene, 3-undecyne, nerol, geranyl acetate, and juniper camphor). Geranial content in lemongrass oil increased when the lemongrass was harvested at 5.5 (37.58%) to 6.5 (45.95%) months after planting. When the plants were harvested at 7.5 months after planting its content decreased slightly to 42.95%.

BIOLOGICAL ACTIVITY OF LEMONGRASS ESSENTIAL OIL

Bioactivity of lemongrass has been extensively studied, including especially its antibacterial [Naik *et al*., 2010; Falcao *et al*., 2012], antioxidant [Anggraeni *et al.,* 2018], antifungal [Sharma *et al*., 2017], insecticidal [Brugger *et al*., 2019], larvicidal [Soonwera *et al.,* 2016], and insect repellent activities [Diabate *et al*., 2019]. Biological activity of essential oils depends on their chemical composition, which can vary dramatically, even within the same species, and also on interactions among their structural components. Even minor essential oil constituents are indispensable and participate in biological functions of the oil due to the synergistic interactions.

Antimicrobial activity

Antibacterial properties of lemongrass essential oil depend on the presence of three main components: geranial, neral, and myrcene [Onawunmi *et al*., 1984]. Geranial and neral individually elicit antibacterial action on Gram-negative and Gram-positive organisms, while myrcene, does not show observable antibacterial activity on its own. However, myrcene was observed to generate enhanced bioactivity when it was mixed with either geranial or neral or both. Naik *et al*. [2010] investigated the effectiveness of lemongrass essential oil against the selected pathogenic bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus, Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* using agar diffusion method and broth dilution method. In general, Gram-positive bacteria were found to be more sensitive to the oil than the Gram-negative ones, which confirmed findings reported earlier by Onawunmi *et al.* [1984]. *P. aeruginosa* turned out to be resistant at all the concentrations of lemongrass oil including undiluted solution. *S. aureus* and *B. cereus* were more sensitive to lemongrass essential oil and were inhibited at 0.03% concentration. *B. subtilis* and *E. coli* were inhibited by the oil at a concentration of 0.06%, whereas *K. pneumoniae* at 0.25%. Moreover, the tested microorganisms, particularly the Gram-negative bacteria, turned out to be more susceptible to lemongrass oil than standard antibiotics. Similar results were reported by Premathilake *et al.* [2018] who investigated such pathogenic bacterial strains as *E. coli*, *B. cereus*, and *S. aureus*. Gram-positive bacterial strains were more sensitive to essential oil of *C citratus* at all of its concentrations than the Gram-negative strain *E. coli*. Lemongrass essential oil was found to significantly inhibit the growth of such pathogenic foodborne bacteria as *Listeria monocytogenes* and *Salmonella* Typhimurium [Reis-Teixeira *et al.,* 2019; Mith *et al.,* 2014]. Food spoilage bacteria (*Brochothrix thermosphacta* and *Pseudomonas fluorescens*) were also sensitive to lemongrass essential oil [Mith *et al.,* 2014]. The antibacterial activity of lemongrass essential oil is due to an interaction between the main oil constituents and the bacterial cell membrane. The lipophilic terpenes can modify the fluidity and permeability of the cell membrane or change intracellular pH and ATP concentrations, which results in cell rupture [Shi *et al.,* 2016]. Nikaido [2003] suggested that the higher resistance of Gram-negative bacteria to essential oils is due to the structure of the outer membrane which protects the bacterial cell against extrinsic chemical agents. The outer membrane is one of the main factors contributing to the resistance of Gram-negative bacteria to hydrophobic antibiotics. However, several studies indicate that lemongrass essential oil can successfully inhibit the growth of numerous multidrug-resistant Gram-negative bacterial strains such as *P. aeruginosa, E. coli, Enterobacter cloaceae, Morganella morganii, Proteus mirabilis* or *Burkholderia cepacia* [Bučková *et al.,* 2018; Vasireddy *et al.,* 2018].

TABLE 1. Composition (%) of selected *Cymbopogon citratus* essential oils obtained by different methods or commercially available in different regions of the world.

Compound	Commercial (South Africa)[a]	Hydrodistillation (China)[b]	SFE CO_2 (China)[b]	Hydrodistillation (Nigeria)[c]	Commercial (Greece)[d]	Hydrodistillation (Kenya)[e]
Aldehydes and ketones						
Camphor	nd	nd	nd	0.12	nd	nd
2-Caren-10-al	0.22	nd	nd	nd	nd	nd
(+)-Carvotanacetone	0.39	nd	nd	nd	nd	nd
trans-Chrysanthemal	0.14	nd	nd	nd	nd	nd
Citronellal	0.34	12.77	12.57	2.37	0.6	0.12
Geranial	48.14	15.12	20.02	0.99	38.5	39.53
Isogeranial	nd	nd	nd	nd	0.7	nd
Lauraldehyde	nd	0.30	0.13	nd	nd	nd
Neral	38.32	11.15	15.11	nd	28.7	33.31
Piperitone	0.99	nd	nd	nd	nd	nd
2-Undecanone	0.57	nd	nd	nd	nd	0.53
Sum	**89.11**	**39.34**	**47.83**	**3.48**	**68.5**	**73.49**
Alcohols						
Borneol	nd	nd	0.76	4.72	nd	nd
α-Cadinol	nd	0.25	1.47	0.37	nd	nd
Carotol	nd	0.37	nd	nd	nd	nd
β-Citronellol	0.09	nd	nd	2.83	0.1	0.34
Cubenol	nd	0.55	3.41	nd	nd	nd
Elemol	nd	nd	nd	1.37	0.1	
α-Eudesmol	nd	nd	nd	0.34	nd	nd
β-Eudesmol	nd	nd	nd	0.32	nd	nd
γ-Eudesmol				0.24		
γ-Eucalyptol	nd	nd	0.80	nd	0.6	nd
trans-Farnesol	nd	nd	1.34	nd	nd	nd
Geraniol	1.34	9.39	6.43	21.86	4.4	3.05
Globulol	nd	1.37	0.55	nd	nd	nd
Hinesol	nd	0.91	2.80	nd	nd	nd
Isoborneol	nd	nd	nd	nd	0.2	nd
Isopulegol	nd	0.35	0.18	nd	nd	nd
Linalool	0.98	nd	nd	0.70	1.6	1.29
3-Methylcyclohexanol	nd	0.71	2.01	nd	nd	nd
E-Methyleugenol	nd	nd	nd	0.19	nd	nd
Methylisoeugenol	nd	nd	nd	7.50	nd	nd
Nerol	nd	0.35	0.19	nd	0.1	0.34
Terpinen-4-ol	nd	nd	nd	2.21	0.1	nd
cis-Verbenol	nd	0.46	0.95	nd	nd	nd
trans-Verbenol	nd	nd	0.33	nd	nd	nd
Sum	**2.41**	**14.71**	**21.22**	**42.65**	**7.2**	**5.02**

Compound	Commercial (South Africa)[a]	Hydrodistillation (China)[b]	SFE CO_2 (China)[b]	Hydrodistillation (Nigeria)[c]	Commercial (Greece)[d]	Hydrodistillation (Kenya)[e]
Esters						
Bornyl acetate	nd	nd	nd	0.71	nd	nd
Citronellyl acetate	nd	nd	0.23	0.98	4.3	nd
Citronellyl isobutyrate	nd	0.61	1.27	nd	nd	nd
Geranyl acetate	0.82	2.24	0.65	3.42	nd	0.24
Geranyl butyrate	nd	nd	nd	nd	0.2	nd
Geranyl formate	0.17	nd	nd	nd	0.2	nd
Terpenyl acetate	nd	nd	nd	0.29	nd	nd
Sum	**0.99**	**2.85**	**2.15**	**5.40**	**4.7**	**0.24**
Hydrocarbon terpenes						
Bicyclogermacrene	nd	nd	nd	2.60	nd	nd
β-Bourbonene	nd	1.97	nd	nd	nd	nd
δ-Cadinene	nd	nd	nd	4.10	1.0	nd
Camphene	nd	nd	nd	7.89	1.0	nd
3-Carene	nd	0.46	nd	nd	nd	nd
β-Caryophyllene	0.17	2.46	0.10	1.86	1.4	0.15
α-Copaene	nd	1.29	1.13	nd	nd	nd
α-Cubebene	nd	0.94	0.46	nd	nd	nd
p-Cymene	nd	nd	nd	0.19	nd	nd
β-Elemene	nd	nd	nd	1.07	0.1	nd
Germacrene D	nd	nd	nd	0.97	0.1	nd
α-Guaiene	nd	0.25	0.45	nd	nd	nd
β-Guaiene	nd	0.14	nd	nd	nd	nd
α-Humulene	nd	nd	nd	0.30	nd	nd
D-Limonene	0.18	0.20	3.55	7.90	6.9	nd
8-Methyl-1-hendecene	nd	nd	0.18	nd	nd	nd
α-Muurolene	nd	0.26	0.30	4.10	nd	nd
γ-Muurolene	nd	1.67	0.20	0.17	nd	nd
Myrcene	nd	nd	nd	0.60	0.3	11.41
β-Ocimene	0.25	nd	0.15	nd	0.3	nd
Perillene	0.19	nd	nd	nd	nd	nd
β-Phellandrene	nd	nd	nd	0.32	nd	nd
α-Pinene	nd	0.71	0.67	2.12	0.6	0.01
β-Pinene	nd	4.31	6.00	nd	0.3	nd
Sabinene	nd	nd	nd	0.14	0.4	nd
Terpinolene	nd	nd	nd	0.50	0.1	nd
(6*E*, 10*E*)-2,6, 12,15-Tetramethylhexadeca--2,6,10,14-tetraen-8-yne	0.44	nd	nd	nd	nd	nd
1-Undecyne	0.51	nd	nd	nd	nd	nd
Sum	**1.74**	**14.66**	**13.19**	**34.83**	**12.5**	**11.57**

nd – not detected; SFE CO_2 – supercritical fluid extraction with CO_2; [a]Mbili *et al*. [2017]; [b]Wu *et al*. [2019]; [c]Moutassem *et al.* [2019]; [d]Hadjilouka *et al.* [2017]; [e]Matasyoh *et al.* [2011].

TABLE 2. Major component contents of *Cymbopogon citratus* essential oil (%) obtained from plants harvested in different regions of the world.

Country/ region	Compound			References
	Geranial	Neral	Myrcene	
Algeria	42.16	31.52	7.45	Boukhatem *et al.* [2014a]
Benin	39.5	35.5	nd	Kpoviessi *et al.* [2014]
Brazil	39.9	30.1	1.59	Andrade *et al.* [2009]
	31.89	24.62	25.37	Farias *et al.* [2019]
Burkina Faso	48.1	34.6	11.0	Bassolé *et al.* [2011]
Cameroon	37.7	21.2	2.5	Nguefack *et al.* [2012]
Egypt	40.72	34.98	15.69	Hanaa *et al.* [2012]
	20.9	16.2	0.8	Mansour *et al.* [2015]
Iran	39.16	30.95	3.57	Amini *et al.* [2016]
Ivory Coast	45.3	32.5	18.1	Sidibé *et al.* [2001]
Malaysia	37.58–45.95	29.44–31.13	3.18–7.68	Tajidin *et al.* [2012]
Mali	30.5	26.3	18.1	Sidibé *et al.* [2001]
Nigeria	33.7	26.5	25.3	Kasali *et al.* [2001]
Saudi Arabia	37.8	33.6	8.4	Mansour *et al.* [2015]
Sri Lanka	35.97	26.5	nd	Premathilake *et al.* [2018]
Zambia	39.0	29.4	18.0	Chisowa *et al.* [1998]

nd – not detected.

The antimicrobial activity of lemongrass essential oil is usually higher against fungi than bacteria. Premathilake *et al.* [2018] found that lemongrass essential oil exhibited fungitoxic activity against *Colletotricum truncatum*, *Fusarium spp.*, *Penicillium spp*., and *Crysosporium spp*. All four different concentrations of the essential oil used in this experiment elicited 100% inhibition of *Fusarium spp., Penicillium spp.,* and *Crysosporium spp. C. truncatum* was also inhibited by the lemongrass essential oil but higher concentrations of the oil were necessary. The essential oil of *C. citratus* was also investigated towards *Candida albicans*, *Candida parapsilosis, Candida tropicalis, Candida glabrata,* and *Candida krusei* [da Silva *et al.,* 2008]. All those strains proved to be sensitive to *C. citratus* essential oil, which indicates new perspectives in the potential application of lemongrass oil in typical *Candida* infections. Tzortzakis & Economakis [2007] reported a broad antifungal activity of essential oil of *C. citratus* against such food pathogens as *Aspergillus niger*, *Colletotrichum coccodes*, *Botrytis cinerea, Cladosporium herbarum,* and *Rhizopus stolonifer*. Amini *et al.* [2016] demonstrated that lemongrass oil effectively controlled mycelium growth of three *Phytophthora* species: *P. capsici*, *P. drechsleri,* and *P. melonis*. The *Phytophora* genus is a plant-damaging mold which causes enormous economic losses in crops as well as environmental destruction in natural ecosystems. Helal *et al.* [2006] investigated the antifungal activity of essential oil of *C. citratus* against *Aspergillus niger* ML2-strain. Lemongrass oil produces a fungi toxic effect against postharvest pathogens of the *Aspergillus* genus: *A. flavus, A. parasiticus,* and *A. clavatus* [Matasyoh *et al.,* 2011; Bozik *et al.,* 2017]. Five mycotoxigenic species isolated from maize samples, including *A. flavus, A. parasiticus, A. ochraceus, A. niger,* and *A. fumigatus*, were also found to be sensitive to lemongrass essential oil [Matasyoh *et al.,* 2011]. The highest activity of the oil was observed against *A. niger* with the minimum inhibitory concentration of 15 mg/mL and the highest resistance was observed from *A. flavus* with an MIC of 118 mg/mL. Sharma *et al.,* [2017] recorded an inhibitory effect of lemongrass essential oil against a pathogenic strain of *Fusarium oxysporum*. The genus *Fusarium* is infamous for infecting plants, leading to economic losses in agriculture and is known to produce mycotoxins in cereal crops. Lemongrass essential oil showed complete inhibition of spore germination at a concentration of 250 ppm, IC_{50} value was found to be 0.98 ppm.

According to Boukhatem *et al*. [2014b], lemongrass oil showed a potent antimicrobial activity against Gram-positive bacteria and exhibited the strongest antifungal effect against *Candida albicans* and *C. parapsilosis*. MIC varied from 0.019 to 1.25 mg/mL for Gram-positive bacteria and yeasts, indicating *S. aureus*, *S. epidermidis,* and *C. albicans* as the most susceptible strains. Moreover, the diameter of inhibition zone (DIZ) increased with an increasing essential oil concentration. Considerably superior antimicrobial activity was observed in the vapour phase. The DIZ resulting from the exposure to essential oil vapour was significantly larger than that from the same volume in the liquid phase. The DIZ

varied from 22 to 90 mm for *Candida* strains. Essential oil in the vapour phase may be an effective antimicrobial system and has advantages over the oil in the liquid phase. These results [Boukhatem *et al.,* 2014b] imply that lemongrass oil could be useful for the development of novel types of natural preservatives for food control.

The high antifungal activity of lemongrass essential oil is attributed to the presence of two isomers of citral [Leite *et al.*, 2014]. According to Harris [2002], citral seems to interact mostly with the cell wall of fungi. Such interaction affects its construction, inhibiting its synthesis, which results in cell death. Earlier literature suggested that the fungitoxic activity of citral stems from its capability to form a charge transfer complex with fungal cell tryptophan, leading to fungi death [Kurita *et al*., 1981]. In recent studies, the antifungal activity of citral was evaluated against *Geotrichum citri-aurantii* [Zhou *et al.,* 2014]. The experiment revealed that citral significantly inhibited mycelial growth. Antifungal properties of citral were attributed to cell membrane destruction and to the subsequent leakage of cellular constituents. The inhibiting activity of lemongrass essential oil may also stem from the synergistic effect of individual minor or major compounds [Nguefack *et al.,* 2012]. Further studies are needed to completely describe its effects especially if it is going to be used in the future as a component of new antifungals. The other major component of lemongrass essential oil – myrcene – is not known to exhibit any antifungal activity. Components with hydroxyl moiety have been known to be active but with differing specificity and levels of activity, which is related not only to the functional group present but also to hydrogen bonding parameters [Skaltsa *et al*., 2003]. The mechanism of action of geraniol, one of the main alcohols found in lemongrass essential oil, probably does not involve complexation with ergosterol or inhibiting the fungal cell wall synthesis [Leite *et al*., 2015]. Whereas, Pereira *et al*. [2015] suggested that the antifungal activity of geraniol and citronellol, *i.e.* two monoterpene alcohols, against *Trichophyton rubrum* involves ergosterol biosynthesis inhibition. In general, high lipophilic nature and low molecular weight of terpenes/terpenoids, that are irreplaceable constituents of lemongrass oil, determine its high antifungal activity which probably involves disrupting the cell membrane, causing cell death or inhibiting the sporulation and germination of fungi. Therefore, many *in vitro* tests have proved terpenes/terpenoids to show much lower antimicrobial activity when used as singular compounds compared to the whole essential oil [Lu *et al.,* 2013].

Antioxidant activity

The antioxidant activity of *C. citratus* essential oil is due to the synergistic effect of all its constituents [Guimarães *et al.,* 2011]. However, recent reports indicate that the main terpenoid component of the essential oil – citral – shows antioxidant activity as well, which is a result of co-oxidation with the target substrate and cross-termination of the oxidative chain [Baschieri *et al.,* 2017]. The antioxidant properties of lemongrass essential oil have been studied by many researchers, though there are some discrepancies between the results, probably due to the different geographical origin of the plant, different extraction methods, and methodology used to evaluate its antioxidant activity. The antioxidant activity assessment requires a combination of various methods because there are significant differences in sample preparation (solvent, temperature *etc*.), selection of end-points, and even the expression of results, hence the comparison between values reported by different scientists may be problematic. A single method will provide only a primary knowledge about antioxidant properties, whereas a combination of methods will produce more detailed information about the antioxidant properties of the sample.

Lawrence *et al*. [2015] thoroughly checked the antioxidant activity of lemongrass leaves oil from India using four different methods: free radical scavenging activity (DPPH method), reducing power assay, nitric oxide scavenging method, and β-carotene bleaching assay. Inhibition constant IC_{50} values recorded for DPPH and NO scavenging method were 0.5 mg/mL and 2.0 mg/mL, respectively. The reducing activity was also significant. The results proved that lemongrass essential oil is effective in scavenging free radical and has the potential to be a powerful antioxidant. Another study was done by Mansour *et al.* [2015] in which higher antioxidant activity was demonstrated for Egyptian lemongrass essential oil with IC_{50} 1.0 mg/mL in comparison to the Saudi Arabian lemongrass volatile oil with IC_{50} 6.9 mg/mL. The strong $DPPH^{\bullet}$ scavenging ability of the Egyptian oil was attributed to its unsaturated alcohols and phenolic compounds such as linalool (5.6%), geraniol (8.3%), terpin-4-ol (2.1%), and eugenol (0.4%). On the other hand, Anggraeni *et al.* [2018] reported a low antioxidant activity of the Indonesian lemongrass essential oil when compared to ascorbic acid and commercial lemongrass oil used as a reference material. Similarly, Viuda-Martos *et al*. [2010] reported a low radical-scavenging capacity of lemongrass oil using a stable DPPH radical with IC_{50} 199.63 mg/mL, whereas the ferric reducing antioxidant capacity (FRAC) of the essential oil was high. The same research group found lemongrass essential oil to be highly effective in chelating iron(II) ions, better than ascorbic acid and BHT. Viuda-Martos *et al.* [2010] also demonstrated that lemongrass EO might show a pro-oxidant activity, which was determined with the Rancimat method.

Various other scientists have also worked on the antioxidant activity of *C. citratus* essential oil. Guimarães *et al.* [2011] reported a low antioxidant activity of Brazilian lemongrass essential oil using the methodology that measures the scavenging of a stable DPPH radical; however, when analysed using the methodology that employs the β-carotene/linoleic acid emulsion system, the oil showed significant antioxidant activities. Using the Folin-Ciocalteau method, Mirghani *et al.* [2012] demonstrated the highest phenolics content in the essential oil extracted from lemongrass stalk, *i.e.* 2100.8 mg GAE/L. The high antioxidant activity of lemongrass stalk oil was also confirmed with the DPPH scavenging assay. The results obtained by Hartartie *et al.* [2019] indicate that the antioxidant activity of lemongrass essential oil depends on the method of distillation and on the part of the plant used. The antioxidant activity measured with DPPH free radical scavenging assay showed that steam-distilled lemongrass stalk oil had the highest antioxidant activity (72.724%), while water-distillated oil from the whole plant of lemongrass had

a lower antioxidant activity reaching up to 70.113%. The antioxidant activity of an essential oil made of leaves using the steam distillation method was higher (60.808%) than that obtained by water distillation (57.331%).

The antioxidant properties of lemongrass essential oils are attributed to the presence of terpenoid and phenolic compounds which may demonstrate their redox properties employing different possible mechanisms, like: hydrogen donation, free radical scavenging activity, transition metal chelating activity, and/or singlet oxygen quenching capacity [Viuda-Martos *et al.,* 2010]. In literature, the antioxidant properties of lemongrass essential oil are attributed to the mixture of different chemical compounds of the oil, because even minor constituents may influence and modulate the activity of the whole oil.

The antioxidant properties of *C. citratus* essential oil might be worth of consideration in terms of essential oil incorporation into formulations of nutraceuticals or/and functional food. Antioxidants can scavenge free radicals and retard lipid oxidation in food products, which is the main cause of food quality deterioration.

Anti-inflammatory properties

Traditionally, lemongrass is also known for analgesic and anti-inflammatory properties, however only few studies have been carried out to substantiate these effects of lemongrass oil. Only recently, a few research groups have undertaken studies to investigate anti-inflammatory properties of lemongrass essential oil and its major constituent – citral. Boukhatem *et al.* [2014a] used carrageenan-induced mouse paw oedema to determine the anti-inflammatory effect of Algerian lemongrass oil. The experiment has proved that lemongrass essential oil is able to considerably inhibit inflammation processes. The degree of oedema inhibition after 90 min of the oral intake turned out to be 82.75% for the dose of 10 mg/kg, while only a little higher degree of oedema inhibition (86.2%) was observed for a higher oil dose (100 mg/kg). The anti-inflammatory activity of the lemongrass oil was compared with the standard drug diclofenac which showed similar oedema inhibition (86.2%) to the lemongrass oil when used in a dose of 50 mg/kg. The detailed mechanism of the anti-inflammatory effect of the lemongrass essential oil remains unexplained. However, it has been indicated that several components of lemongrass oil may cause partial inhibition of the release of inflammation mediator molecules. The main plant constituents, particularly aldehyde monoterpenes (geranial and neral), have already been reported to be able to control inflammatory processes [Perez *et al.,* 2011].

LEMONGRASS ESSENTIAL OIL USES FOR FOOD PRESERVATION

Essential oils are considered as natural substituents of chemical preservatives, since their use in food is regarded as safe and natural. Food products containing essential oils are accepted by consumers and also meet the demands for "green" processing. However, the practical application of essential oils is limited due to their characteristic flavour, possible interactions with food components and structure, which results in reduction of their efficacy [Amany *et al.,* 2010]. Among the various essential oils, the oil of *C. citratus* has been recognised most promising for uses as a food preservative since it exhibits a variety of fundamental and novel bioactivities. Owing to their GRAS status, essential oils are not harmful and are more widely accepted by consumers than the "synthetic" agents.

Currently, the focus of several research laboratories is to investigate the application of lemongrass oil especially in meat preservation. In the meat industry, a number of chemical additives have already been used, but the consumers' growing concern about the safety of food products has contributed to the shift of the interests from synthetic compounds to natural ones. The antimicrobial and antioxidant activity of lemongrass oil can restrain foodborne pathogens and spoilage organisms against their development and subsequent deterioration of meat products. The advantages of such a treatment of meat are that lemongrass essential oil may be useful in extending shelf-life of meat products, preserving meat quality, preventing economic loss, and providing the consumer with meat products containing more acceptable natural additives.

Amany *et al.* [2010] studied the antioxidant and antimicrobial effectiveness of lemongrass oil at various concentrations on the quality of refrigerated fresh minced beef (4°C). The results were compared with the bioactivity of garlic oil and thyme oil, which are widely renowned for their antimicrobial properties against spoilage flora in meat products. Lemongrass oil at a concentration of 1.5% showed a significant effectiveness in decreasing aerobic plate count, coliform count, *Enteriobacteriaceae* count, and *Staphylococci* count. The antimicrobial activity of garlic and thyme oils was not as strong as that of the lemongrass oil. The decreased content of total volatile nitrogen, which determines the degree of meat deterioration during storage, indicated that all oils successfully inhibited the spoilage of minced meat at a comparable level. In the same experiment, lemongrass oil was found to be the most effective in preventing auto-oxidation of meat lipids and bacteriological and/or oxidative rancidity, which was estimated by the evaluation of decrease in thiobarbituric acid values. Different degradation reactions, involving lipid oxidation, influence the sensory profile of meat products. All tested oils proved to enhance the sensory properties of all the refrigerated beef samples to the similar extent. The greatest enhancement was observed for the samples containing 1.5% of lemongrass oil. In general, the best results in refrigerated minced beef preservation were demonstrated by lemongrass oil.

In a recent study, Zaki *et al.* [2018] evaluated the effect of using lemongrass essential oil in the formulation of camel burgers on changes in their physical, chemical, microbiological, and sensory properties during cold storage (4°C). Physical properties (percentage of cooking loss, percentage of reduction in diameter) were improved for camel burgers containing 1% of lemongrass oil. The total volatile nitrogen and thiobarbituric acid values significantly decreased for samples with lemongrass oil when compared to the control ones. The burgers formulated with addition of 1% of lemongrass oil were found to show the lowest total bacterial count, and were given the highest scores for taste, aroma, colour,

texture, and acceptability in the sensory analysis. Lemongrass essential oil antimicrobial properties may support storage stability of meat when combined with other essential oil. The combination of lemongrass and ginger essential oil successfully extended the shelf life of chicken meat [Hartanti *et al.,* 2018].

The antimicrobial potential of lemongrass essential oil was also tested in reducing the microbial population of cream-filled baked goods [Vazirian *et al*., 2012]. Five main food-borne pathogens including: *Bacillus cereus, Candida albicans, Escherichia coli, Salmonella typhimurium*, and *Staphylococcus aureus*, were added manually to cream-filled cakes together with 1 μL/mL of the lemongrass essential oil. After 72 h of storage, no observable microflora was detected in the baked cakes, except for *S. aureus* in the case of which lemongrass essential oil proved ineffective.

The antimicrobial effectiveness of lemongrass oil against *Salmonella enterica* serovar Newport on organic leafy vegetables such as romaine and iceberg lettuce as well as mature and baby spinach was investigated by Moore-Neibel *et al*. [2012]. Samples of leaves inoculated with *Salmonella* Neport were dipped in a lemongrass oil solution for 1 to 2 min and then stored for 1 to 3 days. Significant inhibition of *S.* Neport growth on fresh leafy greens was observed. The antibacterial activity of lemongrass oil depended on oil concentration and storage time. The highest reduction was observed (according to the activity order) on baby spinach, mature spinach, and romaine lettuce. The differences in the effectiveness of lemongrass oil among species of leafy green vegetables may be attributed to the surface morphology, chemical composition, and nutrient content of leaves. However, little differences were reported for the samples stored under refrigeration and abuse temperatures.

At present, research laboratories have focused on investigating the antimicrobial activity of lemongrass essential oil in the vapour phase. Such analyses were undertaken by, *e.g*., Mani-López *et al.* [2018] who evaluated the fungicidal properties of lemongrass oil vapours on the growth of *Penicillium expansum* inoculated on bread. The growth of *P. expansum* was inhibited for 21 days at 20°C with 750 μL of oil/L air. The increasing concentration of the oil resulted in greater inhibition. No significant differences were observed in the sensory analysis for the samples exposed and not exposed to lemongrass oil vapours. Lemongrass essential oil vapours were also reported to control anthracnose disease development on papaya without affecting its natural ripening process [Ali *et al.,* 2015]. A number of studies have demonstrated that the antifungal and antimicrobial activities of essential oils in the vapour phase are more effective than in the liquid phase. These have been extensively reviewed by Reyes-Jurado *et al.* [2019].

Lemongrass oil may also play a vital role in the inhibition of several fundamental postharvest pathogens like *Botrytis cinerea, Colletotrichum coccodes, Cladosporium herbarum*, and *Rhizopus stolonifer* that are important for food preservation [Mbili *et al*., 2017; Tzortzakis & Economaki, 2007]. Lemongrass essential oil as well as powdered plant have been recorded to prevent storage deterioration and aflatoxin contamination of melon seeds infected by *Aspergillus flavus, A. niger, A. tamarii*, and *Penicilium citrinum* [Bankole *et al.,* 2005]. The chemical composition of melon seeds was not affected by lemongrass essential oil and the effects were nearly similar to a commercial fungicide iprodione.

The antifungal activity of essential oil of *C. citratus* grown in Iran was investigated for controlling three species of *Phytophthora*, including *P. capsici, P. drechsleri*, and *P. melonis*, on pepper, cucumber, and melon under *in vitro* and greenhouse conditions, respectively [Amini *et al*., 2016]. Essential oil of *C. citratus* inhibited *in vitro* the growth of *P. capsici* (91.9%), *P. drechsleri* (91.2%), and *P. melonis* (94.6%) and under greenhouse conditions the growth of *P. capsici* (60.5%), *P. drechsleri* (47.4%), and *P. melonis* (55.3%), which affords new perspectives for its application as an alternative to synthetic compounds for integrated control of diseases caused by *Phytophthora* species in crop, vegetable, and ornamental plants after suitable clinical trials.

It was also reported that lemongrass oil increases the shelf life of different fruits like guava [Murmu & Mishra, 2018], strawberries [Kahramanoglu, 2019], table grapes [Sonker *et al.,* 2014], apples [Frankova *et al.,* 2016; Jo *et al.,* 2014] as well as fruit juice [Tyagi *et al.*, 2014]. It was also found effective in preserving processed foods like cheese [Cui *et al.,* 2016]. A recent report has indicated that lemongrass essential oil may influence lactic cultures in dairy products [Farias *et al*., 2019], which may curb its applicability in this sector of food industry. The application of *C. citratus* essential oil in food preservation was comprehensively reviewed by Ekpenyong & Akpan [2017].

Although numerous studies have indicated that lemongrass essential oil may serve as a potential food preservative, little is known about its toxic effects. Some reports suggest that its high levels in food products may have an adverse impact on the gustatory and olfactory systems [Smith *et al.,* 2005]. On the other hand, lemongrass essential oil used in small concentrations is considered safe for human consumption [Sinha *et al*., 2014]. *In vitro* studies have shown that the antimicrobial activity of lemongrass essential oil is predominantly between 0.2 and 10 μl/mL. In food products the concentration of the essential oil is much higher (25 – 100-fold) in order to achieve the comparable antimicrobial activity. The ADI value recommended by the European Union for geraniol is 0.5 mg/kg body weight/ day, hence food products contain excessive doses of the essential oil [Commission Implementing Regulation (EU) No 570/2013]. In many countries, the application of essential oils is not regulated whatsoever. Moreover, inappropriate and random uses of the lemongrass essential oil may result in health problems triggered by genetic damages, carcinogenic effects, and mutations [Sousa *et al.,* 2010]. Thus, further studies on the toxicity of lemongrass essential oil and the comprehensive safety evaluation are needed.

CONCLUSION

There is a growing interest in the application of the essential oil of *C. citratus* in the food system. The oil contains many phytoconstituents such as terpenes, terpenoids, esters, and phenolic compounds, which are responsible for different biological activities, primarily antibacterial and antifungal

ones. Many studies have confirmed that lemongrass essential oil may serve as a natural meat preservative offering protection against various microorganisms, increasing shelf-life of the product, and ensuring its quality. Before industrial application in the meat industry, further research is necessary to explore the efficiency of suitable concentrations of the oil. Additionally, the use of lemongrass oil opens new perspectives to the management of storage fungi, which not only deteriorate the quality of food but also may cause food-borne diseases. In general, lemongrass essential oil is a promising plant product for preserving stored foodstuffs replacing synthetic additives which are associated with various adverse human health effects. Its application in stored food products may be a good solution in remote rural areas which still have no possibility to use modern storage system.

CONFLICT OF INTERESTS

The authors declare they do not have any conflicts of interest.

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Original article
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Changes in the Composition of Aroma and Phenolic Compounds Induced by Different Enological Practices of Croatian White Wine

Marina Tomašević[1], Klemen Lisjak[2], Andreja Vanzo[2], Helena Baša Česnik[2], Leo Gracin[3], Natka Ćurko[1], Karin Kovačević Ganić[1]*

[1]Department of Food Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia
[2]Department of Fruit Growing, Viticulture and Oenology, Agricultural Institute of Slovenia, Hacquetova ulica 17, 1000 Ljubljana, Slovenia
[3]Department of Marine Studies, University of Split University Department of Marine Studies, Ruđera Boškovića 37, 21000 Split, Croatia

Key words: thiol precursors, pre-fermentative maceration, yeast species, antioxidants, Pošip, aroma compounds

The aim of this research was to investigate the effects of pre-fermentative maceration, *Saccharomyces* and non-*Saccharomyces* yeasts during alcoholic fermentation, and antioxidant additions (sulfur dioxide and glutathione) at bottling on the compositions of aroma and phenolic compounds of white wine Pošip (*Vitis vinifera* L.). Additionally, for the first time the insight in volatile thiol precursors in Pošip grape was given, wherein higher concentrations of glutathionylated thiol precursors in comparison to cysteinylated ones were determined. Regarding the applied practices, significant differences among produced wines were established: pre-fermentative maceration resulted in a decrease of 3-sulfanylhexyl acetate (3SHA) and a slight increase of C6 compounds; indigenous yeasts produced higher concentrations of terpenes and esters, while sequential fermentation with *Torulaspora delbrueckii* also influenced higher concentrations of esters. Most abundant phenolic compound was caffeic acid, except for wine produced by indigenous yeasts where *trans*-caftaric acid was predominant. Finally, combination of higher SO_2 and glutathione resulted in higher concentrations of thiols.

INTRODUCTION

The composition of wine and, consequently, its overall quality depends on numerous factors and their interactions. During grape ripening, significant changes occur in the chemical composition of grapes where, primarily, increase of sugar levels along with changes in phenolic and aroma compound profiles take place. Among them, significant increase of volatile thiol precursors, namely 3-*S*-cysteinylhexan-1-ol (Cys-3SH), 3-*S*-glutathionylhexan-1-ol (Glut-3SH), 4-*S*-cysteinyl-4-methylpentan-2-one (Cys-4MSP), and 4-*S*-glutathionyl-4-methylpentan-2-one (Glut-4MSP) also occur during this vine-growing phase [Jeffery, 2016; Roland *et al.*, 2011b]. Recent investigations suggested the presence of new thiol precursors such as cysteinyl-glycine S-conjugate (CysGly) and γ-glutamyl-cysteine S-conjugate (γGluCys) [Bonnaffoux *et al.*, 2017, 2018], as well as *S*-3-(hexanal)-glutathione (Glut-3SH-Al) and its bisulfite (Glut-3SH-SO_3) [Thibon *et al.*, 2016]. Generally, these precursors are odorless compounds that undergo enzymatic cleavage during alcoholic fermentation which result in volatile thiols release. Liberated compounds: 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), 3-sulfanylhexyl acetate (3SHA), are very desirable and important varietal aroma compounds, especially for Sauvignon blanc wines, since they contribute to the enticing boxwood, grapefruit, and passionfruit nuances [Tominaga *et al.*, 1998]. However, it is demonstrated that only a small portion (less than 5%) of thiol precursors are cleaved [Roland *et al.*, 2011b].

Despite the certain concentrations in grapes, transfer of the aroma compounds and their precursors to the must and, subsequently, in wine during wine production is strongly affected by the applied process techniques. For example, in early stage of winemaking, the application of maceration could significantly affect their extraction into grape juice and could lead to modifications in resulting wine. It is known that application of maceration technique could result in improved quality and stability of white wines, due to the increased extraction of aroma compounds and precursors, such as volatile thiols precursors [Olejar *et al.*, 2015], as well as phenolic compounds [Di Lecce *et al.*, 2013]. However, it is necessary to conduct this process under strictly controlled conditions, in order to reduce excessive phenolics extraction and consequently to reduce browning of white wines. Furthermore, release of volatile thiols by yeasts during alcoholic fermentation, as well as

* Corresponding Author: Tel.: +385 1 4605 034;
E-mail: mtomasevic1@pbf.hr (M. Tomašević)

formation of the new aroma compounds as a result of yeast metabolism, significantly affect the final aroma of wine, where the selected yeast strain could have detrimental effect [Belda *et al.*, 2017; Renault *et al.*, 2016; Sadoudi *et al.*, 2012; Zott *et al.*, 2011]. So far, the use of *Saccharomyces cerevisiae* as the most important yeast in winemaking, has been extensively studied in order to understand its role for wine properties [Swiegers *et al.*, 2005]. Recently, non-*Saccharomyces* yeast were arisen as innovative tools for industrial wine production as it has been demonstrated that these yeasts could improve aromatic complexity and distinction of the wines [Azzolini *et al.*, 2012; Loira *et al.*, 2014; Renault *et al.*, 2016]. Several studies showed the advantages of *Torulaspora delbrueckii* in winemaking, such as low production of acetic acid, contribution to the overall aroma profile through the higher esters and volatile thiols production, as well as decreasing the perception of vegetal flavor [Azzolini *et al.*, 2012; Renault *et al.*, 2015, 2016]. Finally, it is well-known that the overall wine aroma depends on the physicochemical reactions that occur during aging, where the oxidation reactions play a significant role and result in a loss of fresh and fruity character of wines. Lately, the importance of antioxidants additions during bottling is increasingly emphasized in a view of longer protection of wine, where the addition of glutathione is particularly highlighted due to its protective effect against aroma loss and browning in white wines [Kritzinger *et al.*, 2013].

The Pošip cultivar is a native grape variety of *Vitis vinifera* L. grown in Croatia southern vine-growing region, primarily on the island of Korčula and, economically, it is the most important white wine variety in this region. Recently, it has provoked the great interest of the winemakers due to its fruity, citrus aroma that resembles the scent often described as 'Sauvignon aroma', presumably due to the presence of volatile thiols, compounds that will be evaluated in this research. The aim of this study was to evaluate the effect of different enological practices on the compositions of aroma and phenolic compounds of produced Pošip wines: the pre-fermentative maceration technique, indigenous yeast strains fermentation, *T. delbrueckii* sequential fermentation and commercial *S. cerevisiae* fermentation, along with antioxidant additions (sulfur dioxide and glutathione) at the bottling. Apart from that, the content of thiol precursors in Pošip grapes will be evaluated for the first time ever.

MATERIALS AND METHODS

Chemicals

Deionized water was produced by a Millipore Milli Q system (Bedford, MA, USA). Ethanol was HPLC grade and purchased from J.T. Baker (Deventer, The Netherlands); sodium chloride p.a., ethyl acetate p.a., hydrochloric acid (37% v/v) and sodium sulfate anhydrous were purchased from Carlo Erba (Val de Reuil, Spain); sodium acetate trihydrate from Gram-mol (Zagreb, Croatia); acetic acid from Alkaloid (Skopje, Macedonia); liquid nitrogen from Messer Croatia (Zagreb, Croatia). Methanol HPLC grade, glutathione reduced, *N*-acetyl-cysteine, 2-aminoethanol, *o*-phthalaldehyde, cysteine hydrochloride hydrate, dichloromethane, *p*-hydroxymercurybenzoate (*p*-HMB), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), Dowex exchange resin, formic acid and sulfuric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethylendiaminotetraacetic acid disodium salt dehydrate (EDTA) p.a. was purchased from Kemika (Zagreb, Croatia); sodium tetraborate decahydrate p.a. and sodium acetate p.a. from Merck (Darmstadt, Germany). The aroma reference standards, with the highest available purity (minimum of 98%), were purchased from Merck, Sigma Aldrich, Fluka (Seelze, Germany) and SAFC (St. Louis, MO, USA) except of 4-methyl-4-sulfanylpentan-2-one (4MSP) and 3-sulfanylhexyl acetate (3SHA) which were purchased from Oxford Chemicals (Hartlepool, UK), as well as 3-sulfanylhexanol (3SH) and 4-methoxy-2-methyl-2-sulfanylbutane which were purchased from Penta Manufacturing Company (Livingston, MONT, USA). Deuterated [$^2H_2$]-3-sulfanylhexyl acetate (d3SHA) and [$^2H_2$]-3-sulfanylhexan-1-ol (d3SH) standards were obtained from University of Auckland, New Zealand. Cysteinylated and glutathionylated thiol precursor standards, together with deuterium labeled analogues (Cys-4MSP-d6 and Glut-4MSP-d10) were synthesized according to the procedures described in details by Vanzo *et al.* [2017]. Chemicals, used for precursor synthesis were analytical or higher grade purity and obtained from Sigma Aldrich, as well as hydroxycinnamic acids reference standards and GRP.

Grapes sampling

Grape samples were collected from the Croatia southern vine-growing region, microlocation Čara, island Korčula. This region is classified in C_3 viticultural climatic zone, as well as in C II wine-growing zone of the European Union, with yearly insolation up to 2700 h and annual precipitation of around 730–1050 mm. The grapes were sampled at their technological maturity; the grape harvest and wine production were carried out the same day (end of August 2015). The physicochemical characteristics of harvested grapes were: reducing sugars 206.5±3.8 g/L; total acidity 5.3±0.1 g/L with pH 3.4±0.1. The berry sampling was conducted in a random way, where three replicates of 100 berries were picked from three different plots of 25 randomly selected vines within the vineyard. Herein, berries were picked from top, the center, and the tip of selected clusters, both exposed to the sun and in shade. Immediately after picking, grapes were rapidly frozen in the liquid nitrogen and transferred to the laboratory in dry ice and stored at -80°C prior to analysis.

Wine production

The grapes were hand-harvested at technological maturity (physicochemical characteristic previously noted), placed in plastic boxes, transported to winery and immediately processed. Winemaking was carried out in Krajančić winery (island Korčula, Croatia). After the grapes were destemmed and crushed, vinification was carried out in four distinct processes, where the first process represents the control wine (wine PK): destemmed and crushed grapes were pressed under inert atmosphere of nitrogen (N_2) with the addition of SO_2 at 50 mg/L, addition of 25 mL/hL of clarifying agent (Hydroclar 30, Enartis, San Martino, Italy) and flotated with nitrogen as the flotation agent. Clarified must was transferred to a stainless steel tank and inoculated with *Saccharomyces cerevisiae* Lalvin

TABLE 1. Physicochemical properties of produced wines.

	Wine PK	Wine MK	Wine MT	Wine MI
Alcohol (%, v/v)	13.1±0.1^{bc}	13.0±0.1^{c}	13.5±0.2^{ab}	13.6±0.2^{a}
Total acidity (g of tartaric acid equivalents/L)	5.3±0.1^{b}	5.7±0.2^{ab}	5.5±0.1^{ab}	5.9±0.2^{a}
Volatile acidity (g of acetic acid equivalents/L)	0.5±0.0^{b}	0.4±0.0^{c}	0.4±0.0^{bc}	0.6±0.1^{a}
pH	3.6±0.0^{ab}	3.6±0.0^{a}	3.5±0.0^{b}	3.6±0.0^{ab}
Reducing sugars (g/L)	2.1±0.1^{b}	3.0±0.1^{a}	2.6±0.2^{a}	2.8±0.2^{a}
Total extract (g/L)	21.0±0.9^{b}	23.8±1.1^{ab}	23.2±1.2^{ab}	23.9±1.1^{a}
Malic acid (g/L)	2.2±0.3^{a}	2.6±0.3^{a}	1.9±0.3^{a}	2.5±0.3^{a}
Lactic acid (g/L)	nd	nd	nd	nd

Concentrations expressed as mean ± standard deviation (n=3). Abbreviations: P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; nd – not detected. Means with different superscript letters in the same row differ significantly ($p<0.05$).

EC-1118 yeasts (25 g/hL, Lallemand, Montreal, Canada) and kept with fermentation temperature below 18°C. Further processes consisted of destemming and crushing and sulfuring (at 50 mg/L), followed by pre-fermentative maceration for 12 h at temperatures below 10°C, pressing under inert atmosphere (N_2) at the same pressing program as in the case of wine PK, addition of 25 mL/hL of a clarifying agent (Hydroclar 30), and flotation with nitrogen. After flotation, clarified must was divided into three stainless steel tanks of 1000 L volume and each tank was subjected to fermentation with different yeasts: (i) first tank (wine MK), was inoculated with 25 g/hL *Saccharomyces cerevisiae* Lalvin EC-1118 yeasts, same as control, PK wine; (ii) second was subjected to sequential inoculation of *Torulaspora delbrueckii* with *Saccharomyces cerevisiae* (25 g/hL, LEVEL2 TD, Lallemand, Montreal, Canada) (wine MT); and finally (iii) third tank was subjected to indigenous yeasts fermentation (wine MI). During sequential fermentation, *T. delbrueckii* was inoculated first followed 48 h later by inoculation of *S. cerevisiae*. Prior to fermentation with indigenous yeasts, a small portion of wine (approx. 60 L) was transferred into a separate tank and slightly heated, at approximately 25°C, in order to accelerate yeast propagation phase. After fermentation started, wine was transferred to the main tank. This procedure assured that *S. cerevisiae* were predominant yeasts for alcoholic fermentation. As in the case of control wine, alcoholic fermentation temperature was kept under 18°C. After fermentation was finished (residual sugars below 3 g/L), SO_2 was added at a concentration level of 20 mg/L. Approximately 50 days after alcoholic fermentation, wines (PK, MK, MT, MI) were bottled with the four distinctive variants of antioxidant additions and closed by screw-cap closures. Physicochemical properties of the produced wines are summarized in Table 1. The antioxidants addition variants were: (i) standard free SO_2 concentration (free SO_2 30 mg/L), (ii) higher SO_2 concentration (free SO_2 45 mg/L), (iii) addition of 20 mg/L of glutathione (free SO_2 30 mg/L), and (iv) higher SO_2 concentration with 20 mg/L of glutathione (free SO_2 45 mg/L). The schematic illustration of wine production is presented in Figure 1. Each variant was bottled in triplicate and the wines were analyzed after 6 months of storage at cellar temperature (15–18°C).

Grape analysis

Analysis of volatile thiol precursors, glutathione (GSH), and oxidized glutathione (GSSG) in grapes

The analysis of volatile thiol precursors, GSH, and GSSG in grapes was carried out using the method described by Vanzo *et al.* [2017]. Briefly, a 10 g aliquot of pulverized frozen grapes was rapidly transferred into cold, deoxygenated methanol (1:4, w/v), spiked with deuterium-labelled internal standards (Cys-4MSP-d6 and Glut-4MSP-d10), vortexed, extracted, and centrifuged. A small aliquot of the extract was filtered and directly injected onto 1290 infinity UHPLC system coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) and separated by 100 mm × 2.1 mm. 1.8 μm column (Acquity HSS T3, Waters, Milford, MA, USA). The chromatographic and mass spectrometry parameters used, were identical as stated in previously mentioned research. Direct injection allowed quantification of GSH and GSSG together with Glut-3SH, which was present in Pošip grape extract above limit of quantification (LOQ). To determine Glut-4MSP, Cys-4MSP, and Cys-3SH which were below LOQ by direct injection, grape extracts were concentrated and purified according to the procedure described in previously mentioned research. Recovery of Glut-4MSP-d10 was used for quantification of Glut-4MSP, whereas recovery of Cys--4MSP-d6 was used for quantification of Cys-3SH and Cys--4MSP. Analyses were conducted in triplicate.

Wine analyses

Glutathione analysis

Concentrations of GSH in wines were analyzed by Agilent 1200 Series high-performance liquid chromatography with fluorescence detection (HPLC-FLD) (Agilent Technologies) and on-line column derivatization. Separation was performed at 25°C using a Synergi Fusion-RP 80A column (150 mm × 2.0 mm, 4 μm) (Phenomenex Inc., Aschaffenburg, Germany) according to the method in detail described by Janeš *et al.* [2010]. Wine samples were, immediately after bottle open-

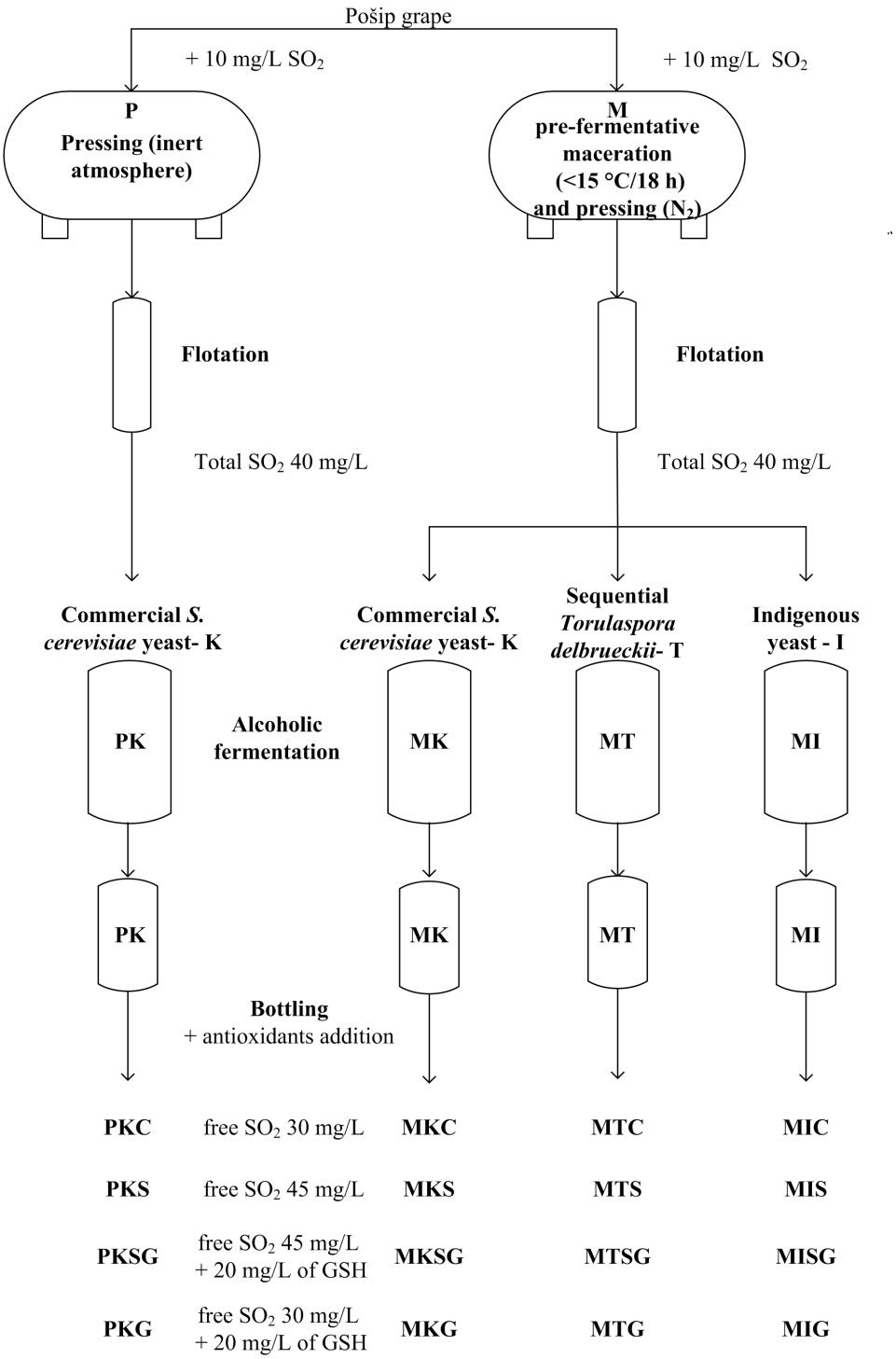

FIGURE 1. Schematic diagram of Pošip wine production.

ing, placed in methanol (1:10), with *N*-acetyl-l-cysteine as the internal standard, filtered through 0.45 μm Minisart RC 25 filters Sartorious (Goettingen, Germany), diluted 1:1 with a 5 mM sodium acetate buffer containing 0.1 mM EDTA, and analyzed as previously described [Janeš *et al.*, 2010]. Analyses were conducted in triplicate.

Volatile thiols analysis

Three thiols: 4MSP, 3SH, and 3SHA, were determined in wines by slightly modified method of Tominaga *et al.* [1998]. The modified method was in detail described by Šuklje *et al.* [2013]. Identification and quantification was performed with a gas chromatograph (Agilent Technologies 7890A) equipped with the MPS 2 automatic sampler (Gerstel, Mülheim an der Ruhr, Germany) and coupled with mass spectrometric detector (Agilent Technologies 5973C upgraded with Triple Axis detector). For quantification of analyzed thiols, single point calibration was used, where water solution of 4MSP at concentration of 70 ng/L, 3SHA at concentration 660 ng/L, and 3SH at concentration of 1654 ng/L was extracted with

the same extraction procedure as samples. Analyses were conducted in triplicate.

Terpenes analysis

Prior to GC/MS analysis, terpenes were extracted and concentrated by headspace solid-phase microextraction (SPME) technique using polydimethylsiloxane/divinylbenzene fiber (Supelco, Bellefonte, PA, USA). Identification and quantification was performed with a gas chromatograph (Agilent Technologies 7890A) equipped with the MPS 2 automatic sampler (Gerstel, Mülheim an der Ruhr, Germany) and coupled with mass spectrometric detector (Agilent Technologies 5973C upgraded with Triple Axis detector). Single point calibration was used for quantification. Sample preparation, as well as extraction and chromatographic conditions were in detail reported by Bavčar *et al.* [2011]. Analyses were conducted in triplicate.

Minor volatiles (without terpenes) analysis

A discontinuous liquid-liquid extraction (LLE) with dichloromethane was used for the extraction of volatile compounds, primarily esters, according to the previously described method [Bavčar *et al.*, 2011]. Identification and quantification were performed with a gas chromatograph (Hewlett Packard 6890, Agilent Technologies) coupled with mass spectrometric detector (Hewlett Packard 5973). For quantification single point calibration was used. Analyses were conducted in triplicate.

Hydroxycinnamic acids and their tartrate esters analysis

Hydroxycinnamic acids and their tartrate esters (HCA), namely *cis*- and *trans*-caftaric, coutaric, fertaric, caffeic, *p*-coumaric and ferulic acids, together with glutathione derivative of caftaric acid (GRP) were analyzed using previously described methods (HPLC analysis) [Šuklje *et al.*, 2012; Vanzo *et al.*, 2007], with the exception of the injection volume of 10 μL. The sample was filtered through a 0.25 μm CA filter (Millipore, Bedford, USA). Separation was performed by Agilent 1100 HPLC with DAD detection (Agilent Technologies), using a 250 × 2.1 mm, 5 μm, ODS Hypersil C18 column (ThermoFisher Scientific, Waltham, MA, USA). The identification of HCA and GRP was carried out by comparison of their spectra and retention time with those of standards, as described by Šuklje *et al*. [2012]. The HCA for which no standards were available (*cis*-caftaric, *cis*-coutaric, and *cis*-fertaric acid) were identified by their retention time and spectral parameters, as reported in previous investigations [Bengoechea *et al.*, 1995; Pena-Neira *et al.*, 2000; Vanzo *et al.*, 2007]. Quantitative determinations were made by using the external standard method with *trans*-caftaric acid, and the respective concentrations of HCA and GRP in the samples were expressed as *trans*-caftaric acid equivalents. As described in Šuklje *et al*. [2012], a calibration curve was prepared by injecting a standard of *trans*-caftaric acid in the range from 1.05 to 500 mg/L. Analyses were conducted in triplicate.

Statistical analysis

The statistical data analysis was carried out using Statistica V.10 software (Statsoft Inc., Tulsa, OK, USA). The one-way analysis of variance (ANOVA) was performed on all independent variables of the analyzed aroma compounds. In order to compare variable means of concentrations of aroma and phenolic compounds, Tukey's HSD test was used when samples were significantly different after ANOVA ($p<0.05$). In order to examine the significance of applied enological treatments, the main effects ANOVA was conducted, while the principal component analysis (PCA) was performed on the correlation matrix using the attributes of all analyzed compounds in order to examine any possible grouping of samples by different applied treatments.

RESULTS AND DISCUSSION

Thiol precursors

Content of four thiol precursors, namely Cys-4MSP, Cys-3SH, Glut-4MSP, Glut-3SH, as well as contents of GSH and GSSG in Pošip grapes are presented in Table 2. The highest content was determined in the analyzed grapes for Glut-3SH and it amounted 7.53 μg/kg, while the Glut-4MSP was found in the lowest content of 0.09 μg/kg. Regarding the contents of these compounds in other grape varieties, only a few investigations deal with their presence in grapes, while the most of the presented studies are focused on their levels in grape juices and musts. Nevertheless, based on available data it can be seen that contents of individual precursors in Pošip grapes, analyzed in the present study, are lower than of those found in Sauvignon blanc grapes. For example, the average contents of thiol precursors in Sauvignon blanc grapes from different vintages (2013–2015) were in the range of 8–16 μg/kg for Glut-3SH, 1–6 μg/kg for Cys-3SH, 1–4 μg/kg for Cys-4MSP, and 0.3 μg/kg for Glut-4MSP [Vanzo *et al.*, 2017]. Furthermore, sum of their contents determined in Sauvignon blanc grapes skin and pulp were in the range of 15–85 μg/kg for Cys-3SH and 17–112 μg/kg of Glut-3SH, while the content of Glut-4MMP amounted 8–11 μg/kg [Roland *et al.*, 2011a]. Furthermore, Capone *et al.* [2011] determined contents of 3SH precursor diastereomers (*R*-/*S*-Glut-3SH, *R*-/*S*-Cys-3SH) at the time of harvest, which amounted around 200 μg/kg of Glut-3SH and 30 μg/kg of Cys-3SH, representing more than 20-fold higher content than those obtained in Pošip grape. But, according to recent investigations [Jeffery, 2016; Vanzo *et al.*, 2017], lower contents are most likely due to the sampling method, where rapid freezing immediately after picking results in significantly lower contents due to the lack of oxygen or enzymatic reaction that impede

TABLE 2. Content of glutathione (mg/kg), oxidized glutathione (mg/kg), and volatile thiol precursors (μg/kg) in Pošip grapes.

Compound	Concentration in Pošip grapes
Glutathione (GSH)	68.37±1.72
Oxidized glutathione (GSSG)	0.75±0.03
Cys-4MSP	0.16±0.00
Cys-3SH	0.30±0.05
Glut-4MSP	0.09±0.00
Glut-3SH	7.53±0.75

Concentrations expressed as mean ± standard deviation (n=3).

precursors formation. Moreover, regarding the proportions among the analyzed precursors, inconsistent results could be found in previous research. Namely, according to Roland *et al.* [2010] cysteinylated precursors were more abundant than glutathionylated ones in Sauvignon blanc grapes, while higher amounts of glutathionylated precursors were found in Australian grape samples [Capone *et al.*, 2010] as well as in Melon B. and Sauvignon blanc grapes from Loire Valley and Montpellier [Roland *et al.*, 2011a]. The latter findings are in accordance with the results obtained in this research. Higher contents of GSH precursors could be due to the higher levels of glutathione in the grape berries, which could participate in Glut-3SH biogenesis [Roland *et al.*, 2011a]. Besides available glutathione, other factors, such as water deficit, assimilable nitrogen, as well as infection of grapes by *Botrytis cinerea* also modulate the final concentrations of precursors [Thibon *et al.*, 2009].

Besides the thiol precursors, concentrations of GSH and GSSG were also determined in the analyzed grape. Generally, GSH is the most abundant thiol compound of low molecular weight and its contents in grapes are closely related to the vine nitrogen status where nitrogen-deficient vines are characterized with its significantly lower levels [Choné *et al.*, 2006]. As presented in Table 2, GSH contents in Pošip grapes reached 68.37 mg/kg, representing a moderate level since it is determined in grapes in ranges of up to 114 mg/kg [Kritzinger *et al.*, 2013]. As stated previously [Roland *et al.*, 2011a], the latter authors [Kritzinger *et al.*, 2013] also indicated the possible relation of a higher GSH content with a higher content of glutathionylated thiol precursors, especially Glut-3SH, which is in accordance with our research. Furthermore, the high GSH:GSSG ratio, as stated in Kritzinger *et al.* [2013], indicates that no oxidative stress occurred in grape berries during the sampling as well as during sample preparation.

Glutathione in wines

The concentrations of GSH, determined in Pošip wines after six months of aging in bottles, are presented in Figure 2, where significant differences among wines could be observed. Primarily, those differences are related to various antioxidant additions at the bottling. For example, in all produced wines, the addition of glutathione (variant G), as well as combination of glutathione with higher SO_2 (variant SG) resulted in significantly higher concentrations of GSH six months after bottling, with more than 2-fold higher concentrations determined in the mentioned variants when compared with wine bottled with only SO_2 additions (variants C and S). It was previously

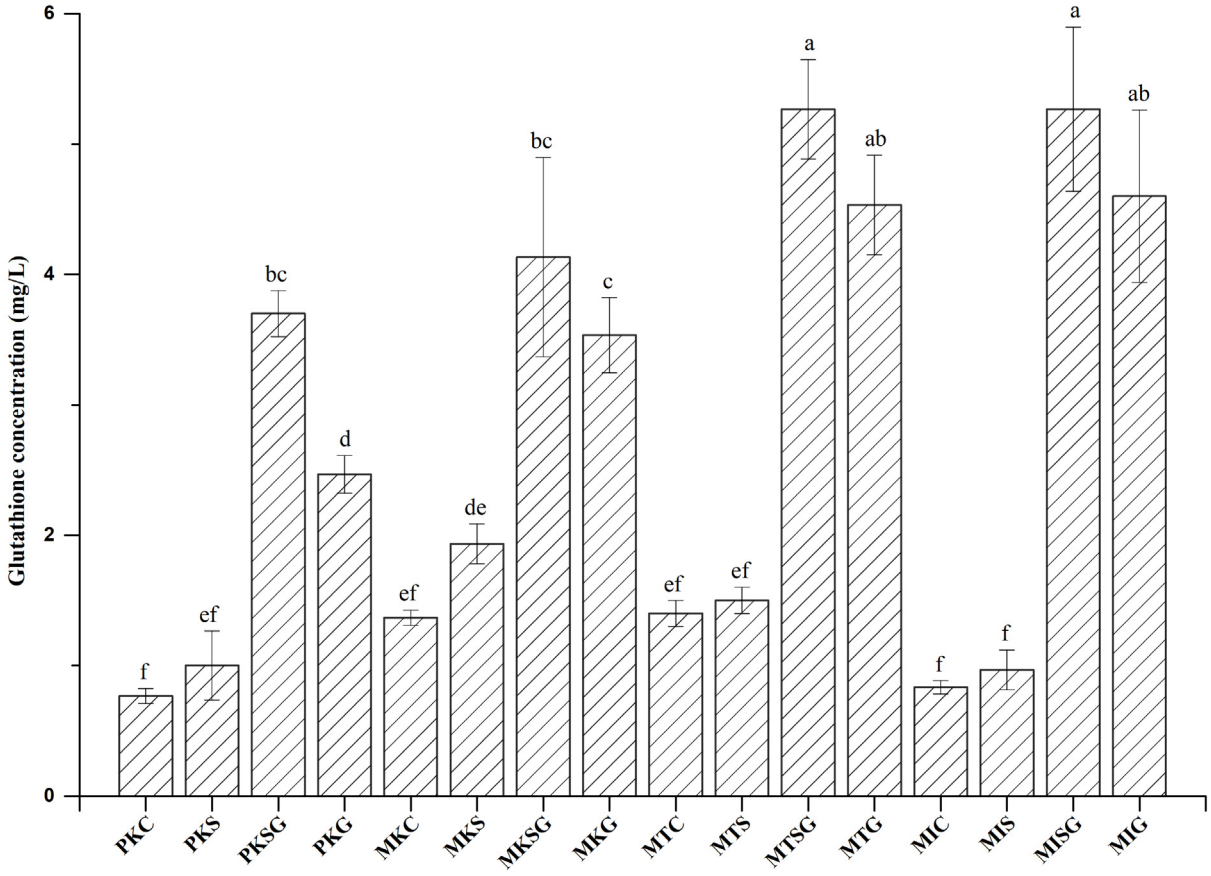

FIGURE 2. Concentration of glutathione (mg/L) in Pošip wines.

Data presented as means ± standard deviation (n=3). P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *T. delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione. Different letters indicate significant differences among wines (Tukey's test, $p<0.05$).

demonstrated that the combination of SO_2 and GSH acted synergistically due to the accelerated consumption of oxygen in white wines [Fracassetti *et al.*, 2013]. Furthermore, it can be seen that wines produced by sequential alcoholic fermentation of *T. delbrueckii* with *S. cerevisiae*, as well as wines fermented by indigenous yeasts resulted in slightly higher concentrations of GSH in comparison to wines fermented by commercial *S. cerevisiae* yeasts. Differences in GSH concentration in wines fermented by distinct yeast strain were also reported previously [Lavigne *et al.*, 2007], implying the importance of selected yeast strain on the final quality of wine. In comparison to grapes, concentrations of GSH in wines were significantly lower. This was expected since only a part of the GSH present in grapes is transferred to the must and wines, as well as considering the fact that its concentration significantly lowers during aging [Fracassetti *et al.*, 2013]. In fact, according to Ferreira-Lima *et al.* [2016], GSH practically disappears after 8 months of wine aging, which is in line with results of our study.

Aroma compounds

Regarding the effect of enological practices on wine aroma, this paper represents a further investigation of the previously published research [Tomašević *et al.*, 2017]. The present study involves different vintage and microlocation, as well as the use of additional enological practices, primarily *Torulaspora delbrueckii* yeasts for sequential alcoholic fermentation. Herein, different aroma compounds were identified in produced Pošip wines, and the results are shown in Tables 3 and 4, wherein Table 3 mostly represents the concentrations of aroma compounds (volatile thiols and terpenes) along with C6 compounds as grape-derived compounds, while Table 4 shows concentrations of esters, as contributors of fermentation aroma. Among aroma compounds, nine compounds were identified: 3 volatile thiols (4MSP, 3SHA and 3SH), four terpenes (linalool, α-terpineol, citronellol, and geraniol), and two C6 compounds (1-hexanol and *cis*-3-hexen-1-ol) (Table 3). Concentrations of the analyzed thiol compounds were in ranges of 29.6–48.0 ng/L (4MSP), 63–157 ng/L (3SHA), and 719–1273 ng/L (3SH). These concentrations are above their perception thresholds, namely 0.8 ng/L (for 4MSP), 4.2 ng/L (for 3SHA), and 60 ng/L (for 3SH) [Tominaga *et al.*, 1998], implying a potential positive influence of these compounds on the sensory characteristics of the produced Pošip wines. Generally, the concentrations of 3MH have been found to be particularly high, up to 18680 ng/L in Sauvignon blanc wines [Lund *et al.*, 2009], while lower concentrations, similar to these determined in Pošip wines, were determined in other grape cultivars, *e.g.* up to 970 ng/L in Riesling wines [Tominaga *et al.*, 2000] and up to 1200 ng/L in Pinot Noir wines [Capone *et al.*, 2015]. Other two compounds were detected in lower concentrations, up to

TABLE 3. Concentration of volatile thiols (ng/L), terpenes (μg/L), and C6 compounds (mg/L) in Pošip wines.

Samples	Volatile thiols			Terpenes				C6 compounds	
	4MSP	3SHA	3SH	Linalool	α-Terpineol	Citronellol	Geraniol	1-Hexanol	*cis*-3-Hexen-1-ol
PKC	43.0±3.3^{a}	104±9^{cd}	1245±27^{a}	40.9±1.5^{bc}	53.0±1.3^{f}	17.1±0.8^{ab}	62.4±5.3^{fgh}	0.70±0.02^{ab}	0.12±0.00^{d}
PKS	33.1±5.9^{a}	100±8^{d}	1161±92^{a}	37.3±0.6^{def}	53.6±1.5^{f}	14.8±0.6^{d}	57.8±5.6^{gh}	0.69±0.03^{ab}	0.12±0.01^{d}
PKSG	38.8±2.3^{a}	100±12^{d}	1273±46^{a}	34.3±0.7^{gh}	56.8±1.4^{f}	13.4±0.3^{e}	53.5±2.4^{gh}	0.66±0.03^{b}	0.11±0.00^{d}
PKG	40.2±8.6^{a}	96±4^{d}	1120±58^{a}	31.5 ±1.0^{h}	61.9±0.6^{e}	12.5±0.7^{a}	55.4±2.2^{gh}	0.67±0.02^{b}	0.11±0.00^{d}
MKC	43.3±4.2^{a}	63±3^{e}	1169±147^{a}	42.6±0.1^{b}	82.5±1.5^{c}	18.1±0.2^{bc}	75.6±5.3^{cde}	0.87±0.03^{a}	0.14±0.00^{ab}
MKS	36.7±6.5^{a}	65±6^{e}	1097±36^{a}	39.4±0.7^{bcd}	82.9±0.9^{c}	16.7±0.4^{cd}	65.4±2.8^{efg}	0.84±0.01^{a}	0.14±0.00^{bc}
MKSG	44.9±7.1^{a}	65±17^{e}	1176±60^{a}	35.1±0.8^{ef}	91.0±0.9^{b}	15.4±0.6^{d}	72.7±3.9^{def}	0.86±0.03^{a}	0.14±0.00^{ab}
MKG	48.0±9.5^{a}	65±9^{e}	1163±116^{a}	34.0±0.6^{gh}	91.9±0.9^{b}	15.2±0.2^{k}	75.2±6.7^{cde}	0.85±0.02^{a}	0.14±0.00^{b}
MTC	37.8±7.3^{a}	99±6^{d}	719±53^{c}	19.1±0.5^{h}	78.6±1.3^{c}	3.6±0.3^{hij}	50.4±2.0^{h}	0.46±0.01^{c}	0.12±0.00^{d}
MTS	39.6±8.6^{a}	100±5^{d}	721±35^{c}	42.0±1.4^{b}	64.8±1.2^{de}	7.1±0.3^{ij}	95.8±4.7^{ab}	0.47±0.03^{c}	0.12±0.01^{d}
MTSG	29.6±3.6^{a}	106±4^{bcd}	739±14^{bc}	38.2±2.5^{cde}	67.5±3.0^{d}	6.4±0.6^{j}	83.3±2.6^{cd}	0.45±0.00^{c}	0.12±0.00^{d}
MTG	31.5±6.1^{a}	104±12^{cd}	763±100^{bc}	35.8±0.5^{ef}	68.8±2.0^{d}	5.9±0.2^{f}	73.5±3.0^{def}	0.48±0.00^{c}	0.12±0.00^{cd}
MIC	43.5±2.8^{a}	134±18^{ab}	1016±193^{ab}	46.4±2.0^{a}	90.6±2.1^{b}	9.5±0.3^{f}	86.7±0.6^{bc}	0.73±0.02^{ab}	0.15±0.00^{ab}
MIS	38.4±5.3^{a}	155±10^{a}	1162±165^{a}	40.7±1.5^{bcd}	91.9±1.2^{b}	8.4±0.3^{fg}	79.3±2.6^{cd}	0.73±0.01^{ab}	0.15±0.00^{ab}
MISG	40.7±6.8^{a}	157±3^{a}	1152±12^{a}	37.4±0.7^{bcde}	94.1±2.9^{ab}	7.7±0.4^{gh}	75.7±0.9^{cde}	0.75±0.04^{a}	0.15±0.01^{d}
MIG	43.7±1.6^{a}	133±15^{abc}	1165±35^{a}	34.5±0.5^{gh}	98.0±1.0^{a}	7.3±0.1^{ghi}	105.5±6.2^{a}	0.70±0.02^{ab}	0.15±0.00^{ab}

Data presented as means ± standard deviation (n=3). Different superscript letters indicate statistical differences among wines (Tukey's test, $p<0.05$). Abbreviations: 4MSP, 4-methyl-4-sulfanylpentan-2-one; 3SHA, 3-sulfanylhexyl acetate; 3SH, 3-sulfanylhexan-1-ol; P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione.

2500 ng/L for 3SHA and up to 50 ng/L for 4MMP [Benkwitz *et al.*, 2012; Piano *et al.*, 2015; Ribéreau-Gayon *et al.*, 2006]. As can be seen in Table 3, there are slight, but not significant differences ($p \geq 0.05$) among samples regarding the concentration of 4MSP, while significant differences ($p < 0.05$) could be observed in 3SHA and 3SH concentrations. Additionally, regarding the applied enological practices, several trends can be drawn. Firstly, the pre-fermentative maceration in combination with commercial *S. cerevisiae* yeasts resulted in lower concentrations of 3SHA, while did not affected other thiols. Secondly, higher concentrations of the previously mentioned thiols were influenced by indigenous yeast fermentation, while sequential fermentation with *T. delbrueckii* resulted in higher concentrations of 3SHA, but lower ones of 3SH when compared to the fermentation by commercial *S. cerevisiae* yeasts. Thirdly, no uniform tendency could be observed regarding the antioxidant additions. But, in majority of the macerated wine samples, the highest concentrations of these compounds, especially of 3SHA, were determined in wines bottled with the combination of higher SO_2 and glutathione addition (variant SG). Regarding the pre-fermentative maceration, it is expected that its application enhances the varietal character of wines due to the improved extraction of aroma compounds and their precursors from grapes [Olejar *et al.*, 2015]. But, as can be seen in the presented results, in our case this process affected lower concentrations of 3SH and 3SHA (wine MK) and practically no changes in concentrations of 4MSP. This could likely be due to the increased extraction of phenolic compounds, and oxidation as well, which occurs during the applied maceration process. These compounds undergo oxidation reactions and form *o*-quinones, being very reactive compounds that easily react with thiols and, subsequently, result in their decrease and loss of varietal aroma [Nikolantonaki & Waterhouse, 2012]. Also, Mattivi *et al.* [2012] have shown that, despite inert pressing, the oxidative loading of grapes into the press resulted in removing a large part of glutathione, and subsequently resulted in lower concentrations of thiols. Since the wine production in our case was similar to the previously described procedure regarding the grape loading, this could be a reason for the obtained trend. Considering the yeast species, previous researches reported different conclusions, primarily regarding the influence of *T. delbrueckii.* For example, several studies investigated the ability of *T. delbrueckii* strain to produce 3SH and 3SHA in sequential as well as in simultaneous inoculation with *S. cerevisiae* and demonstrated that their lower concentrations were produced in comparison to single inoculation with *S. cerevisiae* [Zott *et al.*, 2011]. On the other hand, a recent study showed higher concentrations of 3SH and 4MSP in wines fermented by this yeast [Belda *et al.*, 2017], in comparison to fermentation by *S. cerevisiae.* Moreover, there are different conclusions regarding the assimilation of precursors by *T. delbrueckii* strains. Firstly, this yeasts was reported to be incapable of assimilating cysteinylated precursors [Renault *et al.*, 2016], but in more recent study this conclusion was rebutted [Belda *et al.*, 2017]. These findings suggest that the production of volatile thiols is strain-dependent, since different commercial starter cultures were used in the mentioned investigations. Besides the *T. delbrueckii*, musts were subjected to indigenous yeasts fermentations, as well, and these wines were characterized by more than 2-fold higher concentrations of 3SHA, compared to the wines fermented by commercial *S. cerevisiae* culture. These results are contrary to our previous research regarding the influence of yeast strain on the aroma of Pošip wine [Tomašević *et al.*, 2017], where the commercial *S. cerevisiae* resulted in higher concentrations of thiols in final wine in comparison to indigenous yeasts. The reason of this disagreement could be the starter cultures used, wherein the one used in the current research (Lalvin EC-1118) is characterized by ester-forming ability, while the one used in the previous study (Zymaflore X5) as a thiol-releasing strain. Nevertheless, a higher concentration of 3SHA after indigenous yeast fermentation is most probably due to the higher production of acetate esters by this yeasts.

Among the terpenes, α-terpineol was determined in the highest concentration, ranging from 53.0 to 98.0 μg/L, while citronellol was found in the lowest concentrations, amounting up to 18.1 μg/L (Table 3). The pre-fermentative maceration induced higher concentrations of most of the analyzed terpenes, especially of geraniol. Despite their increased concentrations, only linalool was determined in concentrations above its perception threshold of 25 μg/L [Escudero *et al.*, 2007]. But, even though terpenes are present in concentrations below their perception thresholds, their olfactory impact is synergistic [Ribéreau-Gayon *et al.*, 2006], meaning they could have an important role in the overall aroma of Pošip wine. Regarding the yeast strain used for alcoholic fermentation, commercial yeasts resulted in higher concentrations of citronellol and indigenous yeasts in a higher concentration of α-terpineol, while concentrations of other terpenes were yeast-independent. Generally, terpenes undergo significant changes during alcoholic fermentation, wherein the major transformation concerns the degradation of nerol and geraniol by the enzymatic activity of *S. cerevisiae* yeasts and their reduction to citronellol, α-terpineol, and linalool [Darriet *et al.*, 2012]. This finding was not in line with trends observed in our investigation since both, geraniol and α-terpineol, were determined in higher concentrations, which could probably be due to the different enzymatic activity of indigenous yeasts.

The last group of aroma compounds presented in Table 3 were C6 compounds: 1-hexanol and *cis*-3-hexen-1-ol. The pre-fermentative maceration slightly increased their concentrations, except when *T. delbrueckii* yeasts were used, but in a much lesser extent than that determined in our previous work and other investigations [Cejudo-Bastante *et al.*, 2011; Ribéreau-Gayon *et al.*, 2006; Tomašević *et al.*, 2017], where their concentrations increased significantly after the applied pre-fermentation maceration. This was probably due to the inert conditions which caused a reduction in enzymatic lipid oxidation and thus a decrease in the production of C6 compounds [Petrozziello *et al.*, 2011]. Furthermore, based on the obtained results and reported literature, regarding the perception thresholds, it can be concluded that these compounds have a limited influence on the aroma of the analyzed wines, since it is known that they contribute to the wine aroma in concentrations above 1.1 mg/L (1-hexanol) and 0.4 mg/L (*cis*-3-hexen-1-ol) [Peinado *et al.*, 2004]. Despite the potentially insignificant effect on the sen-

TABLE 4. Concentration of esters (mg/L) in Pošip wines.

Samples	Ethyl butanoate	Ethyl hexanoate	Ethyl octanoate	Ethyl decanoate	Ethyl dodecanoate	Ethyl hexadecanoate	Diethyl butanedioate	3-Methylbutyl acetate	Hexyl acetate	2-Phenyl-ethyl acetate
PKC	0.25±0.01^{cde}	0.54±0.02^{b}	0.96±0.03^{d}	0.39±0.01^{c}	0.02±0.00^{a}	0.06±0.01^{a}	0.54±0.02^{fg}	0.93±0.03^{c}	0.07±0.00^{c}	0.20±0.01^{c}
PKS	0.25±0.01^{de}	0.54±0.01^{b}	0.92±0.01^{d}	0.38±0.02^{c}	0.03±0.00^{a}	0.06±0.02^{a}	0.51±0.01^{g}	0.91±0.02^{c}	0.07±0.00^{cd}	0.20±0.01^{c}
PKSG	0.23±0.05^{e}	0.53±0.01^{b}	0.91±0.01^{d}	0.38±0.01^{c}	0.04±0.02^{a}	0.06±0.01^{a}	0.49±0.00^{g}	0.89±0.09^{c}	0.07±0.00^{cd}	0.20±0.00^{c}
PKG	0.24±0.03^{e}	0.54±0.00^{b}	0.92±0.01^{d}	0.38±0.00^{c}	0.02±0.00^{a}	0.05±0.00^{ab}	0.55±0.01^{efg}	0.91±0.05^{c}	0.07±0.00^{cd}	0.20±0.00^{c}
MKC	0.26±0.00^{abcde}	0.53±0.02^{b}	1.00±0.05^{d}	0.40±0.02^{c}	0.02±0.00^{a}	0.01±0.00^{cd}	0.64±0.03^{d}	1.03±0.03^{c}	0.06±0.00^{cd}	0.21±0.01^{c}
MKS	0.25±0.00^{cde}	0.53±0.01^{b}	0.98±0.01^{d}	0.39±0.00^{c}	0.03±0.01^{a}	0.01±0.00^{cd}	0.61±0.01^{def}	1.00±0.00^{c}	0.06±0.00^{d}	0.20±0.00^{c}
MKSG	0.27±0.01^{abcde}	0.54±0.02^{b}	1.00±0.02^{bcd}	0.40±0.00^{c}	0.02±0.00^{a}	0.02±0.01^{cd}	0.63±0.02^{de}	1.04±0.04^{c}	0.06±0.00^{cd}	0.21±0.00^{c}
MKG	0.26±0.01^{bcde}	0.53±0.01^{b}	0.99±0.01^{d}	0.38±0.00^{c}	0.02±0.00^{a}	0.01±0.00^{cd}	0.64±0.01^{d}	1.01±0.02^{c}	0.06±0.00^{cd}	0.20±0.00^{c}
MTC	0.29±0.01^{abcd}	0.64±0.01^{b}	1.16±0.01^{d}	0.45±0.01^{b}	0.02±0.00^{a}	0.02±0.00^{cd}	0.77±0.01^{bc}	2.98±0.08^{b}	0.11±0.00^{b}	0.73±0.01^{a}
MTS	0.30±0.01^{abc}	0.68±0.03^{a}	1.20±0.02^{a}	0.46±0.03^{b}	0.03±0.01^{a}	0.03±0.01^{bc}	0.75±0.02^{c}	3.10±0.13^{b}	0.11±0.01^{b}	0.75±0.04^{a}
MTSG	0.30±0.01^{abc}	0.66±0.01^{a}	1.16±0.07^{a}	0.45±0.00^{b}	0.02±0.00^{a}	0.02±0.00^{cd}	0.76±0.00^{c}	3.07±0.09^{b}	0.11±0.00^{b}	0.73±0.01^{a}
MTG	0.30±0.01^{a}	0.64±0.01^{a}	1.16±0.02^{a}	0.45±0.01^{b}	0.02±0.00^{a}	0.02±0.00^{cd}	0.90±0.03^{a}	2.99±0.02^{b}	0.11±0.00^{b}	0.72±0.01^{a}
MIC	0.29±0.01^{abc}	0.66±0.02^{a}	1.12±0.03^{a}	0.54±0.04^{a}	0.03±0.01^{a}	0.01±0.00^{cd}	0.84±0.04^{a}	3.42±0.07^{a}	0.23±0.01^{a}	0.32±0.01^{b}
MIS	0.29±0.00^{abcd}	0.65±0.01^{a}	1.10±0.07^{abc}	0.53±0.01^{a}	0.03±0.00^{a}	0.01±0.00^{cd}	0.84±0.02^{a}	3.38±0.02^{a}	0.23±0.00^{a}	0.31±0.01^{b}
MISG	0.30±0.01^{ab}	0.65±0.02^{a}	1.11±0.02^{ab}	0.54±0.01^{a}	0.03±0.01^{a}	0.01±0.00^{cd}	0.83±0.05^{ab}	3.48±0.14^{a}	0.23±0.01^{a}	0.32±0.01^{b}
MIG	0.29±0.01^{abcd}	0.65±0.02^{a}	1.10±0.05^{abc}	0.53±0.02^{a}	0.03±0.00^{a}	0.01±0.00^{d}	0.84±0.02^{a}	3.43±0.12^{a}	0.20±0.01^{a}	0.32±0.01^{b}

Data presented as means ± standard deviation (n=3). Different superscript letters indicate statistical differences among wines (Tukey's test, $p<0.05$). Abbreviations: P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione.

TABLE 5. Concentration of hydroxycinnamic acids (mg/L) in Pošip wines.

Samples	*cis*-Caftaric acid	*trans*-Caftaric acid	GRP	*cis*-Coutaric acid	*trans*-Coutaric acid	*cis*-Fertaric acid	*trans*-Fertaric acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Sum (without GRP)
PKC	2.80±0.47^{efg}	2.74±0.11^{e}	17.05±0.09^{c}	6.76±0.06^{f}	nd	0.68±0.39^{a}	3.31±0.20^{cd}	55.78±0.59^{b}	10.97±0.22^{f}	3.23±0.16^{ab}	86.26±1.56^{d}
PKS	2.63±0.42^{gh}	2.82±0.10^{e}	18.12±0.06^{c}	7.01±0.33^{f}	nd	1.01±0.29^{a}	3.63±0.19^{bcd}	56.38±0.45^{b}	11.31±0.34^{ef}	3.37±0.12^{a}	88.17±0.18^{d}
PKSG	2.19±0.00^{h}	2.90±0.04^{e}	18.01±0.10^{c}	6.66±0.01^{f}	nd	0.44±0.29^{a}	3.43±0.50^{cd}	55.47±1.03^{b}	11.77±0.27^{e}	3.27±0.03^{a}	86.14±1.13^{d}
PKG	2.99±0.03^{cdefg}	2.79±0.10^{e}	18.03±0.04^{c}	7.11±0.29^{f}	nd	0.58±0.32^{a}	3.02±0.39^{d}	55.29±0.87^{b}	11.00±0.17^{f}	3.43±0.18^{a}	86.21±1.63^{d}
MKC	3.44±0.05^{abcd}	5.42±0.04^{c}	24.54±0.09^{b}	9.83±0.97^{cde}	nd	0.59±0.61^{a}	3.52±0.57^{cd}	56.63±1.37^{b}	14.17±0.28^{d}	2.30±0.14^{de}	95.91±3.90^{c}
MKS	2.94±0.06^{cdefg}	5.58±0.03^{c}	25.19±1.13^{b}	10.15±0.47^{bcd}	nd	0.53±0.58^{a}	3.80±0.59^{bcd}	57.33±1.13^{b}	15.03±0.33^{abc}	2.38±0.11^{cde}	97.76±3.19^{abc}
MKSG	2.89±0.09^{defg}	5.49±0.06^{c}	25.93±0.12^{b}	10.31±0.29^{bcd}	nd	0.22±0.03^{a}	3.53±0.03^{cd}	56.95±0.10^{b}	14.93±0.12^{bc}	2.25±0.11^{e}	96.60±0.60^{bc}
MKG	3.50±0.06^{abc}	5.44±0.04^{c}	25.78±0.07^{b}	10.28±0.31^{bcd}	nd	0.61±0.49^{a}	3.64±0.54^{bcd}	57.23±0.98^{b}	14.59±0.25^{cd}	2.38±0.08^{cde}	97.67±1.93^{abc}
MTC	3.56±0.10^{ab}	4.25±0.38^{d}	15.33±0.60^{d}	7.92±0.46^{ef}	nd	0.99±0.51^{a}	4.19±0.50^{bc}	63.49±1.31^{a}	14.86±0.05^{bc}	2.73±0.06^{cd}	102.00±2.48^{abc}
MTS	2.92±0.12^{defg}	4.50±0.46^{d}	16.56±0.69^{cd}	7.82±0.29^{f}	nd	0.76±0.46^{a}	4.44±0.56^{bc}	63.96±1.50^{a}	15.41±0.20^{ab}	2.76±0.11^{bcd}	102.57±2.99^{ab}
MTSG	2.71±0.19^{fgh}	4.52±0.33^{d}	16.75±0.82^{cd}	8.39±1.16^{def}	nd	0.81±0.22^{a}	4.72±0.16^{b}	64.48±0.51^{a}	15.64±0.17^{a}	2.77±0.17^{bc}	104.05±2.38^{a}
MTG	3.70±0.26^{ab}	4.06±0.15^{d}	16.90±1.19^{cd}	8.02±0.13^{ef}	nd	0.69±0.24^{a}	4.18±0.56^{bc}	63.17±1.41^{a}	14.89±0.25^{bc}	2.73±0.21^{cd}	101.44±2.89^{abc}
MIC	3.76±0.04^{ab}	42.01±0.70^{b}	27.09±0.03^{a}	12.02±1.16^{ab}	9.02±0.40^{a}	0.99±0.02^{a}	6.10±0.07^{a}	12.52±0.25^{c}	9.98±0.18^{g}	2.45±0.07^{cde}	98.85±1.46^{abc}
MIS	3.27±0.12^{bcdef}	43.11±0.37^{a}	28.03±0.22^{a}	11.59±1.06^{abc}	9.38±0.44^{a}	0.86±0.02^{a}	6.31±0.05^{a}	12.42±0.11^{c}	10.19±0.17^{g}	2.53±0.03^{cde}	99.66±0.44^{abc}
MISG	3.35±0.05^{bcde}	43.20±0.08^{a}	28.14±0.09^{a}	12.34±1.14^{a}	9.59±0.37^{a}	0.88±0.06^{a}	6.33±0.03^{a}	12.48±0.03^{c}	10.19±0.10^{g}	2.56±0.14^{cde}	100.93±1.40^{abc}
MIG	4.01±0.06^{a}	42.85±0.46^{ab}	28.22±0.16^{a}	13.52±0.26^{a}	9.55±0.44^{a}	1.05±0.02^{a}	6.21±0.08^{a}	12.24±0.18^{c}	9.88±0.14^{g}	2.64±0.15^{cde}	101.95±1.73^{abc}

Data presented as means ± standard deviation (n=3). Different superscript letters indicate statistical differences among wines (Tukey's test, $p<0.05$). Abbreviations: GRP, glutathione derivative of caftaric acid; P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *Torulaspora delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione; nd – not detected.

sory properties of wine, it is important to note the differences in concentrations produced by different yeast species. Thus, the commercial *S. cerevisiae* yeast strain, as well as indigenous yeasts, resulted in their higher concentrations, while sequential fermentation with *T. delbrueckii* resulted in their lowest concentrations, possibly implying that this species uses these compounds as precursors to form other compounds, *e.g.* hexyl acetate through alcohol acetyl transferase activity during fermentation [Sumby *et al.*, 2010]. Finally, regarding the antioxidant additions, no significant influence could be observed on the concentrations of these compounds.

Table 4 represents concentrations of esters determined in wine samples, wherein ten different compounds were quantified. Among them, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, 3-methylbutyl acetate, and 2-phenylethyl acetate are the most significant compounds for the aroma of Pošip wine since they were determined in concentrations above their perception thresholds [Swiegers *et al.*, 2005]. As can be seen in the presented results, the maceration effect is not pronounced (except for ethyl hexadecanoate) although higher concentrations of these compounds in the macerated wines fermented by sequential fermentation and indigenous yeast could be observed, in comparison to the directly pressed sample. These significant differences ($p<0.05$) are probably related to the yeast species used since indigenous yeast as well as sequential fermentation with *T. delbrueckii* resulted in higher concentration of most of the analyzed ester compounds, especially 3-methylbutyl acetate, hexyl acetate, and 2-phenylethyl acetate that were determined in 3-fold and 2-fold higher concentration when compared to commercial *S. cerevisiae* yeasts, respectively. Besides them, indigenous yeast fermentation also resulted in higher concentrations of ethyl hexanoate, ethyl decanoate, and diethyl butanedioate, while sequential fermentation with *T. delbrueckii* resulted in higher concentrations of ethyl hexanoate, ethyl octanoate, and diethyl butanedioate, but in a lesser extent than the previously stated. Similar results, concerning the higher concentration of esters, primarily 3-methylbutyl acetate, were also demonstrated in previous research regarding the Pošip wine aroma where the significant effect of indigenous yeasts was also established [Tomašević *et al.*, 2017]. In relation to the sequential fermentation with *T. delbrueckii*, different results can be found in previous investigations. For example, Azzolini *et al.* [2015] investigated the influence of this strain on aroma of Soave and Chardonnay white wines in comparison to fermentation by *S. cerevisiae* strain and opposite results were obtained, where the higher concentrations of 3-methylbutyl acetate, hexyl acetate, ethyl hexanoate, and ethyl octanoate were determined in wines fermented by single fermentation with *S. cerevisiae.* Also, similar results were obtained in earlier investigation [Azzolini *et al.*, 2012]. On the other hand, higher concentrations of desirable esters produced by *T. delbrueckii*, as presented in this paper, were also previously documented [Loira *et al.*, 2014; Renault *et al.*, 2015]. Finally, as can be seen in the presented results (Table 4) and, similarly to the trend established for aroma compounds, there were no differences among samples according to the different bottling variants.

TABLE 6. Effect of the applied enological practices on the analyzed compounds as indicated by ANOVA.

Compound	Pre-fermentative maceration	Yeast species	Antioxidant addition
4MSP	ns	*	ns
3SH	***	***	ns
3SHA	ns	***	ns
Linalool	ns	*	ns
α-Terpineol	***	***	***
Citronellol	***	***	***
Geraniol	***	***	ns
1-Hexanol	ns	***	ns
cis-3-Hexen-1-ol	***	***	ns
Ethyl butanoate	***	***	ns
Ethyl hexanoate	***	***	ns
Ethyl octanoate	***	***	ns
Ethyl decanoate	***	***	ns
Ethyl dodecanoate	ns	*	ns
Ethyl hexadecanoate	***	***	ns
Diethyl butanedioate	***	***	***
3-Methylbutyl acetate	***	***	ns
Hexyl acetate	***	***	ns
2-Phenyl-ethyl acetate	***	***	*
cis-Caftaric acid	***	***	***
*trans-C*aftaric acid	***	***	ns
GRP	***	***	ns
cis-Coutaric acid	***	***	ns
Coutaric acid	***	***	ns
cis-Fertaric acid	ns	**	ns
Fertaric acid	***	***	ns
Caffeic acid	***	***	ns
p-Coumaric acid	***	***	***
Ferulic acid	***	***	ns

***$p\leq0.001$; **$p\leq0.01$; *$p\leq0.05$; ns, not significant.

Hydroxycinnamic acids and their tartrate esters

Ten HCA were identified and quantified in wine samples: *cis*-caftaric, *trans*-caftaric, *cis*-coutaric, *trans*-coutaric, *cis*-fertaric, *trans*-fertaric, caffeic, *p*-coumaric, and ferulic acid, along with GRP, and the results are shown in Table 5. In most of the analyzed wines the most abundant compound was caffeic acid, except for the wine produced by fermentation with indigenous yeast where the *trans*-caftaric acid was determined in the highest concentration. Generally, it is well-known that hydroxycinnamic esters, such as caftaric, coutaric and fertaric

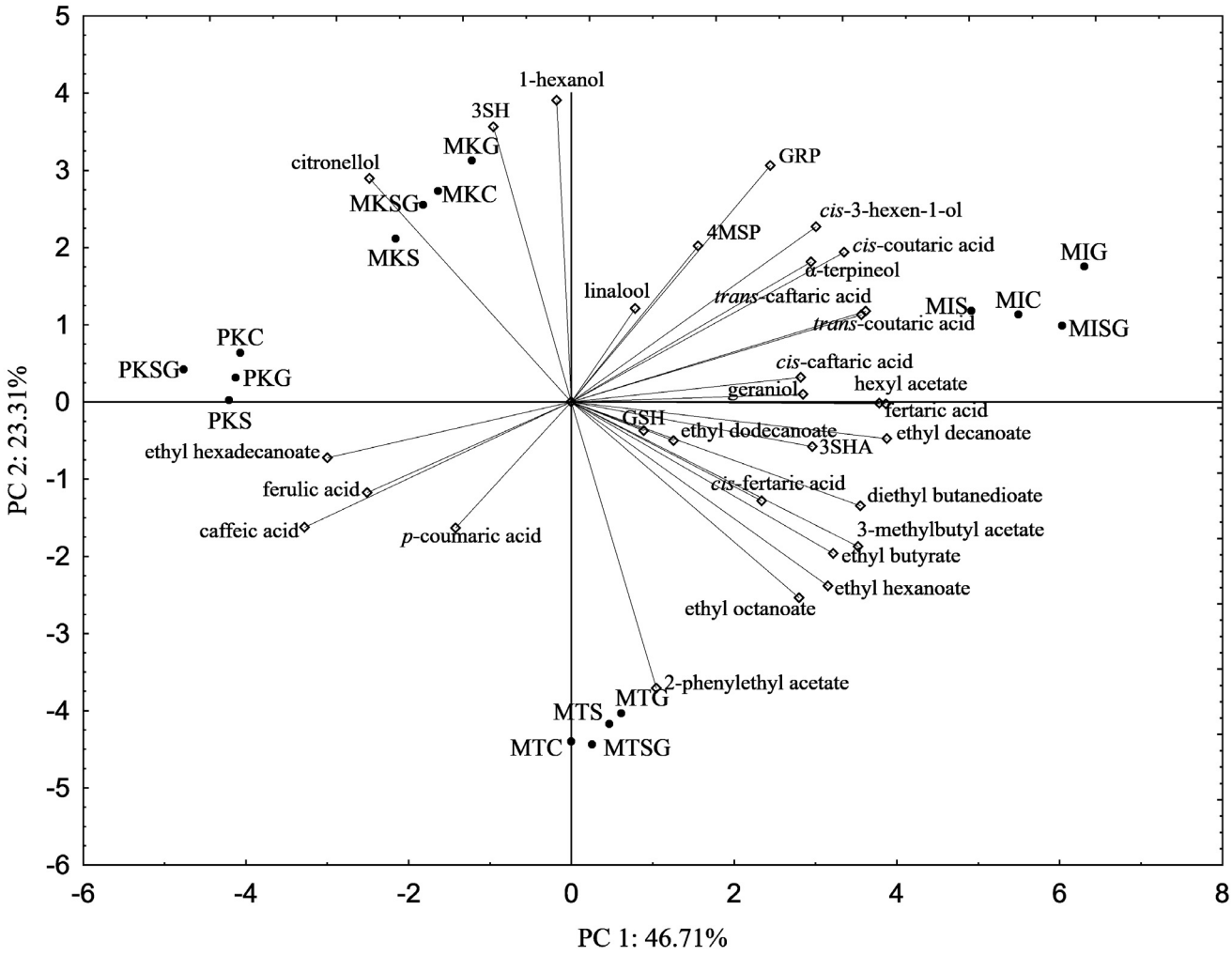

FIGURE 3. Distribution of the wine samples in two dimensional coordinate system defined by the first two principal components (PC1 and PC2) according to the applied practices and concentrations of aroma and phenolic compounds.

P, directly pressed grapes, control; M, pre-fermentative maceration; K, commercial *S. cerevisiae* yeasts; T, *T. delbrueckii* yeasts; I, indigenous yeasts; C, bottling with 30 mg/L of free sulfur dioxide; S, bottling with 45 mg/L of free sulfur dioxide; SG, bottling with 45 mg/L of free sulfur dioxide combined with 20 mg/L of glutathione; G, bottling with 20 mg/L of glutathione.

acid are present in grape skin and pulp and, during alcoholic fermentation and especially during wine maturation and aging, they undergo hydrolysis reaction which results in increased concentrations of their free forms, namely caffeic, coumaric, and ferulic acids. Also, it is known that a yeast strain could affect this hydrolytic activity and result in higher concentrations of their free forms [Monagas *et al.*, 2007], which could be the possible explanation for the obtained results. Beside the *trans*-caftaric acid, in wines produced by indigenous yeasts the highest concentration was also determined for GRP, followed by macerated wine fermented by commercial yeast strain (MK). Higher GRP concentrations potentially implicate that oxidation process occurred in previously mentioned wines. Furthermore, this thesis is additionally supported by lower concentrations of GSH in those wines (Figure 2). Besides oxidation reactions, GSH can possibly be taken up and employed by the yeast or react with other compounds present or formed during fermentation [Sonni *et al.*, 2011]. Similarly to the previously mentioned aroma compounds, the most significant changes in HCA composition were related to the yeast species used. Besides yeast species, the applied pre-fermentative maceration influenced higher concentrations of *cis*-caftaric, *trans*-caftaric, and *cis*-coutaric, as well as lower concentration of ferulic acid. Generally, maceration process influences increase in concentrations of these compounds due to their localization in the grape skin, and the previous investigations also demonstrated their higher concentrations after applied maceration [Di Lecce *et al.*, 2013], but these differences were pronounced to a greater extent than in our research. Furthermore, slightly higher concentrations of these compounds were determined in the case of antioxidants additions but, as in the case of aroma compounds, no differences could be observed among variants of antioxidants additions.

Statistical analyses

In order to evaluate the influence of the applied enological practices on the overall aroma and phenolic compounds in the analyzed Pošip samples, the ANOVA and principal component analysis (PCA) were conducted. The results of ANOVA are presented in Table 6, where the significance

was tested at three levels: p ≤0.001, p ≤0.01, and p ≤0.05. These results confirm previously described trends for individual compounds. The most significant effect showed to be caused by yeast species, since concentrations of all analyzed compounds differed significantly between yeast species variants. The second important factor was pre-fermentative maceration that influenced changes in almost all analyzed compounds (except 3SH, 1-hexanol, ethyl dodecanoate, and *cis*-fertaric acid). The least important factor was antioxidant addition; it caused statistically different differences in concentrations of only few compounds: α-terpineol, citronellol, diethyl butanedionate, 2-phenyl-ethyl acetate, *cis*-fertaric acid, and *p*-coumaric acid.

The results of PCA are shown in Figure 3, where it can be seen that the first two components explained 70.02% of the total variance. First variable (PC 1) showed a strong positive correlation with the content of ethyl decanoate (factor loading: 0.969), fertaric acid (0.966), hexyl acetate (0.946), caftaric acid (0.904); and a highly positive correlation with contents of coutaric acid (0.891), diethyl butanedioate (0.888), 3-methylbutyl acetate (0.880), *cis*-coutaric acid (0.838), ethyl butanoate (0.804), ethyl hexanoate (0.788), *cis*-3-hexen-1-ol (0.751), α-terpineol (0.736), geraniol (0.711), *cis*-caftaric acid (0.705), and ethyl octanoate (0.699); while caffeic acid (-0.820) and ethyl hexadecanoate (-0.749) were highly negatively correlated with this principal component. On the other hand, the second principal component showed a strong positive correlation with 1-hexanol (0.977) and a highly positive correlation with 3SH (0.891), GRP (0.765) and citronellol (0.724), as well as a strong negative correlation with 2-phenylethyl acetate (-0.927). Regarding the distribution of Pošip wines in a two-dimensional coordinate system, a clear separation of the analyzed samples can be observed. Herein, the samples were separated according the yeast species used for their production. As can be seen in Figure 2, wines produced by alcoholic fermentation with commercial yeast strain (both, directly pressed and macerated wines) were placed on the left from the PC1, the ones fermented by indigenous yeast on the right side of the same factorial plane, while wines produced by sequential fermentation of *T. delbrueckii* with *S. cerevisiae* were placed below the second factorial plane. Hence, alcoholic fermentation with commercial yeast strain was resulted in higher concentrations of caffeic acid and ethyl hexadecanoate (directly pressed ones), as well as of 3SH and citronellol (macerated samples). Furthermore, higher concentrations of 2-phenylethyl acetate and ethyl octanoate resulted from the sequential fermentation with *T. delbrueckii.* Also, these wines were characterized with higher concentrations of ethyl hexanoate, ethyl butanoate, 3-methylbutyl acetate, diethyl butanedioate, as well as *cis*-caftaric, *cis*-fertaric, caffeic, and *p*-coumaric acid. Finally, wines produced by indigenous yeast strain were characterized by higher concentrations of the majority of analyzed compounds, where the most significant ones were 3SHA, *cis*-3-hexen-1-ol, ethyl decanoate, diethyl butanedioate, 3-methylbutyl acetate, and hexyl acetate, compounds placed on the right side of the first factorial plane. In addition, these yeast also affected higher concentrations of most of the analyzed hydroxycinnamic acids (*cis*-caftaric, *trans*-caftaric, *cis*-coutaric, coutaric, *cis*-fertaric, and fertaric acids). Besides the yeast species, the influence of pre-fermentative maceration could also be observed, but in a lesser extent. It can be seen that the non-macerated wine samples (wines PK) where placed on the left side of the coordinate system and left of the other, macerated ones. Furthermore, the differences among macerated wines could be also observed where wine produced by commercial yeas strain (MK) showed to be more similar to the control wine (PK) than the other two macerated wines. These results imply the limited influence of this technique and, consequently, do not allow to draw a uniform conclusion of its influence on the analyzed aroma compounds. As stated previously for individual groups of compounds, the influence of antioxidant addition could not be observed.

CONCLUSIONS

Pošip grapes are characterized by higher contents of glutathionylated thiol precursors, especially Glut-3SH, while the applied enological practices resulted in differences in the produced wines. The most significant differences were caused by the yeast species used for alcoholic fermentation, where the indigenous yeast affected higher concentrations of most of the analyzed compounds, primarily 3SHA, α-terpineol, geraniol, *cis*-3-hexen-1-ol, ethyl hexanoate, diethyl butanedioate, 3-methylbutyl acetate, and hexyl acetate, as aroma representatives and most of the analyzed hydroxycinnamic acids. Furthermore, the pre-fermentative maceration found to be very limited since only higher concentrations of C6 compounds and lower concentrations of thiols 3SHA were found in macerated wines. Finally, the initial hypothesis regarding the protective effect of antioxidants during aging was not confirmed by this research, since only slightly higher concentrations of volatile thiols were found in wines bottled with higher SO_2 and glutathione (variant SG). This paper provides a new insight into Pošip grapes composition regarding the aroma precursors, as well as confirmation of a great prospect of indigenous yeast fermentation and sequential fermentation with *T. delbrueckii* yeasts for the potential production of more complex, aromatic wines.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antioxidant Activity of Extracts of Soursop (*Annona muricata* L.) Leaves, Fruit Pulps, Peels, and Seeds

Hakime Hülya Orak[1]*, Ilayda Sevik Bahrisefit[2], Temine Sabudak[2]

[1]Department of Food Technology, Vocational School of Technical Sciences, Namik Kemal University, 59030 Tekirdağ, Turkey
[2]Department of Chemistry, Faculty of Science and Arts, Namik Kemal University, 59030 Tekirdağ, Turkey

Key words: by-products, flavonoids, phenolics, GC-MS analysis, antiradical activity, reducing power

The total phenolic content (TPC), total flavonoid content (TFC), and the antioxidant activity of soursop (*Annona muricata* L.) leaf, fruit pulp, seed, and peel extracts obtained using successive extraction with hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) were determined. The Hxn soursop seed extract was analysed by GC-MS. The highest TPC was determined in MeOH extracts. MeOH and EtOAc extracts were rich sources of flavonoids. Generally, soursop leaf and fruit pulp extracts had the highest and the lowest both TPC and TFC, respectively. Fatty acids were dominant in the Hxn seed extract. Among antioxidants, terpenoids ((*E*)-nerolidolas as dominant) and phytosterols ((3-β)-stigmast-5--en-3-ol with high content) were identified. The soursop seed, followed by leaf and peel extracts (MeOH and EtOAc) had the highest DPPH$^{\bullet}$ scavenging activity, TEAC, FRAP, and CUPRAC. Antioxidant activity of peel extracts (MeOH and EtOAc) was particularly high in β-carotene-linoleic acid emulsion system. Strong correlations were found between TPC, TFC, TEAC, FRAP, and results of DPPH assay. In conclusion, soursop leaves and fruit seeds and peels, which are cheap, waste plant material, could be considered as a source of phenolic antioxidants with a high antioxidant activity.

INTRODUCTION

In recent years, tropical and exotic fruits have been in the focus of researchers interest. Consumer interest in them increases as well. This is due to the potential health benefits of many tropical and exotic plants. One of them is soursop (*Annona muricata* L.), commonly called graviola, belonging to the *Annonacea* family. Soursop is native to the warmest areas of South and North America and is now widely distributed throughout tropical and subtropical regions of Central and South America, Western Africa, and Southeast Asia [Moghadamtousi *et al.*, 2015; Coria-Tellez *et al.,* 2018]. The soursop fruits are quite large (15–20 cm). The pulp contains 55–170 black seeds covered with green peel. Peels and seeds are inedible parts of soursop fruit, there is a high amount of by-products from this fruit that have not been studied as a source of bioactive compounds [Aguilar Hernandez *et al.,* 2019]. However, in recent years, interest in the utilization of fruit and vegetable by-products has increased due to the potential high content of nutrients and bioactive compounds, such as phenolics, dietary fiber, and vitamins, among others [Kosińska *et al*., 2012; Sagar *et al*., 2018; Kuchtová *et al.*, 2018]. The exotic fruit by-products have previously been considered as a source of valuable food additives of natural origin [Ayala-Zavala *et al.*, 2011].

Aromatic soursop fruits are readily used culinary. Pulp is consumed raw and is used to prepare juice, ice-cream or jelly [Benites *et al.*, 2015]. Moreover, different parts of soursop (leaf, bark, root, fruit, and seed) are used in traditional medicine against several ailments including hypertension, inflammation, diabetes, gastrointestinal disorders, respiratory diseases, and cancers [Coria-Tellez *et al.,* 2018; Chamcheu *et al.,* 2018]. The medicinal activities and the health benefits of *A. muricata* L. have been attributed to their phytochemicals including acetogenins, alkaloids, megastigmanes, phenolics, cyclopeptides, and essential oils [Moghadamtousi *et al.,* 2015].

The phenolic compounds are the major phytochemicals responsible for the antioxidant potential of soursop leaves and fruits [Coria-Tellez *et al*., 2018]. Among them, the phenolic acids (mainly hydroxycinnamic acids), flavonoids, and tannins (including procyanidin dimers) were determined in *A. muricata* L. leaves, pulp, and seeds [Marques & Farah, 2009; Huang *et al.*, 2010; Nawwar *et al.*, 2012; Jiménez *et al.*, 2014; Nam *et al.*, 2017]. Solvent extractions are commonly used to obtain plant extracts with phenolic compounds. These conventional techniques were also applied to soursop materials [da Silva *et al.*, 2014; Nam *et al.*, 2017]. The polarity of the solvent is one of the important parameters of the extraction process. There are some reports indicating that the type of solvent affected the bioactivity of *A. muricata* extracts [George *et al.*, 2015; Chamcheu *et al.*, 2018]. Generally, hexane and petroleum ether are suitable for the extraction

* Corresponding Author: E-mail: horak@nku.edu.tr (H.H. Orak)

of phenolic terpenes. Ethyl acetate is used for the extraction of low-molecular-weight phenolics (phenolic acids and flavonoid aglycons). Methanol, ethanol and their mixtures with water allowed extracting high-molecular-weight phenolics and flavonoid glycosides [Oreopoulou & Tzia, 2007].

The aim of our study was to compare extracts obtained by solvents with increasing polarity from soursop fruit pulps, fruit by-products (seeds and peels), and leaves in terms of their total phenolic and flavonoid contents and their antioxidant activity in polar and lipid emulsion systems. Additionally, the antioxidants of hexane seed extract were looked for using GC-MS analysis.

MATERIAL AND METHODS

Plant material

Leaves and mature fruits of soursop (*A. muricata* L.) were obtained from the Dominica Island in December 2017. Nine fruits were sampled at about 0.6–1.4 kg weight. The soursop fruits were harvested from natural grown trees in the Dominica Island and transferred by plane. The fruits were processed for analysis four days after harvest. Peels (Pl), seeds (S), and pulp (P) were manually separated from fruits. All parts of the fruits as well as leaves (L) were frozen at -40°C and dried using a vacuum freeze dryer (FT 33; Armfield, Ringwood, UK).

Extracts preparation

Dried plant materials were grounded and subjected to successive extraction with solvents of increasing polarity. Hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) were used one after the other. Extraction was carried out for twelve hours at room temperature with pure solvent by using Soxhlet extraction method. The ratio of weights of plant material to the solvent was 1:3. Solvents were evaporated under vacuum (R-210 Rotavapor, B-491 heating bath, V-710 vacuum pump; Büchi Labortechnik, Flawil, Switzerland). Samples were stored at -22°C until analysed.

Total phenolics content (TPC)

The content of total phenolics of soursop extracts was evaluated using Folin-Ciocalteu's reagent. The absorbance of reaction mixtures was read at 725 nm (Hitachi U-2000 spectrophotometer 1210002, Tokyo, Japan) [Amarowicz *et al.,* 2004]. The TPC was expressed as mg (+)-catechin equivalents (CE) per g of extract.

Total flavonoids content (TFC)

The content of total flavonoids of soursop extracts was determined according to the procedure described by Zhishen *et al.* [1999]. The extract (250 μL, concentration of 1–10 mg/mL depending on solvent used) was mixed with distilled water (1.25 mL) and sodium nitrite solution (5%, 75 μL). After 6 min of incubation, aluminium chloride (10%, 150 μL) was added to the mixture followed by sodium hydroxide (1 M, 500 μL). Samples were immediately diluted with distilled water (2.5 mL). The absorbance was measured at 510 nm. The TFC was expressed as mg (+)-catechin equivalents (CE) per g of extract.

Antioxidant activities of soursop extracts

DPPH$^{\bullet}$ scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging activity of soursop extracts was determined by the method of Brand-Williams *et al.* [1995]. Firstly, the methanol (2 mL) and methanolic solution of 1 mM DPPH radicals (0.25 mL) were mixed. Then, extracts (0.1 mL) in different concentrations (0.4–2.0 mg/assay) were added. After the reaction in dark (20 min), the absorbance was measured at 517 nm. The EC_{50} value (the half-maximal effective concentration) was determined on the basis of the plot of absorbance *vs.* extract concentrations.

Trolox equivalent antioxidant capacity (TEAC)

Re *et al.* [1999] method was used to determine TEAC. The portions of 2 mL of [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation (ABTS$^{\bullet+}$) reagent and 20 μL of soursop extracts (from 1–2 mg/mL extract concentrations) were mixed and incubated at 30°C for 6 min. The absorbance of samples was determined at 734 nm and the results were expressed as mmol Trolox equivalents per g of extract.

Ferric-reducing antioxidant power (FRAP)

The FRAP assay was carried out according to Benzie & Strain [1996] procedure. The reaction was performed by mixing the extract solution (75 μL), distilled water (225 μL), and FRAP solution (2.25 mL). The FRAP solution was prepared by mixing 2,4,6-Tri(2-pyridyl)-*s*-triazine (10 mM in 40 mM HCl; 6 mL), acetate buffer (300 mM; pH 3.6; 60 mL), and ferric chloride (20 mM; 6 mL). The mixture was incubated at 37°C (for 30 min) and the absorbance was measured at 593 nm. Ferrous sulfate was used to prepare calibration curve and the results were evaluated as μmol Fe^{2+}equivalents per g of extract.

Cupric ion-reducing antioxidant capacity (CUPRAC)

CUPRAC assay was performed according to Apak *et al.* [2004] method. For determination of the antioxidant activity of soursop extracts, 0.5 mL of $CuCl_2$ solution (10 mM), 0.5 mL of neocuproine ethanolic solution (7.5 mM), 0.5 mL of ammonium acetate buffer (1 M; pH7.0), and 0.25 mL of extract solutions (1–2 mg/mL extract concentrations) were added to the test tubes. The volume of the reaction mixtures was adjusted to 2.05 mL with water. Well-mixed tubes were closed and incubated (30 min at ambient temperature). Absorbance readings were done at 450 nm. The results were calculated based on the calibration curve obtained for Trolox and expressed as mmol Trolox equivalents per g of extract.

β-Carotene-linoleic acid bleaching

The β-carotene-linoleic acid emulsion oxidation was carried out according to Miller [1971] procedure with modifications [Orak *et al.*, 2019]. Firstly, the β-carotene (1.0 mg) was dissolved in chloroform (5 mL). Then, Tween40 (400 mg) and linoleic acid (40 μL) were added. The chloroform was evaporated and water (25 mL) was added to the residue with vigorous stirring. For antioxidant activity measurement,

the emulsion (250 μL) was vortexed with extract solution or standard antioxidant (butylated hydroxyanisole, BHA) solution (100 μL collected from 1 mg/mL concentration). The oxidation reaction temperature was 42°C, the absorbance of samples was monitored in 30 min intervals throughout 180 min at 470 nm. The percentage of non-oxidized β-carotene after 180 min of emulsion oxidation was calculated.

GC-MS analysis

GC-MS analysis was done using the HP 6890 instrument (Hewlett-Packard, Palo Alto, CA, USA) combined with a mass selective detector (GCMS-QP2010 Ultra Shimadzu, Kioto, Japan). The HP-5MS capillary column (5% phenyl methyl siloxane, 30 m × 250 μm, film thickness 0.25 μm, Agilent, Palo Alto, CA, USA) was used. Helium was used as a carrier gas. Its flow rate was 1.0 mL/min. The column initial temperature was 180°C (1 min after injection). The temperature increased to 250°C with an 8°C/min heating ramp in a 1 min holding time, and increased to 300°C with 2°C/min heating ramp in 10 min. The injections (5 μL) were done in the split mode with a split ratio of 10:1. For the analysis, the 250°C was interface temperature, the 280°C was injector temperature and running time was 49 min. MS scan range was m/z 20–440 using electron impact (EI) ionization (70 eV) and an ion source temperature of 250°C. Components were identified according to the comparison of their mass spectra with those of Wiley 9 and NIST library. The relative percentage of separated compounds was determined from Total Ion Chromatogram by the computerized integrator.

Statistical analysis

The MSTAT-C software package was used for statistical analyses. The results were subjected to ANOVA with a Fisher's Least Significant Difference (LSD) post hoc test ($p<0.05$). Moreover, the correlations between variables were determined and Pearson correlation coefficients (r) were calculated.

RESULTS AND DISCUSSION

Extraction yield, total phenolic and total flavonoid contents

The yields of hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) extracts of leaves (L), fruit pulp (P), seeds (S), and peels (Pl) of soursop were between 0.23% and 64.14% (Table 1). The largest yield was obtained for P-MeOH extract (64.14%). MeOH was also the most effective solvent for peels (16.50%) and leaves (12.07%). Hxn was able to extract the largest amount of matter from seeds (24.26%). Yields of MeOH extracts determined in our study were in line with those reported for methanol-water extracts of seeds and pulps of some *Annona* species fruits; *e.g.*, *A. coriacea* L. (14.5% and 20.5%, respectively) and *A. sylvatica* L. (8.7% and 5.2%, respectively) [Benites *et al.,* 2015] as well as for methanolic extract of *A. muricata* L. leaves (10.30%) [Nam *et al.,* 2017].

The TPC varied in the range of 10.92–244.61 mg CE/g in leaf extracts; 20.75–187.48 mg CE/g in peel extracts; 19.84–50.15 mg CE/g in pulp extracts; and 5.06–202.17 mg CE/g in seed extracts (Table 1). MeOH extracts had a much higher TPC than the extracts obtained using other solvents (except fruit pulp extracts). In the case of fruit pulp, EtOAc was a more effective ($p<0.05$) phenolic compound extractant. Hxn extracts had the lowest TPC ($p<0.05$). Converting TPC of extracts by extraction yields, it can be noted that peels and leaves were the richest sources of phenolic compounds, followed by pulp and seeds. Higher TPC of soursop fruit pulp compared to that of seeds was in line with literature data [da Silva *et al.*, 2014]. Moreover, higher TPC in the peels than in the pulp of fruits of different *Annona* species (*A. cherimola* L. and *A. squamosal* L.) was previously reported [Loizzo *et al.*, 2012; Huang *et al.*, 2010].

The TFC of extracts is shown in Table 1. The highest TFC was determined in L-MeOH extract (81.32 mg/CEg) and the lowest one in S-Hxn extract (1.54 mg CE/g). Generally, TFC of fruit pulp and by-products decreased in the following order L>S≥Pl>P. When the results were compared based on the extraction solvent used, MeOH and EtOAc extracts had the highest TFC. On the other hand, as could be expected, Hxn was the least effective solvent for flavonoid extraction. Loizzo *et al.* [2012] reported that TFC/TPC ratios of *Annona* fruit peel and pulp ranged from 0.3 to 0.6. In our study, similar values were obtained for MeOH extracts, but TFC/TPC ratios of EtOAc extracts were significantly higher, *i.e.* at about 0.9. This indicates good selectivity of EtOAc for flavonoid extraction from soursop fruits

TABLE 1. The extract yield, total phenolic content (TPC) and total flavonoid content (TFC) of soursop (*A. muricata* L.) leaves (L), fruit pulp (P), peels (Pl) and seeds (S) extracts obtained using hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH).

Extract	Extract yield (%)	TPC (mg CE/g)	TFC (mg CE/g)
L-Hxn	3.66	10.92 ± 1.28^{i}	2.62 ± 0.19^{i}
L-DCM	1.10	30.60 ± 2.71^{f}	26.46 ± 1.57^{e}
L-EtOAc	0.83	73.42 ± 3.48^{d}	65.98 ± 4.79^{b}
L-MeOH	12.07	244.61 ± 7.00^{a}	81.32 ± 3.45^{a}
Pl-Hxn	0.59	20.75 ± 0.20^{h}	1.68 ± 0.09^{j}
Pl-DCM	0.23	27.35 ± 0.50^{g}	15.77 ± 0.22^{f}
Pl-EtOAc	0.25	56.33 ± 4.97^{e}	50.22 ± 2.90^{c}
Pl-MeOH	16.50	187.48 ± 6.78^{c}	36.10 ± 1.04^{cd}
P-Hxn	0.71	19.84 ± 0.90^{h}	2.16 ± 0.12^{i}
P-DCM	0.25	26.23 ± 0.96^{g}	13.34 ± 0.28^{g}
P-EtOAc	0.26	50.15 ± 4.57^{e}	34.41 ± 2.20^{d}
P-MeOH	64.14	38.36 ± 2.12^{f}	13.95 ± 0.19^{g}
S-Hxn	24.26	5.06 ± 1.37^{j}	1.54 ± 0.08^{i}
S-DCM	3.01	20.70 ± 5.00^{h}	11.45 ± 0.59^{h}
S-EtOAc	0.58	53.73 ± 2.81^{e}	48.04 ± 2.11^{c}
S-MeOH	3.66	202.17 ± 12.99^{b}	56.59 ± 5.29^{c}

Data are expressed as the mean ± standard deviation for each extract (n=3). Values in the same column having different superscript letters differ significantly ($p< 0.05$). CE: catechin equivalents.

and leaves. In previous studies, the presence of flavonoids belonging to subclasses of flavan-3-ols and flavonols was determined in soursop leaves, fruit pulp, and peels [Huang *et al.*, 2010; Nawwar *et al.*, 2012; Jiménez *et al.*, 2014; Nam *et al.*, 2017]. Besides flavonoids, hydroxycinnamic acid derivatives were identified in leaves and pulp [Marques & Farah, 2009; Jiménez *et al.*, 2014; Nam *et al.*, 2017]. In turn, phenolic terpenoids were found in soursop seeds [Huang *et al.*, 2010].

Antioxidant activity of soursop leaf and fruit part extracts

Five assays in which antioxidants act as free radical scavengers (TEAC and DPPH assay), as reducing agents (FRAP and CUPRAC) or as inhibitors of the lipid substrate oxidation (β-carotene-linoleic acid bleaching assay) were used to determine the antioxidant activities of extracts of soursop leaves and fruit pulps, peels and seeds.

The DPPH$^{\bullet}$ scavenging activity of the soursop extracts was expressed as EC_{50} values. The results are presented in Table 2. The highest antiradical activity against DPPH$^{\bullet}$ with the lowest EC_{50} value had the S-MeOH extract (0.044 mg/mL). The lowest antiradical activity was determined for the P-Hxn extract (EC_{50} 0.411 mg/mL). In addition to S-MeOH, other methanolic extracts were also characterized by low EC_{50} values, especially in the case of leaf (0.063 mg/mL) and peel (0.090 mg/mL). The extract obtained with use of ethyl acetate and dichloromethane had intermediate EC_{50} values for each of the plant materials except peels where antiradical activity of Pl-Hxn and Pl-DCM extracts as well as Pl-DCM and Pl-EtOAc extracts did not differ significantly ($p>0.05$). Given the type of extracted material, the DPPH$^{\bullet}$ scavenging activity decreased generally in the following order: S>L>Pl>P. The TEAC values shown in Table 2 indicate the ability of soursop extract to inactivate ABTS$^{\bullet+}$. Compared to DPPH$^{\bullet}$ scavenging activity, the highest TEAC was determined for S-MeOH (0.905 mmol Trolox/g) and L-MeOH (0.848 mmol Trolox/g) extracts. Moreover S-EtOAc (0.572 mmol Trolox/g), L-EtOAc (0.474 mmol Trolox/mg), and Pl-MeOH (0.438 mmol Trolox/g) extracts had high TEAC. Plant materials could be ordered as follows: S ≥ L > Pl >P, if decreasing TEAC values of MeOH and EtOAc extracts were considered.

TABLE 2. Ferric-reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC) and DPPH$^{\bullet}$ scavenging activity of soursop (*A. muricata* L.) leaves (L), peels (Pl), fruit pulp (P), and seeds (S) extracts obtained using hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH).

Extract	TEAC (mmolTrolox/g)	FRAP (μmol Fe^{2+}/g)	EC_{50} DPPH (mg/mL)
L-Hxn	0.222±0.029^{i}	66.5±0.48^{l}	0.312±0.04^{c}
L-DCM	0.242±0.008^{hi}	104.0±1.7^{ij}	0.143±0.02^{h}
L-EtOAc	0.474±0.009^{d}	339.7±5.9^{e}	0.136±0.06^{h}
L-MeOH	0.848±0.011^{b}	798.9±2.4^{b}	0.063±0.04^{k}
Pl-Hxn	0.251±0.018^{h}	102.8±4.3^{j}	0.264±0.02^{e}
Pl-DCM	0.253±0.014^{h}	180.7±4.2^{h}	0.286 ±0.02^{d}
Pl-EtOAc	0.300±0.005^{f}	284.1±6.8^{f}	0.277±0.10^{de}
Pl-MeOH	0.438±0.005^{e}	465.2±8.0^{c}	0.090±0.05^{j}
P-Hxn	0.180±0.012^{j}	75.9±3.0^{k}	0.411±0.03^{a}
P-DCM	0.225±0.029^{i}	117.9±1.8^{i}	0.328±0.05^{b}
P-EtOAc	0.280±0.002^{g}	210.4±9.6^{g}	0.307±0.02^{c}
P-MeOH	0.104±0.002^{k}	97.0±2.1^{j}	0.281±0.04^{d}
S-Hxn	0.110±0.009^{kj}	33.2±4.2^{m}	0.231±0.02^{f}
S-DCM	0.201±0.006^{ij}	77.5±3.2^{k}	0.191±0.04^{g}
S-EtOAc	0.572±0.025^{c}	447.4±3.7^{d}	0.115±0.02^{i}
S-MeOH	0.905±0.029^{a}	1100.6± 9.3^{a}	0.044±0.02^{l}

Data are expressed as the mean ± standard deviation for each extract (n=3). Values in the same column having different letters differ significantly ($p < 0.05$).

The ability of extracts to reduce Fe^{3+} (FRAP) and Cu^{2+} (CUPRAC) is shown in Table 2 and Figure 1, respectively. The FRAP ranged from 33.2 to 1100.6 mmol Fe^{2+}/g in seed

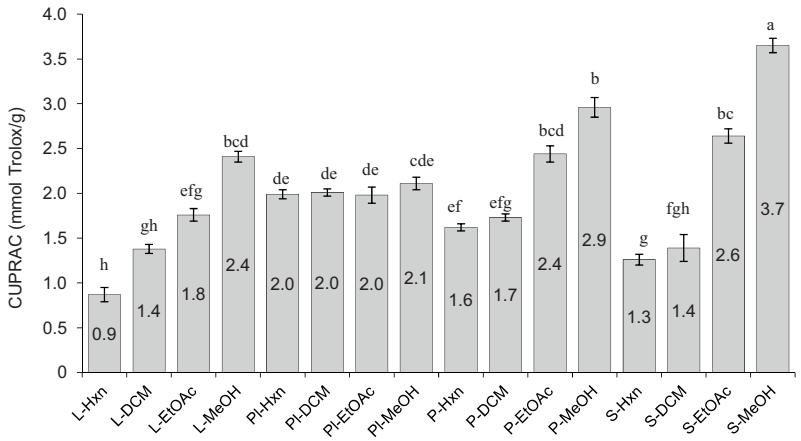

FIGURE 1. Cupric ion reducing antioxidant capacity (CUPRAC) of soursop (*A. muricata* L.) leaves (L), fruit pulp (P), peels (Pl), and seeds (S) extracts obtained using hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH). Data are expressed as mean ± standard deviation (n=3) for each extract. Bars having different letters differ significantly (p<0.05).

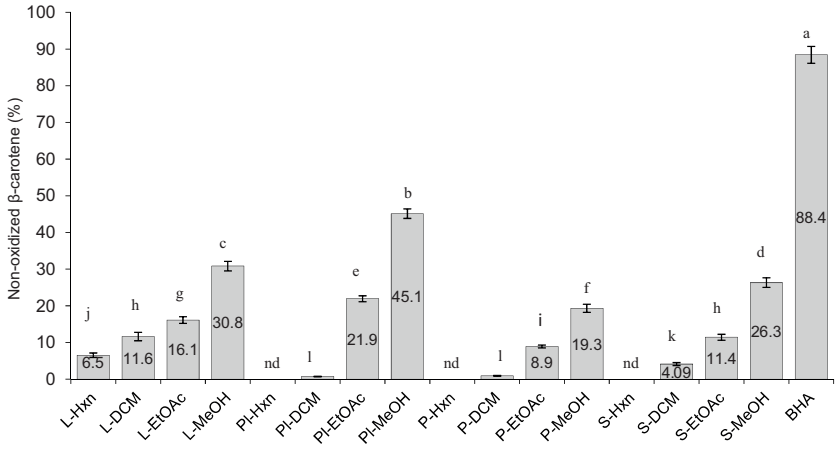

FIGURE 2. Inhibition of β-carotene-linoleic acid emulsion oxidation by soursop (*A. muricata* L.) leaves (L), fruit pulp (P), peels (Pl), and seeds (S) extracts obtained using hexane (Hxn), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH). Data are expressed as mean ± standard deviation (n = 3) for each extract. Bars having different letters differ significantly ($p<0.05$); nd – not detected.

extracts, from 75.9 to 210.4 μmol Fe^{2+}/g in pulp extracts, from 102.8 to 465.2 mmol Fe^{2+}/g in peel extracts, and from 66.4 to 798.9 mmol Fe^{2+}/g in leaf extracts. The differences between CUPRAC of soursop extracts were significant ($p<0.05$). The values ranged from 0.87 mmol Trolox/g to 3.65 mmol Trolox/g. In both assays, again, the S-MeOH and L-MeOH extracts exhibited the highest activity and hexane was the least effective in the extraction of compounds with the ability to reduce metal ions.

Antioxidant activity of soursop extracts determined in the β-carotene-linoleic acid emulsion system is shown in Figure 2. The results are slightly different from those obtained in the previously discussed assays, because after 180 min of oxidation, the most of non-oxidised β-carotene (45.1%) remained in Pl-MeOH extract. Among the EtOAc extracts, the peel extract also had the highest ability to inhibit emulsion oxidation. However, the antioxidant activity of L-MeOH and S-MeOH extracts was also high; the extracts inhibited β-carotene oxidation at 30.8% and 26.3%, respectively. In turn, all Hxn and some of DCM extracts were not able to inhibit the oxidation of the emulsion. All extracts showed a lower antioxidant activity than BHA.

The higher antioxidant activity of soursop seed extracts (MeOH and EtOAc) compared to pulp extracts determined in our studies in all used assays was in line with Benite *et al*. [2015] report in which ABTS, DPPH and β-carotene--linoleic acid bleaching assays of soursop seed and pulp methanol-water extracts were carried out. In turn, Loizzo *et al.* [2012] found that ethanolic extract from *A. cherimola* L. peel had higher FRAP, DPPH$^{\bullet}$ scavenging activity and ability to inhibit oxidation of β-carotene-linoleic acid emulsion than extract from pulp which is also accordance with our finding. However, in mentioned study the significant difference between ABTS results for peel and pulp extracts was not noted.

The results of correlation analysis are shown in Table 3. TPC of extracts of soursop leaves and fruit parts was significantly correlated ($p<0.05$) with TFC ($r=0.761$) as well as with results of antioxidant assays, especially with FRAP ($r=0.899$), TEAC ($r=0.872$), and emulsion oxidation ($r=0.865$). Weaker correlation was noted only between TPC and CUPRAC ($r=0.589$). The correlations of TFC with FRAP, TEAC, and results of emulsion oxidation were also significant ($p<0.05$), and confirmed by high correlation coefficients – 0.900, 0.887 and 0.713, respectively. In a previous study, strong correlations between TPC and antioxidant activities determined by FRAP and DPPH assays were reported for soursop leaf extracts obtained with using different solvents [George *et al.*, 2015]. In turn, Nam *et al.* [2017] found that r values of correlations between TPC and antiox-

TABLE 3. Pearson's correlation coefficients (r) between total phenolic content (TPC), total flavonoid content (TFC), and results of antioxidant assays of extracts of soursop (*A. muricata* L.) leaves and fruit pulp, peel and seed.

	TPC	FRAP	TEAC	CUPRAC	DPPH$^{\bullet}$ (EC_{50})	Emulsion oxidation
TFC	0.761	0.900	0.887	0.646	-0.680	0.713
TPC	1	0.899	0.872	0.589	-0.719	0.865
FRAP		1	0.968	0.724	-0.753	0.739
TEAC			1	0.731	-0.807	0.640
CUPRAC				1	-0.655	0.388
DPPH$^{\bullet}$ (EC_{50})					1	-0.477

FRAP: ferric-reducing antioxidant power; TEAC:Trolox equivalent antioxidant capacity; CUPRAC: cupric ion reducing antioxidant capacity.

TABLE 4. Chemical compounds of soursop (*A. muricata* L.) seed hexane extract identified by GC-MS.

Peak	RT	Compound	%
1	8.83	Decane	0.57
2	12.41	Undecane	0.34
3	16.12	Tridecane	0.45
4	18.29	(*E*)-2-Decenal	1.28
5	19.43	(*E*,*E*)-2,4-Decadienal	3.23
6	23.18	Tetradecane	0.55
7	26.46	Pentadecane	0.14
8	26.78	β-Bisabolene	0.83
9	27.26	β-Sesquiphellandrene	0.53
10	28.46	(*E*)-Nerolidol	3.62
11	29.33	1-Heptadecene	0.24
12	29.57	*n*-Octadecane	0.34
13	32.52	Heptadecane	0.15
14	34.16	Tetradecanoic acid	0.29
15	35.33	Nonadecane	0.13
16	38.01	2-Nonadecanon	0.15
17	38.65	Hexadecenoic acid, methyl ester	0.21
18	39.69	Pentadecanoic acid	19.92
19	40.39	1-Nonadecene	1.17
20	41.04	Hexadecanal	0.26
21	41.99	Heptadecanoic acid	0.16
22	42.83	9,12-Octadecadienoic acid(*Z*,*Z*), methyl ester	0.17
23	42.99	6-Octadecenoic acid, methyl ester	0.31
24	43.89	9,12-Octadecadienoic acid (*Z*,*Z*)	15.58
25	44.06	9-Octadecenoic acid	27.82
26	44.47	Octadecanoic acid	3.40
27	44.59	(*Z*)-9-Octadecenoic acid, ethyl ester	1.70
28	44.84	Hexadecanamide	0.64
29	45.01	Hexadecanoic acid, butyl ester	0.20
30	45.20	1-Nonadecene	0.94
31	45.88	Octadecanal	0.23
32	47.57	Heneicosane	0.20
33	48.72	6,9-Octadecadienoic acid, methyl ester	0.39
34	48.84	(*Z*)-9-Octadecenamide	3.04
35	49.38	Octadecanamide	0.20
36	49.61	1-Eicosanol	0.61
37	51.31	Di-(9-octadecenoyl)-glycerol	0.13
38	53.04	Phthalic acid mono-2-ethylhexyl ester	0.27
39	53.40	Oxirane, hexadecyl	0.27
40	56.12	Humulane-1,6-dien-3-ol	0.52
41	57.18	Glyceryl trioleate	0.67
42	58.39	(3-β)-Stigmast-5-en-3-ol	7.45
43	59.29	Bis (2-ethylhexyl) phthalate	0.34
44	59.90	Urs-12-ene	0.28

RT: Retention times

TABLE 5. The chemical class distribution of the compounds of soursop (*A. muricata* L.) seed hexane extract.

Chemical class of compounds	Distribution (%)
Fatty acids	67.17
Unsaturated fatty acids	43.40
Saturated fatty acids	23.77
Terpenoids	13.23
Alkanes	2.87
Alkenes	2.35
Aldehydes and ketones	5.15
Alcohols	0.74
Esters	4.26
Amides	3.88
Epoxides	0.27
Total	99.92

idant activity (FRAP, ABTS, and DPPH assays) of extracts of different parts of *A. muricata* L. were higher compared to those determined for the TFC – antioxidant activity correlation. In the present study, the FRAP, TEAC, and CUPRAC values were significantly ($p<0.05$) correlated with each other, wherein the highest r value (0.968) was noted for TEAC and FRAP correlation (Table 3). Strong, negative correlations were found between EC_{50} values of DPPH assay and FRAP and TEAC. This finding was in line with literature data [Nam *et al.*, 2017]. Additionally, the lower r value was determined for correlations between emulsion oxidation results and results of CUPRAC ($r=0.388$) and DPPH assay ($r=-0.477$) (Table 3).

GC-MS analysis of hexane extract of soursop seeds

The hexane extract of soursop seeds was obtained with a high yield (Table 1). The TPC and TFC of this extract were low. Despite this, it showed some antioxidant activity. Therefore, GC-MS analysis of hexane seed extract was carried out in search of potential antioxidants.

The GC-MS analysis allowed identifying 44 compounds in the hexane extract. These compounds were characterized by their retention time (RT), their molecular formula, and contents which were calculated based on peak area (%) (Table 4). According to chemical class distribution, fatty acids were most abundant (67.17%), followed by terpenoids (13.23%), aliphatic hydrocarbons (alkanes/alkenes) (5.22%), aldehydes and ketones (5.15%), esters (4.26%), alcohols (0.74%), and amides (3.88%) (Table 5). Unsaturated fatty acids constituted 43.40% of all determined compounds. The content of saturated fatty acids was 23.77%. Oleic acid (9-octadecenoic acid; 27.82 %) and linoleic acid (9,12-octadecadienoic acid; 15.58%) were the major unsaturated fatty acids in the soursop seed hexane extract. Especially, linoleic acid is known as an essential fatty acid with an important metabolic role [Eromosele & Eromosele, 2002]. The high

content of oleic and linoleic acids in the hexane seed extract confirmed previous findings. Both acids were found as dominant in *A. muricata* L. seed oil [da Silva & Jorge, 2017; Pinto *et al.*, 2018]. In turn, the percentage of stearic acid (pentadecanoic acid) in total fatty acids was low compared to the result presented in our study (19.92%). Among phytosterols, the content of 3-β-stigmast-5-en-3-ol (7.45%) was the highest in the hexane extract (Table 4). da Silva & Jorge [2017] noted that this compound was the major phytosterol of soursop seed oil. The antioxidant activity of 3-β-stigmast-5-en-3-ol examined both *in vitro* (DPPH and ABTS assays) and *in vivo* had already been reported [Ayaz *et al*., 2017]. Its anti-proliferative properties were noted as well [Moon *et al.,* 2008]. Terpenoids are another class of compounds with recognized antioxidant activity; they were detected in the analysed soursop hexane seed extract. The main terpenoid in the extract was (*E*)-nerolidol (Table 4). Chan *et al.* [2016] reviewed various biological activities of this sesquiterpene alcohol, including its antioxidant activity. The major aldehydes in the extract were identified as (*E*)-2-decenal (1.28%) and (*E*,*E*)-2,4-decadienal (3.23%). Caboni *et al.* [2012] reported a high nematicidal activity of both compounds. In turn, Cheng *et al.* [2008] suggested antioxidant activity of (*Z*)-9-octadecenamide, which was also present in soursop seed hexane extract (Table 4).

CONCLUSIONS

The successive extraction of soursop (*Annona muricata* L.) leaves, fruit pulps, seeds, and peels with hexane, dichloromethane, ethyl acetate, and methanol allowed obtaining extracts with different antioxidant activity. More polar solvents were better extractants of antioxidants; methanol extracts were characterised by the highest total phenolics content while both methanol and ethyl acetate extracts were rich sources of flavonoids. Considering each plant material individually (leaves, seeds, and peels), the methanol and ethyl acetate extracts had the highest antioxidant activity examined as antiradical activity (TEAC and DPPH assay), as ability to reduce metal ions (FRAP and CUPRAC), and as ability to inhibit β-carotene-linoleic acid emulsion oxidation. Besides, while ethyl acetate was a good solvent for the extraction of antioxidants from pulp and peel, methanol was better for leaves and seeds. Low total phenolics and especially total flavonoids contents of the hexane extract was correlated with their low antioxidant activity. However, bioactive constituents of the hexane seed extract, such as terpenoids and phytosterols, could positively influence its antioxidant activity. (*E*)-Nerolidol and (3-β)-stigmast-5-en-3-ol, both with previously recognised antioxidant activity, were determined in the hexane seed extract as major terpenoid and phytosterol, respectively.

When considering the total phenolic and flavonoid contents of extracts, the best material turned out to be leaves and seeds. Methanol and ethyl acetate extracts of these materials had a high antioxidant activity in non-lipid assays (DPPH$^{\bullet}$ scavenging activity, TEAC, FRAP, and CUPRAC). Antioxidant activity of peel extracts was particularly high in the β-carotene-linoleic acid emulsion system. Pulp extracts showed the lowest antioxidant activity with lower total phenolic and flavonoid contents. Strong correlations were found between total phenolic and flavonoid contents and antioxidant activity determined as TEAC, FRAP, and DPPH$^{\bullet}$ scavenging activity.

Our study showed that soursop leaves and soursop by-products from fruit processing (seeds and peels) have the potential to be used to obtain extracts with a high antioxidant activity.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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Original article
Section: Food Chemistry

Variation in the Phenolic Compounds Profile and Antioxidant Activity in Different Parts of Hawthorn (*Crataegus pentagyna* Willd.) During Harvest Periods

Jovana Pavlović[1], Snežana Mitić[1], Milan Mitić[1], Gordana Kocić[2], Aleksandra Pavlović[2], Snežana Tošić[1]*

[1]Department of Chemistry, Faculty of Science and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia
[2]Department of Biochemistry, Faculty of Medicine, University of Niš, Bulevar dr Zorana Đinđića 81, 18 000 Niš, Serbia

Key words: phenolic compounds, *Crataegus pentagyna* Willd., RP-HPLC, principal component analysis, leaves, flowers, fruits

Plants of the genus *Crataegus*, Rosaceae, are widely distributed and have long been used for food and in folk medicine for the treatment of various ailments. This study focused on HPLC-DAD-FLD identification of phenolic compounds of flowers, fruits, and leaves of hawthorn (*Crataegus pentagyna* Willd.), on monitoring changes in the content of phenolic compounds during different harvesting periods, as well as on the *in vitro* testing of the antioxidant activity. Study results showed that neochlorogenic and chlorogenic acids (5.2 and 4.85 mg/g dry weight, respectively) were the most abundant phenolic compounds in the flowers. (-)-Epicatechin and procyanidin B2 were the most abundant phenolic compounds in leaves and fruits. The highest content of phenolic compounds was found in leaves harvested in the early maturity stages, with a tendency of declining in the later stages of maturity. On the other hand, in the case of fruits, the most noticeable changes were found in the content of flavan-3-ols. The highest content of (-)-epicatechin (21.1 mg/g fresh weight) in fruit was in August, and those of procyanidins B2 and B5 in September (10.6 and 3.74 mg/g fresh weight, respectively). Statistical analysis showed a strong correlation between the total phenolics content and the antioxidant activity. This study has demonstrated that there is seasonal variation in both the quantity and the type of phenolic compound as well as the antioxidant activity of different parts of hawthorn.

INTRODUCTION

Knowledge and experience of herbal treatment acquired for centuries have been held up to this day as traditional medicine. In addition to the application in health care, medicinal and aromatic plants are increasingly used in various industries: pharmaceutical, food, cosmetic, chemical, and others. Such extensive application has contributed to the increase in economic significance of medicinal and aromatic plants and provided the possibility of revenue on different levels of production, processing, and distribution. Frequent and widespread use of herbs throughout the world has increased significant concerns over their quality, safety, and efficacy. Thus, sound scientific evidence or assessment have become the criteria for acceptance of traditional health claims. The genus *Crataegus* comprises of a complex group of trees and shrubs native to Northern temperate zones, mostly between latitudes 30° and 50° N. *Crataegus* belongs to the subfamily Maloideae in the Rosaceae, a natural group of complex genera with the ability to interbreed freely [Albarouki & Peterson, 2007; Kumar *et al*., 2012]. There are over 1000 species of *Crataegus* distributed primarily in Asia, Europe, and North America [Zhao & Tian, 1996]. Different species dominate and different parts of the plant are used in these regions. For example, *Crataegus pinnatifida* Bge. and *Crataegus scabrifolia* are the most important species in China, whereas *Crataegus laevigata* Poir. (syn: *Crataegus oxyacantha* L.), *Crataegus monogyna* Jacq., *Crataegus pentagyna* Waldst., *Crataegus nigra* Waldst., and *Crataegus azarolus* L. are the major species in Europe [Rayyan *et al*., 2005; Liu *et al*., 2011].

Crataegus species have long been used in traditional Chinese and European herbal medicine. In addition to being highly recommended in folk medicine, hawthorn berries are valuable food ingredient due to the content of nutrients and compounds with health benefits [Bernatoniene *et al*., 2008; Tadić *et al*., 2008]. Hawthorn berries are used to prepare jams, jelly, drinks, wine, and canned fruit.

Fruits and leaves of hawthorn are rich in phenolic compounds, which are considered to be the key bioactive compounds in this plant, accounting for its antioxidant activity and other beneficial biological effects [Bleske *et al*., 2008]. The main components of hawthorn are thought to be flavones, flavonols, flavan-3-ols (especially (-)-epicatechin), and flavan-3-ol oligomers (procyanidins) [Liu *et al*., 2011; Svedström *et al*., 2006; Yang & Liu, 2012]. Oligomeric procyanidins and their glycosides are the major phenolic compounds in fruits, whereas flavonols, flavonol glycosides, and *C*-glycosyl flavones dominate in leaves [Liu *et al*., 2011]. On the basis of literature data, quercetin, kaempferol, and sexangularetin are the major flavonols in hawthorn [Yang & Liu, 2012]. Rutin and hyperoside are the predominant flavonol glycosides, while vitexin, vitexin-2''-*O*-rhamnoside,

* Corresponding Author: E-mail: jovanapavlovic@ymail.com (J. Pavlović)

and acetylvitexin-2''-*O*-rhamnoside are the most prevalent flavone glycosides [Liu *et al*., 2011].

Various factors (climatic, agronomic, genomic, pre- and post-harvest conditions, and processing) may affect the chemical composition of plant foods and may have a significant role in determining the phenolic composition and the bioactivity of these compounds [el Amira *et al*., 2012; Imeh & Khokhar, 2002]. Contents of phenolics are often dependent on pre- and post-harvest factors such as species, environmental characteristics, agronomic features, ripeness, harvesting, storage, *etc.* [Routray & Orsat, 2014; Tiwari & Cummins, 2013]. Owing to all the above mentioned, many types of researches have addressed changes in the content of bioactive components of plants [Ben Ahmed *et al*., 2017; Cezarotto *et al*., 2017; Hlel *et al*., 2017; Rimkiene *et al*., 2017].

As mentioned above, the composition and the content of phenolic compounds in hawthorn vary between different parts of the same plant and differ significantly depending on the origin and species of hawthorn. The maturity stage is also an important factor that may influence the compositional quality of hawthorn. Bearing in mind that the plant chemical composition is conditioned by all of the above, it is essential to evaluate the optimal cultivation time in order to obtain the highest amount of bioactive compounds. The aim of our research was to investigate the qualitative and quantitative composition and the antioxidant activity of phenolic compounds in different parts of hawthorn plant (*C. pentagyna* Willd.) and also to provide information about composition changes within different harvest periods.

MATERIAL AND METHODS

Chemical and reagents

The following reagents were used: 2,2'-azino-bis(3--ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteu's phenol reagent, neocuproine, potassium peroxodisulfate, ammonium iron(II) sulfate hexahydrate, iron(III) chloride, potassium hexacyanoferrate(III), sodium hydroxide, sodium carbonate, sodium dihydrogen phosphate, sodium hydrogen phosphate, trichloroacetic acid, hydrochloric acid, acetic acid, formic acid, and acetonitrile (Merck, Darmstadt, Germany). The following certification standards were also used: *p*-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, quercetin, quercetin 3-*O*-glucoside (isoquercetin), quercetin 3-*O*-rutinoside (rutin), kaempferol 3-*O*-glucoside, (-)-epicatechin, hyperoside, luteolin, apigenin, cyanidin 3-*O*-glucoside, procyanidins B1 and B2 (Sigma Aldrich, Steinheim, Germany). 6-Hydroxy--2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Acros Organics (Morris Plains, New Jersey, USA). Solutions were prepared using deionized water (specific conductivity 0.05 μS/cm) which was produced using MicroMed high purity water systems (TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany).

Plant material

Hawthorn (*C. pentagyna* Willd.) flowers, fruits, and leaves were picked from the field in Bela Palanka, Serbia, from May 19 to October 6, 2015, at roughly one-month intervals. One flower sample, four fruit samples, and five leaf samples were collected at each harvest date. Three shrubs were randomly chosen in the field for the uniformity of tree development, light exposure, and health. Leaves were collected in May and June, from the apical position of ten young growing shoots of comparable length and position near the base of each shrub. The leaves from each shoot were pooled together. Leaves from July to September were collected from three different positions on three different shrubs: basal, middle, and terminal. Leaves from each shrub were pooled together. Immediately after the harvest, the fruits were frozen and stored at -18°C until analysis, while flowers and leaves were air dried in shade and ground.

Extraction

Plant material was extracted by maceration, combined with ultrasound-assisted solvent extraction. Hawthorn fruit was defrosted and blended. Defrosted tissue, dried flower, and leaves (0.2 g of each) were mixed with 15 mL of 80% acetone and kept at room temperature for 48 h. Afterwards, liquid was decanted, while the residual plant material (marc) and the rest of the liquid were transferred to a Büchner funnel (diameter 55 mm) for pressure-assisted filtration and pressed with porcelain discs (500 g) of the same diameter as the Büchner funnel for 30 min. The obtained decanted, filtrated, and pressed out liquids were mixed together. Subsequently, the marc was subjected to ultrasound-assisted extraction for 15 min with 10 mL of 80% acetone, five successive times. The liquid after each ultrasound-assisted extraction step was collected and pooled together with the liquid obtained previously from maceration. The pooled extraction liquid was filtrated through Whatman filter paper (black ribbon) and supplemented with 80% acetone to the final volume of 50 mL [Bravo & Mateos, 2008; Katalinic *et al*., 2010; Munhoz *et al*., 2014].

Determination of total phenolics content

Total phenolics (TP) content was determined with the spectrophotometric method using the Folin-Ciocalteu's phenol reagent [Singleton *et al*., 1999]. Briefly, 50 μL of each acetone extract was mixed with 0.25 mL of the Folin-Ciocalteu reagent and 1 mL of 20% (w/v) sodium carbonate solution, and made up to 10 mL with deionized water. After standing for 2 h at room temperature, absorbance was measured at 760 nm using an Agilent UV/VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Results were expressed as mg of gallic acid equivalents per g of dry weight (mg GAE/g dry weight) for leaves and flowers and as mg of gallic acid equivalents per g of fresh weight (mg GAE/g fresh weight) for fruits. All measurements were performed in triplicate.

High-performance liquid chromatography (HPLC)

Qualitative analysis of phenolic acids, flavonols, flavan-3-ols, flavones, and anthocyanins was performed with the HPLC, employing a direct injection method [Mitić *et al*., 2012]. The analysis was performed using the HPLC system (Agilent 1200 series, Agilent Technology, Santa Clara, CA, USA), equipped with four solvent delivery units G1354A, diode array detector (DAD) G1315D, fluorescence detec-

tor (FAD) G1321A, and HP Chemstation chromatography workstation. Chromatographic analyses were performed on 150 mm x 4.6 mm i.d., Zorbax Eclipse XDB C_{18} column (Agilent Technologies, Santa Clara, CA, USA). The column was thermostated at 30°C. The flow rate was 0.8 mL/min and the injection volume was 5 μL. The mobile phase were: A: H_2O-HCOOH (95:5, v/v) and B: ACN-HCOOH-H_2O (80:5:15, v/v/v). The gradient procedure was: 0–10 min with 0% B, 10–28 min gradually increases 0–25% B, from 28 to 30 min 25% B, from 30 to 35 min gradually increases 25–50% B, from 35 to 40 min gradually increases 50–80% B, and finally for the last 5 min gradually decreases 80–0% B. Eluate was monitored at 280 nm, 320 nm, 360 nm, and 520 nm with DAD, for the identification of flavones, flavonols, hydroxycinnamic acids, and anthocyanins. Four flavan-3-ols including: (-)-epicatechin, procyanidin B, procyanidin B5, and procyanidin B2, were monitored at 275/322 nm ($\lambda_{Ex}/\lambda_{Em}$) with FLD. Phenolics present in the samples were identified by comparing their retention times and spectra with those of pure components. Quantification of components was performed using external standards. For each standard, stock solutions (1.0 mg/mL) were prepared in 10% (v/v) methanol. Concentrations of components in the samples were calculated from the equation obtained from calibration graphs, constructed for each standard, while for components without the appropriate standard, quantification was performed based on a calibration graph plotted for structurally similar standards, *i.e*: *p*-coumaric acid was used to express content of 4-*O*-coumaroylquinic acid; chlorogenic acid was used as the standard for 4-*O*-caffeoylquinic acid (cryptochlorogenic acid) and 5-*O*-caffeoylquinic acid (neochlorogenic acid); kaempferol 3-*O*-glucoside, quercetin 3-*O*-rutinoside, luteolin and apigenin were used to express contents of kaempferol rutinoside, quercetin rhamnoside, luteolin and apigenin derivatives, respectively; (-)-epicatechin was the standard for procyanidin B2, B5 and PC derivative; and cyanidin 3-*O*-glucoside for ideain (cyanidin 3-*O*-galactoside). All chemicals and solvents were of analytical or HPLC purity. All analyses were performed in triplicate.

Antioxidant activity assays

The antioxidant capacities of hawthorn parts (flowers, fruits, and leaves) were studied in four antioxidant assays: scavenging DPPH radical [Brand-Williams *et al*., 1995], scavenging ABTS radical cation [Re *et al*., 1999], iron (III) to iron(II) reduction power (FRP) assay [Oyaizu, 1986], and cupric ion reducing antioxidant capacity (CUPRAC) assay [Apak *et al*., 2006]. The antioxidant activities of hawthorn from all the assays were expressed as μmol of Trolox equivalent (TE) per g of fresh weight of fruits and dry weight of flowers and leaves.

Statistical analysis

Correlation statistical analysis was performed using Statistica software (StatSoft 8.0, Tulsa, Oklahoma, USA). Data were expressed as mean values and standard deviations (SD). Correlation analysis between the total phenolics content, the antioxidant activities of four independent assays (DPPH, ABTS, CUPRAC, and FRP) and the sum of contents of compounds from different classes of phenolics was performed with the bivariate (Pearson's) correlation test at a significance level of $p<0.05$.

The analysis of variance (one-way ANOVA) was applied on the following set of variables: quantitative variables (total phenolics content, content of individual phenolics, the sum of the contents of phenolic compounds belonging to different classes, and the antioxidant activity measured by four different assays) and qualitative (explanatory) variable: harvest period (leaves and fruit samples harvested in different periods). Tukey's Honestly Significantly Different (HSD) test was applied to pairwise all differences between means at $p<0.05$.

The principal component analysis (PCA) was applied on the following set of variables: content of individual phenolic compounds in flower, fruit, and leaves, and the sum of the contents of individual phenolic compounds belonging to different classes (hydroxycinnamic acids, flavonols, flavones, flavan-3-ols, and anthocyanins), as well on the sum of the contents of individual phenolic compounds belonging to different classes in leaves and fruits and the antioxidant activity measured by four different methods. Each principal component was calculated by using linear combination of eigenvectors of the correlation matrix with original values. PCA and ANOVA were performed using Excel plug-in program XLSTAT, version 2014.4 (Addinsoft, New York, NY, USA).

RESULTS AND DISCUSSION

Total and major classes phenolics content

Total phenolics content of hawthorn flowers, fruits, and leaves is presented in Table 1. The sum of the contents of phenolic compounds from particular classes is presented in Tables 2 and 3.

Measured with a colorimetric method, the content of total phenolics varied between 84 and 128 mg/g dry weight in flowering tops (flowers and leaves) and between 36.7 and 53.8 mg/g fresh weight in fruits. The sum of the contents of the phenolic compounds measured with the HPLC method was the highest in fruits harvested in August-September (37.8–39 mg/g dry weight) (Table 2) and in leaves harvested in June (41.6 mg/g dry weight) (Table 3). The majority of phenolic compounds in leaves reached the maximum in mid-June, with a clear declining pattern between June and September. Although the sum of the contents of hydroxycinnamic acids and flavonols was the highest in June, the sum of the contents of flavan-3-ols overshadowed the contents of other phenolic compounds. In the case of fruit phenolics: the sum of the contents of hydroxycinnamic acids, flavonols, and flavan-3-ols was the highest in early August, while the sum of the contents of flavones and anthocyanins was the highest in mid-September. According to our results, the highest content of different classes of phenolics was in relatively young fruit, which decreased steadily during growth. A similar decrease was reported in apple [Burda *et al*., 1990], peach [Lee *et al*., 1990], grape [Romeyer *et al*., 1983], and loquat fruit [Ding *et al*., 2001]. Seasonal decrease in phenolics content may be due to their conversion into insoluble cell wall components, or transformation into oligo and polymeric compounds, *e.g.* tannins or lignans [Nurmi *et al*., 1996].

TABLE 1. Total phenolics (TP) content and the antioxidant activity of extracts from *C. pentagyna* Willd. flowers, fruits, and leaves represented as mean ± standard deviation (SD).

Harvest date	Sample	TP content	DPPH assay	ABTS assay	CUPRAC assay	FRP assay
		(mg GAE/g dry weight)	(μmol TE/g dry weight)			
19th May	Flowers	84.5±1.2	466±10	477±2	579±5	311±2
19th May		85.7±1.1^{e*}	499±5^{d}	485±1^{e}	580±5^{d}	438±5^{e}
10th June		89.4±1.5^{d}	533±5^{cd}	507±3^{d}	670±5^{d}	486±5^{d}
13th July	Leaves	98.3±0.5^{c}	570±8^{bc}	562±5^{c}	690±5^{c}	527±3^{c}
5th August		107±2^{b}	612±12^{b}	579±5^{b}	705±5^{b}	621±2^{b}
16th September		128±1^{a}	749±5^{a}	641±2^{a}	760±2^{a}	668±3^{a}
		(mg GAE/g fresh weigh)		(μmol TE/g fresh weight)		
13th July	Fruits	36.7±0.5^{c}	14.6±0.2^{d}	26.4±0.2^{c}	50.3±1.4^{a}	33.8±0.6^{c}
5th August		45.1±0.6^{b}	19.7±0.1^{c}	28.9±0.1^{b}	58.1±0.5^{a}	61.1±0.6^{b}
16th September		45.9±0.9^{b}	20.1±0.1^{b}	28.8±0.1^{b}	70.0±0.3^{a}	113±6^{a}
10th October		53.8±0.5^{a}	21.1±0.1^{a}	30.7±0.2^{a}	72.0±0.3^{a}	109±2^{a}

* Values for leaves or fruits in the same column with different superscripts (a-e) are significantly different ($p<0.05$) based on the analysis of variance and Tukey's test.

According to our results, the highest content of different classes of phenolics was found in leaves harvested at the early maturity stage (Table 3), while the total phenolics content was the highest in leaves harvested at the end of the growing season (Table 1). If we look at other studies, there are some opposed claims. According to Wang & Lin [2000], young leaves from blackberry, raspberry, and strawberry had a higher content of total phenolics than mature leaves. Similarly to our results, Vaigiri *et al*. [2015] found that young leaves do not always express a higher content of total phenolics, compared to mature leaves. Also, the sum of contents of phenolic compounds was lower than the content of total phenolics determined using the method with the Folin-Ciocalteu's phenol reagent, which is understandable. The Folin-Ciocalteu's method is less selective and specific (as the reagent is reacting not merely with phenolics but with some nitrogen-containing compounds) and tends to overestimate the content of total phenolics. In addition, there might be some high molecular weight phenolic compounds in hawthorn extracts, which can react with the reagent. Gao *et al*. [2010] identified seven lignin--glycosides in the leaves of *C. pinnatifida*. Amel *et al*. [2014] found that total tannins content in the leaves of *C. azarolus* (115.33 mg/g, expressed as tannic acid equivalents) was comparable to total phenolics content (188.91 mg/g, expressed as gallic acid equivalents). Both, lignans and tannins are high molecular weight polymers, containing aromatic hydroxyl groups that react with the Folin-Ciocalteu's phenol reagent, hence discrepancies between the content of total phenolics and the sum of phenolics can be explained in this manner.

Antioxidant activity

A substantial number of reactive chemical species with various mechanisms of action can damage the homeostasis of the cell [Cecarini *et al*., 2007]. Because of that, it is impossible to define one method for determining antioxidant activity, and it is necessary to choose a combination of several tests based on different principles to demonstrate the antioxidative potential through various mechanisms of action such as direct bonding of free radicals, electron transfer, inhibition of prooxidative enzymes, activating antioxidant enzymes, chelating of prooxidative metal ions *etc*. [Huang *et al*., 2005]. The results of different antioxidant activity assays are presented in Table 1. Results from this study showed that the antioxidant activities follow the clear pattern: throughout the harvest season, there is an evident increase of antioxidant activity with an increase in the total phenolics content.

Variation in the content of individual phenolic compounds during harvesting period

Twenty-two compounds belonging to five groups of phenolic compounds (hydroxycinnamic acids, flavonols, flavones, flavan-3-ols, and anthocyanins) were detected and identified or tentatively identified in flowers, fruits, and leaves of *C. pentagyna* Willd. Their contents are presented in Table 2 and 3.

Hydroxycinnamic acids were the major phenolic compounds in the flower (13.3 mg/g dry weight), and accounted for 64.5% of the total phenolic compounds (20.6 mg/g dry weight). Isomeric acids, neochlorogenic (5-*O*-caffeoylquinic acid) and chlorogenic (3-*O*-caffeoylquinic acid), were the most abundant in flowers (5.20 and 4.85 mg/g dry weight, respectively). Compared with the content of hydroxycinnamic acids, the content of flavan-3-ols was quite low (2.02 mg/g dry weight). (-)-Epicatechin was the most abundant flavan--3-ol (1.54 mg/g dry weight), which accounted for 76% of total flavan-3-ols. Similar results were reported for the content of (-)-epicatechin in flowers of *C. laevigata*, lower ones for the flowers of *C. pinnatifida* var. major [Chen & Liu, 2005], and higher ones for the flowers of *C. monogyna* [Bernato-

TABLE 2. The content of hydroxycinnamic acids, flavonols, flavones, anthocyanins, and flavan-3-ols in flowers (mg/g dry weight) and fruit (mg/g fresh weight) of *C. pentagyna* Willd. at different harvest times.

Phenolic compounds	Harvest date				
	19th May	13th July	5th August	16th September	6th October
	Flowers	Fruits			
Neochlorogenic acid	5.20±0.30	–	–	–	–
Caffeic acid	1.01±0.02	0.45±0.01^{b*}	1.13±0.01^{a}	0.14±0.01^{d}	0.23±0.01^{c}
Chlorogenic Acid	4.85±0.03	0.10±0.01^{d}	1.21±0.02^{b}	1.52±0.01^{a}	0.84±0.02^{c}
4-*O*-Caffeoylquinic acid	1.24±0.05	–	–	0.45±0.02^{a}	0.46±0.01^{a}
4-*O*-*p*-Coumaroylquinic acid	–	0.041±0.001^{d}	0.061±0.004^{c}	0.17±0.01^{b}	0.24±0.01^{a}
p-Coumaric acid	1.01±0.03	0.020±0.001	–	–	–
Ferulic acid	–	1.16±0.01^{a}	0.85±0.02^{b}	0.41±0.01^{c}	–
∑ Hydroxycinnamic acids	**13.3**	**1.78±0.01**^{c}	**3.26±0.04**^{a}	**2.69±0.03**^{b}	**1.77±0.04**^{c}
Rutin	0.50±0.01	0.140±0.001^{c}	0.078±0.002^{d}	0.18±0.01^{b}	0.22±0.01^{a}
Hyperoside	0.46±0.01	1.12±0.03^{b}	1.54±0.03^{a}	0.75±0.02^{c}	0.74±0.04^{c}
Isoquercetin	3.14±0.05	0.21±0.02^{d}	0.47±0.01^{c}	0.61±0.01^{b}	0.64±0.01^{a}
Quercetin pentoside	–	0.059±0.001^{a}	0.019±0.001^{c}	0.030±0.001^{b}	–
Kaempferol rutinoside	0.17±0.02	–	–	–	–
Quercetin rhamnoside	0.09±0.00	–	–	–	–
Quercetin	–	–	–	–	–
Kaempferol	0.07±0.00	–	–	–	–
∑ Flavonols	**4.27**	**1.53±0.03**^{bc}	**2.11±0.02**^{a}	**1.57±0.02**$^{b, c}$	**1.60±0.05**^{b}
Luteolin glycoside	0.31±0.02	0.050±0.002^{d}	0.060±0.001^{b}	0.090±0.001^{a}	0.060±0.001^{b}
Apigenin glycoside	0.74±0.04	0.12±0.01^{c}	0.060±0.002^{d}	0.23±0.02^{a}	0.19±0.01^{b}
∑ Flavones	**1.05**	**0.17±0.01**^{c}	**0.120±0.003**^{d}	**0.32±0.02**^{a}	**0.25±0.01**^{b}
PC derivative	–	0.47±0.01^{c}	0.32±0.01^{d}	2.27±0.02^{a}	0.77±0.03^{b}
Procyanidin B2	0.48±0.02	2.68±0.03^{c}	9.01±0.02^{b}	10.6±0.1^{a}	9.05±0.01^{b}
(-)-Epicatechin	1.54±0.08	6.29±0.02^{d}	21.1±0.1^{a}	13.1±0.1^{c}	14.3±0.2^{b}
Procyanidin B5	–	0.75±0.01^{d}	1.17±0.02^{c}	3.74±0.06^{a}	2.65±0.02^{b}
∑ Flavan-3-ols (monomer + dimers)	**2.02**	**10.2±0.1**^{d}	**31.6±0.1**^{a}	**29.7±0.1**^{b}	**26.8±0.2**^{c}
Ideain	–	0.32±0.02^{d}	0.67±0.02^{c}	4.70±0.05^{b}	6.21±0.04^{a}
∑ Anthocyanins	**–**	**0.32±0.02**^{d}	**0.67±0.02**^{c}	**4.70±0.05**^{b}	**6.21±0.04**^{a}
∑ Indiv. phenolics	**20.6**	**14.0±0.1**^{d}	**37.7±0.1**^{b}	**39.1±0.2**^{a}	**36.6±0.2**^{c}

*Values for leaves or fruits in the same row with different superscripts (a-d) are significantly different ($p<0.05$) based on the analysis of variance and Tukey's test.

niene *et al*., 2008]. Isoquercetin, rutin, and hyperoside represented 92.5% of total flavonols. Isoquercetin was the major flavonol glycoside (3.14 mg/g) accounting for 70.9% of total flavonols. According to an extensive literature review, previous studies were mainly focused on the isolation of flavones and flavonols from the *C. pentagyna*. According to Nikolov [1975], besides flavone *C*-monoglycosides (vitexin, saponaretin, orientin, and homoorientin) vitexin rhamnoside was isolated from the *C. pentagyna*. From the leaves and flowers of the *C. penatgyna* W. et K., two flavonol glycosides were isolated: crateside (quercetin 3'-*β*-L-arabofuranoside) [Nikolov *et al*., 1973a] and glogoside (3-*O*-methylherbacetin-8-*β*-D-glucopyranoside) [Nikolov *et al*., 1973b]. Besides rutin, various glycosides of vitexin and orientin have been isolated from the leaves and flowers of the *C. pentagyna*, and identified as: 2"-*O*-rhamnosyl-isovitexin, 2"-*O*-rhamnosyl-vitexin, 2"-*O*-rhamnosyl-(4"'-*O*-acetyl)-vitexin, 2"-*O*-rhamnosyl-orientin, and 2"-*O*-rhamnosyl-isoorientin [Nikolov *et al*., 1982].

TABLE 3. The content of hydroxycinnamic acids, flavonols, flavones, anthocyanins, and flavan-3-ols in leaves (mg/g dry weight) of *C. pentagyna* Willd. at different harvest times.

Phenolic compounds	Harvest date				
	19th May	10th June	13th July	5th August	16th September
Neochlorogenic acid	2.26±0.03[a*]	1.11±0.05[b]	0.39±0.03[d]	0.47±0.03[c]	0.15±0.01[e]
Caffeic acid	0.65±0.02[a]	0.21±0.01[b]	0.14±0.01[c]	0.14±0.01[c]	0.10±0.01[d]
Chlorogenic acid	5.30±0.20[a]	5.03±0.06[a]	1.79±0.09[c]	2.18±0.02[b]	1.32±0.02[d]
4-*O*-Caffeoylquinic acid	0.49±0.01[a]	0.44±0.01[b]	0.20±0.01[d]	0.248±0.002[c]	0.12±0.01[e]
4-*O*-*p*-Coumaroilquinic acid	0.73±0.02[a]	0.54±0.01[b]	0.078±0.003[d]	0.118±0.002[c]	0.048±0.003[e]
p-Coumaric acid	1.56±0.09[a]	1.25±0.04[b]	0.57±0.04[c, d]	0.67±0.03[c]	0.45±0.01[d]
Ferulic acid	1.13±0.08[a]	0.73±0.02[b]	0.56±0.05[c]	0.39±0.02[d]	0.34±0.01[d]
∑ Hydroxycinnamic acids	**12.1±0.3**[a]	**9.31±0.12**[b]	**3.74±0.10**[d]	**4.22±0.06**[c]	**2.53±0.06**[e]
Rutin	1.23±0.03[b]	1.00±0.01[d]	1.20±0.02[b]	1.46±0.01[a]	1.12±0.02[c]
Hyperoside	4.54±0.05[a]	3.00±0.03[b]	2.43±0.01[d]	2.15±0.02[e]	2.68±0.01[c]
Isoquercetin	0.24±0.01[b]	0.058±0.002[e]	0.26±0.01[a]	0.150±0.002[c]	0.120±0.005[d]
Quercetin pentoside	0.53±0.01	–	–	–	–
Kaempferol rutinoside	0.30±0.01[a]	0.14±0.01[b]	–	–	–
Quercetin rhamnoside	0.17±0.01[c]	0.049±0.002[e]	0.10±0.01[d]	0.24±0.01[b]	0.49±0.01[a]
Quercetin	0.36±0.00[b]	0.45±0.02[a]	0.32±0.02[c]	0.01±0.01[e]	0.058±0.002[d]
Kaempferol	–	–	–	–	–
∑ Flavonols	**7.37±0.09**[a]	**4.70±0.03**[b]	**4.31±0.01**[d]	**4.01±0.03**[e]	**4.47±0.01**[c]
Luteolin glycoside	–	–	–	–	0.089±0.003
Apigenin glycoside	0.030±0.002[d]	0.82±0.02[c]	1.61±0.01[a]	1.46±0.02[b]	1.58±0.02[a]
∑ Flavones	**0.030±0.002**[c]	**0.82±0.01**[d]	**1.61±0.01**[b]	**1.46±0.02**[c]	**1.67±0.02**[a]
PC derivative	0.80±0.02[d]	1.90±0.02[a]	1.18±0.02[b]	1.00±0.07[c]	0.99±0.02[c]
Procyanidin B2	3.20±0.01[c]	5.88±0.13[a]	3.03±0.10[d]	2.94±0.02[d]	3.60±0.05[b]
Epicatechin	9.75±0.11[d]	15.1±0.1[a]	12.7±0.2[b]	11.3±0.3[c]	12.8±0.2[b]
Procyanidin B5	2.20±0.01[bc]	3.92±0.08[a]	2.03±0.03[c]	2.31±0.09[b]	0.53±0.04[d]
∑ Flavan-3-ols (monomer + dimers)	**15.9±0.1**[d]	**26.8±0.3**[a]	**18.9±0.3**[b]	**17.6±0.3**[c]	**18.0±0.2**[c]
∑ Indiv. phenolics	**35.5±0.4**[b]	**41.6±0.4**[a]	**28.6±0.4**[c]	**27.3±0.3**[d]	**26.6±0.3**[d]

*Values for leaves or fruits in the same row with different superscripts (a-e) are significantly different ($p<0.05$) based on the analysis of variance and Tukey's test.

According to Prinz *et al*. [2007], vitexin, isovitexin, rutin, hyperoside, and isoquercitrin are the compounds which can be found in *C. pentagyna*, *C. monogyna* as well in *C. laevigata*., while isoorientin, isoquercitrin, and 8-methoxykaempferol-3-*O*-glucoside were found only in *C. pentagyna* and could serve as markers for the differentiation of *C. pentagyna* from *C. monogyna* and *C. laevigata*.

Procyanidins B2 and B5 showed similar changing trends during the harvest period. The highest contents of procyanidins B2 and B5 were found in the fruits collected in September (10.6 and 3.74 mg/g fresh weight, respectively); their contents were observed to increase successively from July. From mid-September, the content of procyanidins decreased. Monomers and dimers of flavan-3-ols were the major phenolic compounds in the fruits of *C. pentagyna* Willd (Table 2). The sum of the contents of flavan-3-ols varied from 10.2 to 29.7 mg/g fresh weight during the harvest period. (-)-Epicatechin (6.29–21.1 mg/g fresh weight) and procyanidin B2 (2.68–10.6 mg/g fresh weight) were the most abundant. Ideain (cyanidin 3-*O*-galactoside) was the second most abundant phenolic compound in fruits, and its content increased from 0.32 to 6.21 mg/g fresh weight during the growing season. Compared to the contents of flavan-3-ols, those of hydroxycinnamic acids and flavonols were quite low. The major flavonols were hyperoside and isoquercetin. Chlorogenic acid was the most abundant hydroxycinnamic acid with content rang-

ing from 0.10 to 1.52 mg/g fresh weight. On the other hand, (-)-epicatechin content was at the highest level in August (21.1 mg/g fresh weight). Afterwards, it decreased dramatically. Chlorogenic and caffeic acids displayed quite similar changing trends during the harvest period. The highest contents of those acids were found in the fruits collected in August and September, which is consistent with fruit ripening season (August-October). The content of hyperoside, which is the most abundant flavonol glycoside, was the highest in August and afterwards it decreased by 50%. In most cases, the contents of flavonol glycosides were the highest in early stages of fruit development. Similar behavior of flavonol glycosides was detected in apples [Awad *et al*., 2001] and blueberries [Jaakola *et al*., 2002]. With the exception of ideain, the majority of phenolic compounds in fruits of *C. pentagyna* Willd. reached the highest content in August and September. The content of ideain in fruits increased continuously throughout the harvest period (0.32–6.21 mg/g fresh weight). Similar results were reported earlier in other hawthorn species and fruit [Elmastas *et al*., 2017; Jaakola *et al*., 2002]. There is evidence that the flavonoid biosynthesis is closely related to the stage of fruit development and that the activity of the enzyme is controlled due to diverse ecological and developmental conditions [Jaakola *et al*., 2002]. During the development of blueberry fruit (*Vaccinium myrtillus*), studying the expression of five genes involved in the flavonoid biosynthesis, Jaakola *et al*. [2002] concluded the following: coordinated expression of the genes involved in the flavonoid biosynthesis influences the accumulation of flavonols, proanthocyanidins, and anthocyanins during fetal development. As well, expression of these genes occurs in two phases: during the flowering stage and earlier stages of ripening when the color of fruit steel develops. Gene expression is most pronounced in the ripening stage when the fruit is still immature from the inside, with certain colored stains and then in the fully mature fruit the expression of the gene is declining. Similar enzymatic activities were also observed by Manning [1998] during the development of strawberry fruit and by Kobayashi *et al*. [2001] during the development of grapes.

The sum of flavan-3-ols in the leaves ranged from 16 to 26.8 mg/g dry weight (Table 3). As in the fruits, (-)-epicatechin, procyanidin B2, and procyanidin B5 were the major flavan-3-ols. Hydroxycinnamic acids were the second most abundant compounds. Chlorogenic, neochlorogenic, *p*-coumaric, and ferulic acids were present at 5.30, 2.26, 1.56, and 1.13 mg/g dry weight, respectively, at the beginning of the harvest period. The content of hyperoside, the most abundant flavonol glycoside (4.54–2.68 mg/g dry weight), decreased from May to August, and then slightly increased in September. This increase in the content of hyperoside may be related to a drop in temperature [He *et al*., 2016].

The changing patterns for hydroxycinnamic acids were quite similar: at the beginning of the harvest period (May) the content of all hydroxycinnamic acids was at the highest level, and decreased until July. Afterwards, the content of individual hydroxycinnamic acids fluctuated: content of individual hydroxycinnamic acids remained constant or slightly increased in August and decreased after that. Flavan-3-ol contents of leaves increased progressively from May until reaching the highest levels in June. Afterwards, it decreased until August, and slightly increased in September. A literature review indicates that phenolic composition of plant tissue is determined by many factors, such as genotype, ontogenesis, environment, stage of the development, analysis time, processing and storage conditions, as well as the method of analysis itself [Krüger *et al*., 2011; Martz *et al*., 2010].

One-way ANOVA was used in order to examine the effect of harvesting date on total phenolic content, content of individual phenolics, and the sum of the contents of phenolic compounds belonging to different classes in fruits and leaves of *C. pentagyna* Willd. The results of Tukey's HSD test (confidence interval 95%, $p<0.05$), which was used to show a significant difference in the content of the total pheholics, individual phenolic compounds, the sum of the contents of different classes of phenolics, and the antioxidant activity in fruits and leaves harvested in different periods, are represented in Table 1, 2, and 3.

The coefficient of determination ($R^2=0.997$) has shown that 99.7% of the variability of the total phenolics content in the *C. pentagyna* Willd. leaves is being explained by the explanatory variables (harvest period). According to the results of the Tukey's HSD test, we have concluded with confidence that there is a significant effect of the harvesting date on the total phenolics content in the leaves of *C. pentagyna* Willd (Table 1). The ANOVA results for the effect of the harvesting date on the antioxidant activity of leaves, measured by DPPH ($R^2=0.984$), ABTS ($R^2=0.997$), CUPRAC ($R^2=0.997$), and FRP ($R^2=0.998$) have shown that there was a significant difference in the antioxidant activity in different harvesting periods (Table 1). Same results were obtained for the effect of harvest date on the content of individual phenolics and the sum of the contents of different classes of phenolics in the leaves. The coefficient of determination (R^2) ranged from 0.984 to 0.999 for different quantitative variables (content of individual phenolics and the sum of the content of the different classes of phenolics) so the 98.4%-99.9% of the variability in the content of individual phenolics and the sum of the content of different classes has been explained by the harvest period (Table 3). Similarly, ANOVA results for the total phenolics content, the antioxidant activity, and the content of individual phenolics in fruits have shown a significant effect of the harvest date (Table 2).

Correlation analysis

Correlation analysis was used to explore the relationships amongst the sum of the contents of phenolic compounds belonging to individual classes and the antioxidant activity determined by different assays for fruits and leaves of *C. pentagyna* Willd. Pearson's correlation coefficients are represented in Table 4.

Based on the Pearson's correlation coefficients, it is clear that for fruit samples there are direct correlations between the total flavone content and the antioxidant activities determined particularly with CUPRAC ($r=0.769$) and FRP ($r=0.825$) methods. Flavan-3-ol content showed a significant simple correlation with the antioxidant activity determined by the ABTS method ($r=0.761$), as well as a very strong correlation with the antioxidant activity determined by the DPPH

TABLE 4. Pearson's correlation coefficient (r) between: ∑ hydroxycinnamic acids, ∑ flavonols, ∑ flavones, ∑ flavan-3-ols, and ∑ anthocyanin, total phenolic content (TPC) and antioxidant activity determined with DPPH, ABTS, CUPRAC, and FRP assays in fruits and leaves of *C. pentagyna* Willd.

	Leaves					Fruits				
	TPC	DPPH	ABTS	CUPRAC	FRP	TPC	DPPH	ABTS	CUPRAC	FRP
∑ Hydroxycinnamic acids	**0.827***	**-0.810**	**-0.928**	**-0.916**	**-0.879**	0.005	0.368	0.100	0.008	0.046
∑ Flavonols	-0.543	-0.549	-0.671	**-0.860**	**-0.701**	0.079	0.286	0.180	-0.193	-0.228
∑ Flavones	**0.753**	**0.746**	**0.871**	**0.928**	**0.828**	0.415	0.417	0.354	**0.769**	**0.825**
∑ Flavan-3-ols	-0.297	0.023	0.028	0.397	0.074	**0.703**	**0.919**	**0.761**	0.682	0.677
∑ Anthocyanins						**0.843**	**0.733**	**0.792**	**0.956**	**0.939**

*Values in bold refer to statistically significant correlations at $p < 0.05$

method (r=0.919). The content of anthocyanins was well correlated with the antioxidant activity of the fruit when assayed with all methods (r=0.733–0.956). The best correlation was found between the content of ideain in the fruit and the antioxidant activity determined with the CUPRAC method (r=0.956). A weak correlation between the sum of the contents of hydroxycinnamic acids, flavonols, and the antioxidant activity of the fruit could be the consequence of the structure of the phenolic compounds themselves since only the flavonoids of the certain structure and the exact position of the OH group in the molecule determine the antioxidant properties [Heim *et al*., 2002]. Both the configuration and the total number of hydroxyl groups substantially influence the mechanism of the antioxidant activity. The glycosylation of flavonoids reduces their *in vitro* antioxidant activity when compared to the corresponding aglycons [Rice-Evans *et al*., 1996; Mishra *et al*., 2003]. For example, the comparison of TEAC values of quercetin and rutin (quercetin 3-*O*-rutinoside), shows that glycosylation of the 3-OH group has a strong suppressive effect on the antioxidant activity [Rice-Evans *et al*, 1996]. Similar results were observed for other pairs of flavonoid aglycon and glycoside (hesperetin – hesperidin, luteolin – luteolin-4'--glucoside; luteolin – luteolin-7-glucoside; quercetin – quercitrin) [Rice-Evans *et al*., 1996] also. Based on these results, we can say that flavones contribute most to the antioxidant activity determined with the FRP method, the flavan-3-ols to the antioxidant activity determined with the DPPH method, and the anthocyanins to the antioxidant activity determined with the CUPRAC method. As flavan-3-ols ((-)-epicatechin as the most preferred flavan-3-ol) and ideaine are the most abundant compounds in the fruit during the entire harvesting period, it can be expected that they contribute most to the antioxidant activity. Unlike the fruit, in which no correlation between the sum of the contents of hydroxycinnamic acids, flavonols and antioxidant activity was observed, with leaf an inverse correlation was noticed between the sum of the contents of hydroxycinnamic acids and antioxidant activity. Content of hydroxycinnamic acids in the leaf showed strong negative correlation with the antioxidant activity determined with all methods (r=-0.810 to -0.928), while flavonols showed a strong correlation with the antioxidant activity determined with CUPRAC (r=-0.860) and FRP (r=-0.701) methods. The sum of the contents of hydroxycinnamic acids and flavonols decreased during the harvest period, and this could be the consequence of the strong negative correlation with antioxidant activity since the antioxidant activity increased during the harvest period. Hence, it might be inferred that hydroxycinnamic acids and flavonols have no impact on the antioxidant activity. Only flavones showed a positive correlation with the antioxidant activity of the leaf (r=0.746–0.928).

A strong correlation was observed between the total phenolics content and the sum of the contents of anthocyanins (r=0.843) and flavan-3-ols (r=0.703) in fruits (Table 4). Based on the obtained results, the strong correlation between these two classes of phenolic compounds and total phenolics content, may be due to the fact that the compounds from those two classes are the predominant related to other compounds. A strong simple correlation between total phenolics content and the sum of the contents of flavones (r=0.753) and a strong negative correlation between total phenolics content and the sum of the contents of hydroxycinnamic acids (r=- 0.827) were observed in the case of leaves.

Principal Component Analysis

PCA was conducted in order to explain the relationship between different conditions, in this case: harvest period, the variation in the sum of the contents of the different classes of phenolic compounds and the antioxidant activity of *C. pentagyna* Willd. leaves and fruits. Factor loadings >0.90 are typically regarded as excellent and <0.7 as very poor [Gucia *et al*., 2012]. In this study, three principal components with eigenvalues higher than 1.0 were extracted, as suggested by the Kaiser criterion [1960]. The number of principal components, initial eigenvalues, total variance (percent), and factor loadings are presented in Table 5.

The results of PCA for data sets indicate that three factors explained approximately 96% of the total variance in the fruits and 98.7% of the total variance in leaves. The results of the principal component analysis conducted for fruits and leaves are represented in Figure 1A and 1B. Due to a large number of variables compared to the number of samples, the components PC1 and PC2 explained about 87% and 94% of the variability of the fruit and leaves samples, respectively. Distinct grouping of the fruit (HF3 and HF4 – HF represents hawthorn fruit samples) samples in the lower right quadrant along with the sum of the content of the anthocyanins, as well as the leaves (HL3, HL4, and HL5 – HL represents hawthorn leaves samples) samples with the sum

TABLE 5. Principal components and factor loadings for the ∑ hydroxycinnamic acids, ∑ flavonols, ∑ flavones, ∑ anthocyanins, ∑ flavan-3-ols and result of antioxidative assays in fruits and leaves of *C. pentagyna* Willd.

	Fruits			Leaves		
	PC 1	**PC 2**	**PC 3**	**PC 1**	**PC 2**	**PC 3**
∑ Hydroxycinnamic acids	**-0.976***	0.178	-0.082	**-0.968**	0.032	0.228
∑ Flavonols	-0.072	**0.984**	0.117	**-0.832**	-0.488	0.237
∑ Flavones	**0.716**	-0.633	-0.095	**0.954**	0.152	-0.240
∑ Flavan-3-ols	**0.769**	0.560	0.072	-0.098	**0.958**	0.269
∑ Anthocyanins	**0.908**	-0.354	0.067			
DPPH	**0.925**	0.358	0.118	**0.892**	-0.236	0.375
ABTS	**0.889**	0.270	0.156	**0.968**	-0.218	0.101
CUPRAC	-0.516	-0.324	**0.790**	**0.978**	0.163	0.124
FRP	**0.979**	-0.169	0.041	**0.950**	-0.172	0.127
Eigenvalue	5.762	2.198	0.703	6.140	1.338	0.420
Total variance (%)	64.023	24.420	7.805	76.746	16.727	5.253
Cumulative (%)	64.023	88.442	96.247	76.746	93.473	98.726

* Values in bold refer to statistically significant correlations at $p<0.05$.

of the content of the flavones is noticeable. PC2 is highly correlated with the sum of the contents of flavonols in the fruit and the sum of the contents of flavan-3-ols in leaves samples. PC1 is strongly correlated with the sum of the contents of flavones and anthocyanins and strongly negatively correlated with the sum of the contents of flavonols and the sum of the contents of hydroxycinnamic acids in leaf samples. Variability in the contents of phenolic compounds during harvesting period can be a consequence of different tissue, growing and environmental conditions or changes in the metabolism of phenolic compounds [Francini & Sebastiani, 2013].

From the PCA graphs (Figure 1) it can be concluded that PC1 of fruit samples is highly correlated with DPPH, FRP, and ABTS, as well as that FRP and the sum of the contents of anthocyanins are well correlated. It can be assumed that flavan-3-ols and anthocyanins in fruits, as well the later harvest period (HF3 and HF4), have the major effect on the antioxidant activity of *C. pentagyna* Willd. fruits.

In the leaves, flavones as well as antioxidant activity assayed with FRP, DPPH, CUPRAC, and ABTS, are highly correlated with PC1. The grouping of HL3 along with the CUPRAC and flavones, and HL4 and HL5 in the vicinity of FRP

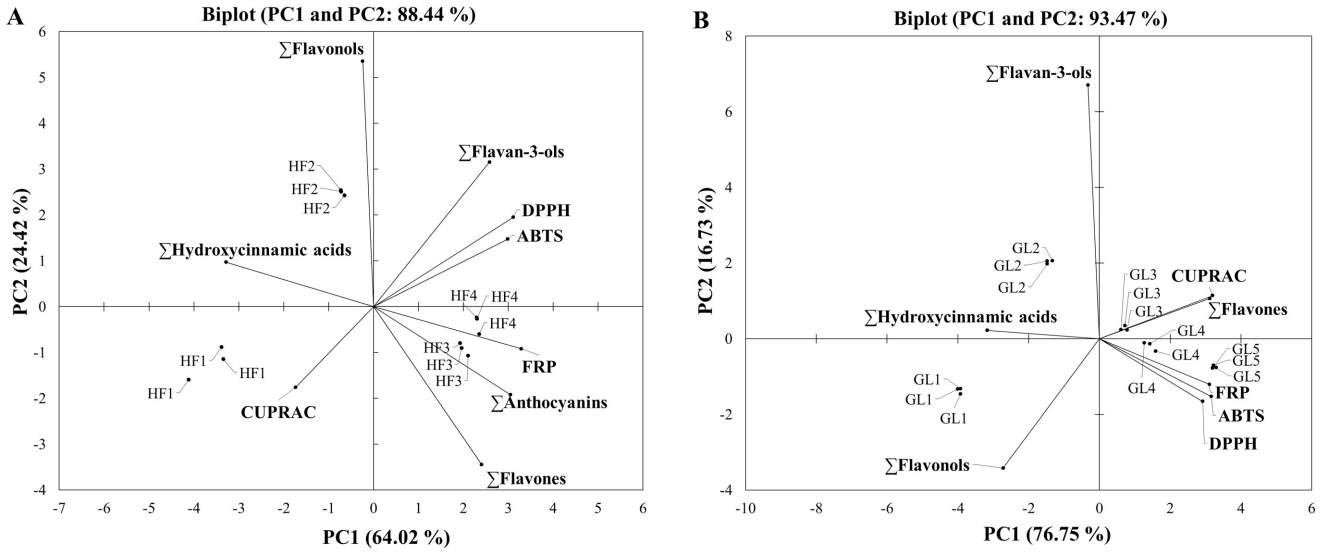

FIGURE 1. Principal component analysis of hawthorn: A) fruits and B) leaves harvested in different periods.

HF*1–4 hawthorn fruits harvesting date: 13th July, 5th August, 16th September, 6th October, respectively; HL*1–5 hawthorn leaves harvesting date: 19th May, 10th June, 13th July, 5th August, 16th September, respectively.

is noticeable. Also, flavonols and hydroxycinnamic acids are negatively correlated with PC1, as well as with DPPH, ABTS, and FRP. Flavones and later harvest period (HL3, HL4, and HL5) could be the most meritorious for the antioxidant activity of leaves.

CONCLUSION

Relying on the results of this research, especially on the content of the individual phenolic compounds in the flowers, leaves, and fruits of *C. pentagyna* Willd., it can be inferred that this study contributes to the better understanding of the influence of different harvest periods on the composition of phenolics within different parts of *C. pentagyna* Willd. plant. As the results of the study show, it can be concluded that there is a seasonal variation in both the quantity and the type of phenolic compounds, which might be due to fluctuations in temperature, precipitation, and conditions of plant habitat. The present work can be useful in finding the appropriate growth conditions and harvest time of *C. pentagyna* Willd. to ensure desired contents of particular valuable compounds.

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Concentrations of Blood Serum and Urinal Ellagitannin Metabolites Depend Largely on the Post-Intake Time and Duration of Strawberry Phenolics Ingestion in Rats

Ewa Żary-Sikorska[1], Monika Kosmala[2], Joanna Milala[2], Bartosz Fotschki[3], Katarzyna Ognik[4], Jerzy Juśkiewicz[3]*

[1]Department of Microbiology and Food Technology, Faculty of Agriculture and Biotechnology University of Science and Technology, Kaliskiego 7, 85–796 Bydgoszcz, Poland
[2]Institute of Food Technology and Analysis, Łódź University of Technology, Stefanowskiego 4/10, 90–924 Łódź, Poland
[3]Department of Biological Functions of Food, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10–748 Olsztyn, Poland
[4]Department of Biochemistry and Toxicology, Faculty of Biology, Animal Sciences and Bioeconomy, University of Life Sciences, Akademicka 13, 20–950 Lublin, Poland

Key words: strawberry, ellagitannins, metabolites, urine, serum, rat

The different duration of a strawberry phenolic fraction intake and different post-intake time were experimental factors affecting the concentrations of ellagitannin metabolites in the urine and blood serum of rats. For four days, the animals were gavaged once a day as follows: group C (water, days 1–4), group F1–4 (fraction, days 1–4), group F1–3 (fraction, days 1–3; water, day 4), group F1–2 (fraction, days 1, 2; water, days 3, 4), group F3–4 (water, days 1, 2; fraction, days 3, 4), and group F4 (water, days 1–3; and fraction, day 4). The daily dosage of the fraction gavaged to one rat was 20 mg/kg of body weight. The fraction contained monomers and dimers of ellagitannins as well as proanthocyanidins. The caecal, urinal, and blood serum ellagitannin metabolites were analysed 12 h after the last treatment. Ellagic acid, urolithin A, and nasutin A were detected in the caecal digesta. In turn, urolithin A, nasutin A, and their glucuronide conjugates were detected in the urine, while urolithin A glucuronide, nasutin A glucuronide, and ellagic acid dimethyl ether glucuronide were found in the serum. The highest caecal and urinal concentrations of ellagitannin metabolites followed the F1–4 treatment. In the serum, the highest concentrations of the metabolites were determined in the rats administered the phenolic fraction during days 1–4 and 3–4. No metabolites were found in the rats following the C and F1–2 treatments. The results suggest that the presence of ellagitannin metabolites in the rat's urine and serum largely reflects the concentrations of caecal metabolites and the number of subsequent days of strawberry phenolic fraction administration.

INTRODUCTION

Worldwide, ellagitannins (ET) along with ellagic acid (EA) are ingested every day by consumers of fruit, seeds, and nuts. It is well known that pomegranates, berries, walnuts, almonds, grapes, sea buckthorn, and plenty of other plant sources contain high amounts of ET, being the hydrolysable class of tannins [Landete, 2011]. Ellagic acid, ET, and their derived metabolites are claimed to evoke a range of numerous beneficial physiological effects and are mostly referred as antioxidant, anti-inflammatory, and anti-pathogenic molecules that can act both locally in the gastrointestinal tract and systemically in the peripheral tissues of the host [Tomás-Barberán *et al*., 2017; Van de Velde *et al*., 2019]. From the chemical perspective, the ellagitannins are hexahydroxydiphenoyl esters of carbohydrates and cyclitols that may be subject to oligomerisation transformations [Milala *et al*., 2017]. The metabolic fate of ET in humans remains quite unknown although some animal experiments have partly revealed those processes. It has been shown that part of plant ET may undergo hydrolysis to EA already in the stomach [Milala *et al*., 2017], while other researchers claimed ET to remain stable under conditions of that gastrointestinal segment [Sandhu *et al*., 2018]. There is no doubt that the large intestinal microbiota population with its huge enzymatic power acts as a paramount agent facilitating the hydrolysis of ET [Cerdá *et al*., 2005a,b; Ito *et al*., 2008; Sandhu *et al*., 2018]. Several researchers have reported that ET are not absorbed and prior to absorption must be metabolised by intestinal microbiota first into EA and then into other molecules such as urolithins and nasutin [Milala *et al*., 2017; Saha *et al*., 2016]. Urolithins contain a 6H-dibenzo[b,d]-pyran-6-one nucleus with different phenolic hydroxyl groups. There are several types of urolithins, namely urolithins D, C, A, B with a decreasing number of the phenolic hydroxyl groups, respectively [Toney *et al*., 2019]. Following intestinal absorption and hepatic biotransformation, the main metabolites circulating in blood stream are urolithin A glucuronide and urolithin A aglycone [Kawabata *et al*., 2019]. The production of urolithins is a common result of consuming ET with

* Corresponding Author:
E-mail: ezary@utp.edu.pl (E. Żary-Sikorska)

a diet in almost all mammals, but some species (beavers, pigs, rats) are able to produce dehydroxyellagic acid derivatives, namely nasutins [González-Barrio *et al*., 2011].

Most *in vivo* experiments have shown data with regard to a continuous prolonged ingestion of berry ET [Larrosa *et al*., 2010; Giampieri *et al*., 2012]. Some authors reported that urolithins reach the maximum levels in human blood between 24 h and 48 h after ingestion of a meal containing ET, and their metabolites might be present in the blood serum and/or urine for the next 24 h [Larrosa *et al*., 2006] or even longer [Garcia-Munoz *et al*., 2014]. To our best knowledge, there are no results regarding the urinal and blood ET metabolites presence after different duration of polyphenols administration taking into account varied post-intake time points as well. Those knowledge gaps justify the need for a better recognition of the physiological and metabolic consequences of dietary ET administration. It should be noted that the chemical structure of ET and subsequently that of ET metabolites largely depends on the plant source [Landete, 2011; Karlińska *et al*., 2019]. The main strawberry ET is agrimoniin, a GOG-type dimer composed of two α-1-*O*-galloyl-2,3:4,6--bis-hexahydroxydiphenoyl-D-glucose units linked by a C-O-C bond between two gallic acid residues [Fotschki *et al*., 2018; Chiabrando *et al*., 2018; Nowicka *et al*., 2019]. The presence of sanguiin H-6 and lambertianin C, but as minor ET, has been also detected in strawberry fruit [Vrhovsek *et al*., 2012]. In the present study, it was hypothesised that the presence of ellagitannin metabolites in the blood serum and urine is strongly determined by their large intestinal metabolism following the post-intake time and the duration of strawberry phenolic fraction administration, and that these effects may be rapidly reversible after phenolic fraction withdrawal from a diet.

MATERIAL AND METHODS

Preparation of strawberry phenolic fraction and its analysis

The polyphenol-rich fraction was obtained from strawberry pomace, a common by-product obtained during juice production and provided by ALPEX Co. (Łęczeszyce, Poland). The details of strawberry phenolic fraction preparation were presented in the previous paper by Juśkiewicz *et al*. [2015]. In brief, the strawberry pomace was subjected to industrial vacuum drying at 70±2°C and then a seedless fraction was obtained with the aid of an appropriate set of sieves. That fraction was treated by a two-stage extraction with a 60% (v/v) aqueous solution of acetone. Next, after partial removal of the solvent *via* distillation, the resultant solutions were transferred onto a column packed with polymeric resin (Amberlite XAD 16, Sigma-Aldrich, Poznań, Poland). The water was used to elute the sugars and other water-soluble compounds from the extract loaded onto the column. The ellagitannin-rich fraction (F) was desorbed with a 40% aqueous solution of ethanol, concentrated to *ca*. 15% of dry matter, and then lyophilized. The detailed composition of the fraction was analysed based on the methods described below.

The official AOAC methods [Horwitz & Latimer, 2007] were used to determine contents of: dry matter and ash (940.26), protein (920.152), fat (930.09), and total dietary fibre (985.29). The concentration of ET, ellagic acid, anthocyanins, and flavonols were determined in the fraction after its dilution in methanol (1 mg/mL) using an HPLC system (Knauer Smartline system with photodiode array detector, Berlin, Germany) coupled with a Gemini C18 column (110 Å, 250×4.60 mm; 5 μm, Phenomenex, Torrance, CA, USA). Phase A was 0.05% (v/v) phosphoric acid in water, phase B was 0.05% (v/v) phosphoric acid in 80% (v/v) acetonitrile, the flow rate was 1.25 mL/min, the sample volume was 20 μL, and the temperature was 35°C. Gradient: stabilization for 5 min with 4% of phase B, 4–15% B for 5–12.5 min, 15–40% B for 12.5–42.5 min, 40–50% B for 42.5–51.8 min, 50–55% B for 51.8–53.4 min and 4% B for 53.4–55 min. The following standards were used for phenolics identification: ellagic acid, flavonols (quercetin-3-*O*--glucoside, kaempferol-3-*O*-glucoside, quercetin, kaempferol, tiliroside), pelargonidin-3-*O*-glucoside (all from Extrasynthese, Genay, France), *p*-coumaric acid (Sigma-Aldrich), and ETs (hexahydroxydiphenoyl-D-glucose and agrimoniin) obtained by semi-preparative HPLC as described by Sójka *et al*. [2013]. The absorbance was measured at 280 nm (*p*-coumaric acid, tiliroside, hexahydroxydiphenoyl-D-glucose and agrimoniin), 360 nm (ellagic acid, quercetin, kaempferol and kaempferol glycosides), and 520 nm (anthocyanins).

The concentration of proanthocyanidins in the fraction was determined with the HPLC method after their hydrolysis in an acidic environment with an excess of phloroglucinol, according to Kennedy & Jones [2001]. The obtained reaction products were analysed using a Knauer Smartline chromatograph (Berlin, Germany) equipped with a UV–Vis detector (PDA 280, Knauer, Berlin, Germany) and a fluorescence detector (Shimadzu RF-10Axl, Kyoto, Japan), and coupled with a Gemini C18 column (110 Å, 250×4.60 mm; 5 μm, Phenomenex, Torrance, CA, USA). The separation conditions were as earlier described by Kosmala *et al*. [2015]. The identification was performed at 280 nm using a UV–Vis detector and the following standards: (–)-epicatechin, (+)-catechin, (–)-epigallocatechin and their respective phloroglucinol adducts. Quantification was conducted based on peak areas recorded using a fluorescence detector (excitation wavelength: 278 nm; emission wavelength: 360 nm). Standard curves of (–)-epicatechin and (+)-catechin for terminal units and (–)-epicatechin--phloroglucinol adduct for extender units were used to quantify the proanthocyanidin breakdown products.

The gavage *in vivo* experiment

The *in vivo* experiment was conducted on 48 adult male Wistar outbred rats (*Rattus norvegicus*, Cmdb:WI). The animals were used in compliance with the European Guidelines for the Care and Use of Laboratory Animals [Directive 63 EU, 2010]. The experimental protocol was approved by the Local Animal Care and Use Committee (Approval No. 10/2018; Olsztyn, Poland). All efforts were made to minimise the suffering of experimental animals. Two weeks before and during the gavage experiment, the rats were fed with a polyphenol--free diet prepared in a laboratory scale based on recommendation of AIN-1993 [Reeves, 1997]. This diet contained about 14.8% casein (Lacpol Co., Murowana Goślina, Poland),

TABLE 1. Experimental treatments# with the strawberry phenolic fraction gavaged to rats for 4 days.

Group	Day 1; 8 p.m.	Day 2; 8 p.m.	Day 3; 8 p.m.	Day 4; 8 p.m.	Day 5; 8 a.m.
C	Water	Water	Water	Water	*
F1–4	Fraction	Fraction	Fraction	Fraction	*
F1–3	Fraction	Fraction	Fraction	Water	*
F1–2	Fraction	Fraction	Water	Water	*
F3–4	Water	Water	Fraction	Fraction	*
F4	Water	Water	Water	Fraction	*

*Urine, blood, and caecal digesta collection (at the termination of the study). #The dosage of the strawberry phenolic fraction gavaged to a rat was 20 mg/kg of BW.

0.2% DL-methionine (SIGMA, Poznań, Poland), 8% rapeseed oil, 6% cellulose, standard mineral and vitamin mixes (3.5 and 1%, respectively; according to AIN-93 requirements of laboratory adult rats), 0.2% choline chloride, and corn starch up to 100%. The energy contribution of main dietary components was as follows: 16.1% by protein, 63.9 by N-free extractives, and 20% by fat.

The rats were similar in the body weight (BW) values (245±12.6 g). The gavage experiment lasted 5 days (see experimental scheme presented in Table 1). A fresh diet was served every day *ad libitum* and access to water was continuous. Standard conditions at a temperature of 21–22°C and relative air humidity of 50% with intensive room ventilation (15 × per h), and a 12 h lighting/12 h dark regimen were applied. During the period of 1–4 days, every day at 8 p.m., the rats were subjected to the following treatments (n=8) without or with the strawberry ellagitannin-rich fraction gavaged intragastrically: group C (control with water all four days), group F1–4 (fraction administered all four days), group F1–3 (fraction gavaged days 1, 2, 3, and water gavaged day 4), group F1–2 (fraction gavaged days 1, 2, and water gavaged days 3, 4), group F3–4 (water gavaged days 1, 2, and fraction gavaged days 3, 4), and group F4 (water gavaged days 1, 2, 3, and fraction gavaged day 4). The dosage of the strawberry phenolic fraction gavaged to a rat was 20 mg/kg of BW and it reflected an ingestion of a half kilogram of fresh strawberry with average phenolics concentration by an adult weighing 65 kg (calculated with the aid of Body Surface Area method; Wang & Hihara, [2004]). The amount of water or a phenolic fraction solution gavaged to a rat was prepared in a way that an animal weighing 250 g was treated with 1 mL. The animals were treated according to recommendations for refinements for intragastric gavage in rats [Okva *et al*., 2006].

At the termination of the study, on the fifth day 8 a.m., the rats were anaesthetised with a ketamine/xylazine solution in 0.9% NaCl (100 and 10 mg/kg BW, respectively). After laparotomy, blood samples were taken from the caudal vena cava. The caecum was removed and fresh digesta was frozen in liquid nitrogen, then stored at -80°C until analyses. The serum was obtained by blood centrifugation (MPW-352R; MPW MED. INSTRUMENTS, Warsaw Poland) for 15 min at 380 × *g*. The urine was collected directly from the urinal bladder, then serum and urinal samples were stored under the same conditions as caecal digesta. In the digesta samples, free ellagic acid and ET metabolites were extracted twice with the aid of 100% and 70% (v/v) acetone. The details of extraction and HPLC analyses of caecal metabolites were as earlier described by Milala *et al*. [2017]. Urine samples (1–2 mL) were acidified with 0.125 mL of 1 mol/L H_3PO_4 and injected to a 60 mg SPE Strata X-33 column conditioned according to the manufacturer's instructions (Phenomenex, Torrance, CA). The column was washed with 3 mL of water, 3 mL of 0.1 mol/L acetate buffer, and 6 mL of water, and dried. The analyte was eluted with methanol (2×0.5 mL) and analysed by UHPLC-MS using a Dionex UltiMate 3000 UHPLC and a Thermo Scientific Q Exactive series quadrupole ion trap mass spectrometer. The column was a Kinetex C18 (100 Å; 150×2.1 mm; 2.6 μm, Phenomenex, Torrance, CA, USA) kept at 35°C. A binary gradient of 0.1% (v/v) formic acid in water as a mobile phase A and 0.1% (v/v) formic acid in acetonitrile as a mobile phase B was used at a flow rate of 0.5 mL/min. The gradient program was: 5% of mobile phase B for the first 1.44 min, 5−15% B from 1.44 to 2.98 min, 15−40% B from 2.98 to 10.1 min, 40−73% B from 10.1 to 11.5 min, isocratic conditions at 73% B from 11.55 to 12.7 min, 73−5% B from 12.7 to 13.28 min, and column equilibration at 5% B from 13.28 to 18 min. Mass spectrometry analysis was performed in a negative ion mode under the following conditions: capillary voltage, +4 kV; sheath gas pressure, 60 au (arbitrary units); auxiliary gas, 10 au; and scan range from 120 to 1200 m/z. Blood serum samples: 1 mL of acetone was added to 0.5 mL of serum; the mixture was mixed, sonicated for 10 min, and centrifuged at 900 rad/s (ScanSpeed 40, LaboGene, Allerød, Denmark). The procedure was repeated with 1.0 mL of acetone, and the extracts were combined and evaporated to dryness under vacuum in a ScanSpeed 40 low speed centrifuge (LaboGene, Allerød, Denmark), lyophilised in an Alpha 1–2 LD plus freeze−dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), solubilised in 0.2 mL of methanol, and then analysed by UHPLC-MS (parameters as in the analysis of urine). Detection of ellagitannin metabolites was performed at 360 nm. Standards used were ellagic acid, urolithin A, and urolithin B (Sigma-Aldrich, Steinheim, Germany). Urolithin A glucuronide was prepared as described by Cerdá *et al*. [2005a]. The raw material for the isolation of urolithin A glucuronide was two litres of urine from three healthy volunteers (25−45 years old) who consumed 400 g of strawberry per day, as described in Fotschki *et al*. [2014]. Nasutin was isolated from the faeces of rats fed ellagitannin preparation in the same manner. Identification of metabolites was confirmed by HPLC-MS and compared with literature findings [González-Barrio *et al*., 2011; García-Villalba *et al*., 2016; Jurgoński *et al*., 2017; Milala *et al*., 2017].

Statistical analysis

The results are expressed as the means±SEM except for the chemical composition of the strawberry phenolic fraction, which is expressed as the means±SD. The Kruskal-Wallis one-way ANOVA by ranks was used followed by Dunn's post hoc test ($P \leq 0.05$). The statistical analysis was performed

using STATISTICA software, version 12.0 (StatSoft Corp., Kraków, Poland).

RESULTS AND DISCUSSION

Ellagitannins are abundant in many berries, including strawberries, and after consumption those polyphenolic compounds are degraded to their monomers throughout the gastrointestinal segments, and then metabolised by gut microbiota to 6*H*-dibenzo[*b,d*]pyran-6-one derivatives, *i.e.*, urolithins, nasutins, depending on the host species [Fotschki *et al*., 2015; Kosmala *et al*., 2017; Milala *et al*., 2017]. The ET metabolites could be further conjugated to glucuronides and sulfates in the intestine and liver before entering the systemic circulation [Sandhu *et al*., 2018]. Some authors reported that large intestinal bacteria were able to directly metabolise the native ET molecules without their hydrolysis in the upper gastrointestinal tract [Piwowarski *et al*., 2016]. The recent experiment conducted by Milala *et al*. [2017] demonstrated that the metabolism of ellagitannins with different degrees of polymerization may result in some changes in the urinal and blood serum metabolite profile. Despite those differences, the aforementioned and other authors clearly showed that after strawberry consumption the main metabolites in the rat blood serum are urolithin A glucuronide, nasutin A glucuronide, and ellagic acid dimethyl ether glucuronide (DMEAG) while the urinal metabolites are represented by urolithin A, nasutin A, urolithin A glucuronide, nasutin A glucuronide, and nasutin iso-glucuronide [Fotschki *et al*., 2016]. The analysis of the current knowledge allows to conclude that the resultant production of ET metabolites is difficult to predict accurately. But, if the ET composition of a certain natural product is known, the formation of gut microbiota metabolites might be quite precisely assessed [Piwowarski *et al*., 2016]. In the present experiment, the strawberry phenolic fraction gavaged to rats contained monomeric and dimeric ellagitannins, and proanthocyanidins as the main polyphenolic constituents, and small amounts of ellagic acid and flavonols (Table 2).

TABLE 2. Chemical composition of the strawberry phenolic fraction gavaged to experimental rats.

Components	Content (g/100 g)
Dry matter	91.31±0.05
Ash	0.03±0.04
Fat	0.00±0.00
Protein	1.83±0.03
Other components*	7.17±0.01
Total polyphenols	82.3±0.10
Ellagic acid	0.20±0.00
ETs	57.3±0.10
Monomers	23.3±0.10
Dimers	34.0±0.10
Proanthocyanidins	23.9±0.20
Anthocyanins	0.00±0.00
Flavonols	0.90±0.00

The results are expressed as the means±SD, n=3. ETs, ellagitannins. *Low-molecular carbohydrates and structural components of plant cell walls, including dietary fibre.

The formation of urolithin A and nasutin A in the caecal digesta followed the experimental gavage treatments (Table 3). In the digesta, ellagic acid was also present, and it could have originated both directly from the phenolic fraction and from the bacterial hydrolysis of ET. The dominant caecal metabolite was nasutin A followed by urolithin A. Such results were in agreement with other studies conducted on laboratory rats fed diets containing strawberry products or extracts [Fotschki *et al*., 2014, 2016; Jurgoński *et al*., 2017; Kosmala *et al*., 2015; Milala *et al*., 2017; Tomás-Barberán *et al*., 2017]. The caecal concentration of total ET metabolites was the highest in the F1–4 rats ($P<0.05$ *vs*.

TABLE 3. Caecal EA and ET metabolites presence in rats differently gavaged the strawberry phenolic fraction for four days (μg/g digesta).

Group*	Ellagic acid	Urolithin A	Nasutin A	Sum of metabolites
C	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
F1–4	0.856±0.089^{a}	45.1±7.22^{a}	122±18.5^{a}	168±17.2^{a}
F1–3	0.422±0.066^{b}	18.2±11.1^{c}	25.6±5.12^{c}	44.2±10.4^{c}
F1–2	0.056±0.043^{c}	1.02±0.891^{d}	1.25±0.880^{d}	2.33±0.885^{d}
F3–4	0.815±0.095^{a}	39.2±7.89^{ab}	96.1±11.8^{ab}	136.1±10.4^{ab}
F4	0.803±0.102^{a}	31.2±10.9^{b}	85.9±10.4^{b}	117.9±9.22^{b}
P value	<0.001	<0.001	<0.001	<0.001

*The rats were subjected to the following treatments (n=8) without or with the strawberry phenolic fraction (see Table 1) gavaged intragastrically: group C (control with water all four days), group F1–4 (fraction administered all four days), group F1–3 (fraction gavaged days 1, 2, 3, and water gavaged day 4), group F1–2 (fraction gavaged days 1, 2, and water gavaged days 3, 4), group F3–4 (water gavaged days 1, 2, and fraction gavaged days 3, 4), and group F4 (water gavaged days 1, 2, 3, and fraction gavaged day 4). The caecal samples were collected on the 5th day 12 h after the last gavage treatment. Values (presented as mean±SEM) within each column with the same superscript letter are not different at P<0.05. EA, ellagic acid; ET, ellagitannins.

TABLE 4. Urinal EA and ET metabolites presence in rats differently gavaged the strawberry phenolic fraction for four days (μg/L).

Group*	Urolithin A	Nasutin A	Urolithin A glucuronide	Nasutin A glucuronide	Sum of metabolites
C	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
F1–4	111±56.0^{a}	70.1±12.4^{a}	889±25.9^{a}	569±12.2^{a}	1639±234^{a}
F1–3	22.0±12.4^{b}	18.2±11.1^{c}	77.4±29.0^{c}	88.9±32.1^{c}	206±27.4^{c}
F1–2	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
F3–4	102±45.8^{a}	69.2±21.8^{a}	758±288^{ab}	469±174^{ab}	1398±233^{ab}
F4	95.1±27.7^{a}	44.2±20.9^{b}	655±201^{b}	409±137^{b}	1203±174^{b}
P value	0.009	<0.001	<0.001	<0.001	<0.001

*The rats were subjected to the following treatments (n=8) without or with the strawberry phenolic fraction (see Table 1) gavaged intragastrically: group C (control with water all four days), group F1–4 (fraction administered all four days), group F1–3 (fraction gavaged days 1, 2, 3, and water gavaged day 4), group F1–2 (fraction gavaged days 1, 2, and water gavaged days 3, 4), group F3–4 (water gavaged days 1, 2, and fraction gavaged days 3, 4), and group F4 (water gavaged days 1, 2, 3, and fraction gavaged day 4). The caecal samples were collected on the 5th day 12 h after the last gavage treatment. Values (presented as mean±SEM) within each column with the same superscript letter are not different at $P<0.05$. EA, ellagic acid; ET, ellagitannins.

other groups except F3–4 rats; Table 3). In regard to the respective caecal ET metabolites, the observed significance of differences in nasutin A and urolithin A concentrations between the treatments was similar to that noted for the sum of all caecal metabolites. The highest concentration of EA in the caecal digesta was in the F1–4, F3–4, and F4 groups ($P<0.05$ *vs*. remaining groups). Additionally, the F1–3 rats had significantly higher caecal EA concentration than those from the C and F1–2 groups.

Nasutin A has been hardly often detected in intestinal digesta and faeces of humans, and urolithin A and urolithin B are predominant ET bacterial metabolites [González-Barrio *et al*., 2011]. Certainly, some variations among ET consumers have been observed, and those differences are ascribed to the different profile of the intestinal bacteria population [Cerda *et al*., 2005a; Romo-Vaquero *et al*., 2019]. In the intestine of several species producing nasutins, *e.g.* termites, beavers, rats, pigs, free or released EA is undergoing microbial dihydroxylation but without lactone ring opening leading to nasutins formation, *i.e.* metabolites with two lactone rings (as in EA) but without two hydroxyl groups [Espín *et al*., 2013]. The products of bacterial ET metabolism absorbed in the upper and lower gastrointestinal tract, then glucuronidated in the portal circulation, eventually turn up in the urine [Milala *et al*., 2017]. In the present experiment, urolithin A, nasutin A, urolithin A glucuronide, nasutin A glucuronide were detected in the urine of rats treated with the strawberry phenolic fraction, while urolithin A glucuronide, nasutin A glucuronide and DMEAG were found in their blood serum (Tables 4 and 5). It has been reported that in the case of rats the production of nasutin A and its glucuronide was elevated upon the consumption of bis-hexahydroxydiphenoyl--D-glucose (monomeric ET) and agrimoniin (dimeric ET), while dietary EA caused increased concentrations of urolithin A and its glucuronide in the gastrointestinal digesta as well as the inner biological fluids: blood, lymph, and urine [Jurgoński *et al*., 2017]. Our results showed that urolithin A and urolithin A glucuronide were present in the urine of rats despite the fact that the phenolic fraction contained only trace amounts of EA. Urolithin A was also the main metabolite detected in the blood serum. In the urine, the predominant metabolites were glucuronidated urolithin A and nasutin A. It has been reported that glucuronidation enhances the solubility of these molecules and facilitates their urinal excretion [Fotschki *et al*., 2018].

Many authors while conducting studies on human volunteers but also on laboratory rodents observed huge differences between individuals within a group in relation to concentrations of ET metabolites [Kosmala *et al*., 2017; Tomás-Barberán *et al*., 2017]. In the present experiment, the standard error of the mean (SEM) values calculated for each group, and each metabolite were not as high as expected, hence the differences observed between treatments were statistically significant, thus giving the opportunity to observe biological relationships in the ET metabolites formation.

The animals from the F1–4 group had the highest total ET metabolites concentration in the urine ($P<0.05$ *vs*. other groups except F3–4 rats; Table 4). Considering respective ET metabolites, the urinal urolithin A concentrations determined in groups F1–4, F3–4, and F4 significantly excelled those determined in the remaining groups ($P<0.05$); additionally, the F1–4 group had a significantly higher concentration of total ET metabolites in the urine *vs*. F4 group. It should be noted that apart from control rats (group C) treated only with water, the animals from group F1–2 had no metabolites in their urine and blood serum as well. As for nasutin A in the urine, the F4 rats had a significantly lower concentration of that metabolite in comparison to the rats from groups F1–4 and F3–4, but significantly higher one compared to the rats following C, F1–2, and F1–3 treatments ($P<0.05$). The significance of differences in urinal urolithin A glucuronide and nasutin A glucuronide concentrations between the treatments was identical as it was for the sum of metabolites in the urine ($F1–4^{a}>F3–4^{ab}>F4^{b}>F1–3^{c}>F1–2^{d}$, C^{d}).

The blood serum analyses of ET metabolites revealed that the total concentration metabolites, as well as concentrations of urolithin A glucuronide and DMEAG were the highest

TABLE 5. Blood serum EA and ET metabolites presence in rats differently gavaged the strawberry phenolic fraction for four days (μg/L).

Group*	Urolithin A glucuronide	Nasutin A glucuronide	Ellagic acid dimethyl ether glucuronide DMEAG	Sum of metabolites
C	0.00±0.00[c]	0.00±0.00[d]	0.00±0.00[c]	0.00±0.00[c]
F1–4	44.4±15.9[a]	14.0±8.22[a]	9.11±2.99[a]	67.1±18.8[a]
F1–3	7.32±2.22[c]	1.01±0.00[d]	0.00±0.00[c]	8.08±2.09[c]
F1–2	0.00±0.00[c]	0.00±0.00[d]	0.00±0.00[c]	0.00±0.00[c]
F3–4	41.4±25.9[a]	9.04±1.00[b]	8.05±3.12[a]	58.3±26.5[a]
F4	25.1±8.19[b]	4.04±1.02[c]	5.13±1.22[b]	34.4±9.49[b]
P value	0.004	0.041	0.024	0.021

*The rats were subjected to the following treatments (n=8) without or with the strawberry phenolic fraction (see Table 1) gavaged intragastrically: group C (control with water all four days), group F1–4 (fraction administered all four days), group F1–3 (fraction gavaged days 1, 2, 3, and water gavaged day 4), group F1–2 (fraction gavaged days 1, 2, and water gavaged days 3, 4), group F3–4 (water gavaged days 1, 2, and fraction gavaged days 3, 4), and group F4 (water gavaged days 1, 2, 3, and fraction gavaged day 4). The caecal samples were collected on the 5th day 12 h after the last gavage treatment. Values (presented as mean±SEM) within each column with the same superscript letter are not different at $P<0.05$. EA, ellagic acid; ET, ellagitannins.

in groups F1–4 and F3–4 ($P<0.05$ *vs*. remaining treatments), followed by the F4 group ($P<0.05$ *vs*. all other groups; Table 5). In the case of blood serum, the highest concentration of nasutin A glucuronide was determined in the F1–4 rats ($P<0.05$ *vs*. others), followed by F3–4 ($P<0.05$ vs. others), and F4 ($P<0.05$ *vs*. others) rats. The above-mentioned results support the accepted hypothesis that the presence of ellagitannin metabolites in the blood serum and urine is strongly determined by the post-intake time and the duration of strawberry phenolic fraction administration, as well as that these effects may be rapidly reversible after phenolic fraction withdrawal from a diet. The latter statement is justified especially by results obtained in the F1–2 treatment which was accompanied by no ET metabolites detected both in the urine and the blood serum. That statement was also supported by comparison of results achieved for rats from group F4 *vs*. F1–3, the former had significantly more ET metabolites despite ingesting the strawberry phenolic fraction only once but 12 h prior analyses, and the F1–3 rats were treated three times with the fraction but the last treatment before ET metabolites analysis in that group was with water. In that context, other experiments clearly showed that ellagitannin and ellagic acid metabolites are detected in blood and urine already one day after consumption of a diet containing strawberry polyphenols [Milala *et al*., 2017]. In the study reported by Ito [2011], the urinal excretion of metabolites continued up to 72 h after oral administration of geraniin but the dosage (50 mg/rat) was much more higher than it was in our study (20 mg of strawberry phenolic fraction per kg of BW).

CONCLUSIONS

In conclusion, the performed experiment on rats gavaged once a day the strawberry phenolic fraction or water showed that the concentration of strawberry ellagitannin metabolites in the rat's biological fluids, *i.e.* urine and blood serum, reflected metabolites content in the caecal digesta and depended on the number of subsequent days of phenolic fraction administration. The presence of those metabolites in the caecal digesta, urine, and serum is quickly reversible after phenolic fraction withdrawal from a diet.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Original article
Section: Food Quality and Functionality

Effect of Transglutaminase and Bacterial Concentrates on the Development of Functional and Technological Properties of Minced Meat

Svetlana Merenkova[1], Oksana Zinina[1], Olga Loretz[2], Olga Neverova[2], Pavel Sharaviev[2]*

[1]South Ural State University (National Research University), 76 Lenin Avenue, Chelyabinsk – 454080, Russian Federation
[2]Department of Technology, Ural State Agrarian University, Ekaterinburg, Russian Federation

Key words: minced meat, transglutaminase, bacterial concentrate, technological properties, rheological properties

The results of the effects of transglutaminase (TG) and bacterial concentrates (BC) of different strains of microorganisms on the functional and technological properties of minced meat obtained from beef trimming (80/20) are presented. A control and seven experimental samples of minced meat were obtained – three samples with the addition of 10% BC: Bifilact-Pro, Bifilact-AD, Bifilact-Kefir (S-Pro, S-AD, SK), a sample with the addition of 0.2% TG (S-TG) and three samples with simultaneous addition of 5% BC: Bifilact-Pro, Bifilact-AD, Bifilact-Kefir and 0.1% TG (S-Pro + TG, S-AD + TG, S-K + TG). The control and experimental samples were analyzed for: pH, water binding capacity (WBC), water holding capacity (WHC), cooking loss, as well as physicochemical and rheological properties. The highest WHC was observed in the S-TG samples during the 6–12 h of ripening, whereas in the S-Pro+TG, S-AD+TG and S-K+TG samples during the ripening period of 12–18 h. The data obtained correlated with an increase in the pH value in the samples during the indicated periods. Significant differences were noted in the changes in WBC and WHC when BCs were added to the minced meat, as dependent on the composition of the microorganism cultures. The lowest cooking losses were determined in the S-TG sample, *i.e.* 19.03–19.99% ($p<0.05$), whereas in the S-AD+TG and S-K+TG samples they reached 17.91–18.61% ($p<0.05$) and 18.96–19.58% ($p<0.05$), respectively. The most pronounced elastic properties were found in the samples with TG. Thus, the results showed that the combination of BC and TG demonstrates a synergistic effect on the functional and technological properties of meat systems.

ABBREVIATIONS

TG – Transglutaminase; BC – Bacterial concentrate; WBC – Water binding capacity; WHC – Water holding capacity; EPS – Exopolysaccharides; LAB – Lactic acid bacteria.

INTRODUCTION

The rising cost of beef products has led to the need for the rational use of low-value meat cuts and beef trimmings as restructured meat, as prepared from small cuts of meat. This approach allows for an increase in the yield of marketable products and enterprises to gain additional profits [Sorapukdee & Tangwatcharin, 2018].

In recent years, enzymatic processing of raw materials has become particularly popular with the intensive development of biotechnology [Cheng *et al*., 2009; Liu *et al*., 2012; Rawdkuen & Benjakul, 2012]. Enzymatic modification of food components is more acceptable to the food industry than the more commonly used chemical methods [Kieliszek & Misiewicz, 2014]. The experience of the practical use of enzymes for the processing of raw meat, as accumulated in many countries, suggests that this method of processing is very effective at improving the properties of raw materials and increasing the productive yield of meat products [Rivier, 2007; Rawdkuen & Benjakul, 2012; Uran & Yilmaz, 2018]. Transglutaminase (TG) plays an important role among these enzymes [Uran & Yilmaz, 2018], with its potential to improve the firmness, viscosity, elasticity, and water binding capacity of food products [Kieliszek & Misiewicz, 2014].

The use of TG in the meat industry improves the functional properties of proteins in meat products, contributes to the strong cohesion of a block of meat without the further need for the addition of salt or phosphates, and also has a positive effect on the texture of the final product, which results in an increase in its hardness [Kieliszek & Misiewicz, 2014; Atilgan & Kilic, 2017; Uran & Yilmaz, 2018]. Meat products contain a high level of protein, including the myofibrillar proteins: actin and myosin, which affect the development of product texture and at the same time serve as good substrates for transglutaminase [Tseng *et al.*, 2002].

TG helps solving technological problems in the production of both emulsified sausages and fine and coarse-minced sausages. The ability to bind proteins of different origins can be used in the production of combined meat products, allowing for the inclusion of lower-quality raw materials and ingredients, such as mechanically deboned meat, skimmed milk powder, soy or wheat flour. The use of TG allows for the production of lower fat content meat products which do not dif-

* Corresponding Author:
E-mail: merenkovasp@susu.ru (S. Merenkova)

fer in organoleptic properties from the conventional products [Kieliszek & Misiewicz, 2014].

The results obtained by Sorapukdee & Tangwatcharin [2018] and Baugreet *et al*. [2018] showed that pieces of restructured beef processed with microbial TG could be bound together *via* cross-linking of myosin chains and actin.

Many researchers point to the widespread use of bacterial preparations and starter microorganisms to improve the properties of raw meat [Campbell-Platt, 1995; Hammes & Hertel, 1998; Ruiz-Moyano *et al.*, 2008]. Many of these bacterial preparations include bifidobacteria and propionic acid bacteria [Ruiz-Moyano *et al.,* 2008; Danylenko *et al*., 2014].

Exopolysaccharides (EPS) have an immense commercial value because of their industrially useful physico-chemical properties [Patel *et al.*, 2012]. The polysaccharides excreted by lactic acid bacteria (LAB) during the fermentation processes result in the particular viscosity of certain foods [Sanni *et al.*, 2002].

EPS are complex carbohydrates located outside the cell. Many strains of LAB produce extracellular polysaccharides which may be tightly associated with the bacterial cell wall as capsules or otherwise liberated into the growth medium as a loose slime [Cerning, 1995]. Some Bifidobacterium strains, as well as many other microorganisms, are able to produce these polymers [Ruas-Madiedo *et al.*, 2007]. Dairy propionibacteria are also capable of producing EPS [Cerning, 1995].

EPS-producing lactic acid bacteria (EPS-producing cultures) are used to improve dairy product functionality by binding free water. EPS have the ability to bind water and to increase moisture retention by water binding or entrapment within their three-dimensional networks [El Soda, 2014]. EPS derived from LAB play a crucial role in improving the rheology, texture, and mouth feel of fermented food formulations and conferring beneficial physiological effects to human health [Doleyres *et al.*, 2005].

However, the influence of TG in combination with bacterial concentrates (BC) on physicochemical and technological properties of meat products has not been reported to date in the relevant literature. Thus, the aim of this research was to determine the effect of transglutaminase and bacterial concentrates composed of different strains of microorganisms on the functional and technological properties of minced meat during the ripening process.

MATERIALS AND METHODS

Materials

Beef was collected 48 h after slaughtering from a breeding plant "Dubrovsky" (Chelyabinsk, Russia). Fresh beef trimmings (80/20) (66.42 g/100 g moisture, 16.10 g/100 g protein, and 17.64 g/100 g fat) were obtained from rib and round portions. Beef trimmings were transported in a refrigerator at 4°C to the laboratory of the Food and Biotechnology Department (South Ural State University, Chelyabinsk, Russia) within 2 h for further processing. Then, beef trimming was analyzed for pH using a portable pH-meter (HANNA HI83141, Woonsocket, RI, USA). An electrode was inserted to a depth of 5±1 cm, where the values obtained ranged from 5.56 to 5.62.

Bacterial concentrates (BCs): Bifilact–Pro, Bifilact–AD and BK–Kefir, were produced by the Federal State Unitary Enterprise "An Experimental Biofactory" of the Russian Agricultural Academy (Uglich, Russian Federation):

- concentrate Bifilact–Pro consists of *Lactococcus lactis subsp. diacetilactis, Streptococcus thermophilus, Propionibacterium freudenreichii, Bifidobacterium Bifidum, Bifidobacterium longum, Bifidobacterium adolescentis* species;
- concentrate Bifilact–AD consists of *Lactococcus lactis subsp. diacetilactis, Streptococcus thermophilus, Lactobacillus acidophilus, Bifidobacterium Bifidum, Bifidobacterium longum, Bifidobacterium adolescentis* species;
- concentrate BK–Kefir consists of *Lactococcus lactis subsp. Lactis, Lac. lactis subsp. Cremoris, Lac. lactis subsp. Diacetilactis, Leuconostoc mesenteroides subsp. Cremoris, Streptococcus thermophiles, Lactobacillus plantarum, Lbc. fermentum, L. casei, Acetobacter aceti*.

Transglutaminase is an enzyme preparation produced by cultivation of *Streptomyces mobaraensis* (BioBond Shanghai's Kinry Pharmaceutical Co., Ltd. Shanghai, China).

Preparation of minced meat samples

The beef trimmings (80/20) were passed through a grinder (Fimar 32/RS Unger, Italy) fitted with a plate with 6 mm diameter holes.

BCs were activated in 1 L of sterilized skimmed milk at a temperature of 37±1°C. Microbial TG was added to the meat in a dry form, and water was added to activate it. For the research, control and test samples were made – experimental samples of minced meat with the introduction of 10% BCs by weight of raw materials (S-Pro – sample with concentrate Bifilact–Pro, S-AD – sample with concentrate Bifilact–AD, S-K – sample with concentrate BK-Kefir), experimental samples of minced meat with the addition of 0.2% TG by weight of raw materials (S-TG), and experimental samples

TABLE 1. Formulation of samples.

Samples	Components (kg per 100 kg of minced meat)		Biotechnological additives (kg per 100 kg of minced meat)	
	beef trimmings (80/20)	water	TG	bacterial concentrate
S-C	90	10	0	0
S-Pro	90	0	0	10
S-AD	90	0	0	10
S-K	90	0	0	10
S-TG	90	10	0.2	0
S-Pro+TG	90	5	0.1	5
S-AD+TG	90	5	0.1	5
S-K+TG	90	5	0.1	5

Note: S-C – control sample; S-Pro, S-AD, S-K – samples with the addition of 10% concentrate Bifilact–Pro, Bifilact–AD, Bifilact-Kefir; S-TG– samples with the addition of 0.2% transglutaminase; S-Pro+TG, S-AD+TG, S-K+TG – samples with the addition of 0.1% transglutaminase and 5% Bifilact–Pro, Bifilact–AD, Bifilact-Kefir.

with simultaneous addition of 0.1% enzyme TG and 5% BC by weight of raw materials (S-Pro+TG – sample with concentrate Bifilact-Pro and TG, S-AD+TG – sample with concentrate Bifilact–AD and TG, S-K+TG – sample with concentrate BK-Kefir and TG). A sample of the minced meat from the trimmings was taken as the control sample (S-C). Thus, a total of eight formulations were taken (Table 1), where five samples of minced meat were made for each formulation.

Prepared minced meat samples were kept for ripening at a temperature of 2±2°C for 24 h, and parts of the samples were taken to determine indicators characterizing the functional and technological properties of minced meat (pH, water binding capacity (WBC), water holding capacity (WHC), cooking loss and rheological properties) every 6 h (0, 6, 12, 18, 24 h).

Technological properties measurements

Water binding capacity

The WBC was determined *via* the filter paper method [Grau & Hamm, 1956], which is based on determining the amount of separated moisture after lightly pressing and calculating the spot area formed on a piece of filter paper using a planimeter. The mass fraction of the bound moisture in a given sample was calculated using the following formula:

$$X = (A - 8.4 \times B) \times 100\ A,$$

where: X – mass fraction of bound water in a sample (% total moisture), A – total mass of moisture in a sample (mg), and B – area of the wet spot (mm^2).

Water holding capacity

The WHC was determined gravimetrically as described by Zhang *et al.* [2018]. Samples were placed in sealed centrifuge tubes and centrifuged at 70×*g* for 15 min at 4°C (Laboratory centrifuge "OKA", Sibagroprom, Novosibirsk, Russia). After heating at 75°C, the supernatant was decanted, and samples were weighed. The samples were then stored at 4°C for 24 h and centrifuged at 110×*g* for 10 min at 4°C (Laboratory centrifuge "OKA", Sibagroprom, Novosibirsk, Russia). The resulting supernatant was removed using filter paper and samples were weighed. Water holding capacity (%) was expressed using the following formula:

$$WHC(\%) = [(W_2\text{-}W)/(W_1\text{-}W)] \times 100\%$$

where: W – mass of the sample (g), W_1 – mass of the sample after heating and decanting the supernatant (g), and W_2 – mass of the sample after centrifuging and removal of resulting supernatant (g).

Cooking loss

Weight loss of minced meat during heat treatment was determined using the gravimetric method, based on measuring the mass of a sample before and after heat treatment. To determine cooking loss, the minced meat was formed into meatballs weighing 11±1 g each, which were weighed before heating at 75°C in the air-o-steam (Rational AG, Landsberg am Lech, Germany) for 10 min. Cooking loss was calculated using the following formula:

Cooking loss (%) = [(weight of raw sample (g) –weight of cooked sample (g) / weight of raw sample (g)] × 100.

pH

To determine pH values, 5 g of minced meat sample was blended with 20 mL of distilled water for 1 min using a chopper [Bosch MMR 0801, Gerlingen, Germany]. The pH was measured with a stationary pH meter (model HANNA HI 2210 (PH/T), Woonsocket, RI, USA) equipped with a pH and temperature electrode HI 1131B and HI 7662 (HANNA) at 20°C. Before measuring pH, the detector was calibrated with pH 4.01 and pH 6.86 buffers.

Rheological measurements

Rheological measurements were conducted using a texture analyzer "Structurometer ST2" (LAB, Quality Laboratory, Moscow, Russia) by compressing it with an indenter "Cylinder Ø36" (duralumin, mass 42.5 g). The analysis of the mechanical tension induced on a cylindrical indenter during its introduction into the product was carried out under the following loading mode: contact force (Fc = 7 g), strain rate (Vd = 0.5 mm/s), and the introduction of the indenter continued until the effort Fmax = 500 g. Total, plastic, and elastic deformations were determined. Measurements were carried out at a sample temperature of 2±2°C.

Physicochemical analyses

Chemical analyses of the minced meat samples were performed 24 h after their preparation. Total nitrogen content was assayed using the Kjeldahl method [ISO 937:1978]. Nitrogen was converted to equivalent protein content using a factor of 6.25. Moisture was determined according to ISO standard [ISO 1442:1997]. Total fat was determined by the Soxhlet method according to ISO standard [ISO 1444:2000].

Statistical analysis

The experiment was carried out in five replicates and each analysis was performed in three repetitions. All measurements were conducted in triplicate. Results were expressed as mean values of five replicates ± standard deviation. Probability values $p \leq 0.05$ were taken to indicate statistical significance. The data was analyzed *via* one-way ANOVA and the Tukey test using the free web-based software offered by Assaad *et al.* [2014].

RESULTS AND DISCUSSION

pH, WBC, WHC and cooking loss

TG maintains its maximal enzymatic activity at temperatures close to 0°C [Yokoyama *et al.,* 2004], allowing it to be used at the low temperatures required for meat ripening.

The research results (Table 2) demonstrated that the pH of minced meat changed depending on the concentration of biotechnological components and length of the ripening period. In the control sample, a gradual increase in the pH to 5.82 by the end of the ripening period was observed, which

TABLE 2. The pH value of minced meat samples during ripening.

Samples	0 h	6 h	12 h	18 h	24 h
S-C	5.53 ± 0.004^{eA}	5.56 ± 0.005^{dD}	5.66 ± 0.004^{cE}	5.76 ± 0.005^{bD}	5.82 ± 0.005^{aC}
S-Pro	5.46 ± 0.005^{bcB}	5.48 ± 0.007^{bE}	5.50 ± 0.004^{aF}	5.46 ± 0.005^{cF}	5.34 ± 0.004^{dE}
S-AD	5.45 ± 0.004^{dB}	5.49 ± 0.004^{cE}	5.52 ± 0.005^{bF}	5.56 ± 0.004^{aE}	5.46 ± 0.004^{dD}
S-K	5.45 ± 0.004^{bB}	5.50 ± 0.004^{aE}	5.44 ± 0.003^{bG}	5.40 ± 0.005^{cG}	5.37 ± 0.003^{dE}
S-TG	5.51 ± 0.004^{cA}	5.87 ± 0.005^{bA}	5.93 ± 0.004^{aC}	5.88 ± 0.007^{bC}	5.86 ± 0.004^{bB}
S-Pro+TG	5.45 ± 0.002^{dB}	5.65 ± 0.005^{cC}	5.88 ± 0.005^{aD}	5.86 ± 0.005^{bC}	5.84 ± 0.004^{bBC}
S-AD+TG	5.45 ± 0.004^{eB}	5.69 ± 0.005^{dC}	5.97 ± 0.004^{aB}	5.95 ± 0.004^{bB}	5.90 ± 0.007^{cA}
S-K+TG	5.46 ± 0.005^{dB}	5.76 ± 0.004^{cB}	6.02 ± 0.004^{aA}	6.00 ± 0.007^{aA}	5.94 ± 0.006^{bA}

Note: data are the mean values of five replicates ± standard deviation (n=5). Means in a row without a common superscript letter a, b, c – are statistically significantly different ($p<0.05$). Means in a column without a common superscript letter A, B, C – are statistically significantly different ($p<0.05$) as analyzed by one-way ANOVA and the TUKEY test.

S-C – control sample; S-Pro, S-AD, S-K – samples with the addition of 10% concentrate Bifilact–Pro, Bifilact–AD, Bifilact-Kefir; S-TG – samples with the addition of 0.2% transglutaminase; S-Pro+TG, S-AD+TG, S-K+TG – samples with the addition of 0.1% transglutaminase and 5% Bifilact–Pro, Bifilact–AD, Bifilact-Kefir.

is typical of a meat system that does not contain technological additives.

The pH values of minced systems varied from 5.45 to 5.53 before the start of the ripening process. From results presented in Table 2, it can be concluded that the introduction of BC in a 10% concentration led to a successive increase in the acidity of the meat system and a decrease in pH to 5.34–5.46 after 24 h of fermentation. This effect is due to the accumulation of acidic metabolic products of bacteria that will reduce the pH as a fact of their acidity [Mejri *et al.,* 2017]. Moreover, the accumulation of the acidic products of microbial activity in minced meat was irregular throughout the ripening period; in the samples containing BC – S-AD and S-Pro (10%), there was a significant increase in pH to 5.50–5.56 ($p<0.05$) after 12–18 h of ripening, followed by a successive decrease.

There was a significant increase in pH in the samples containing TG compared with the control samples after 12–18 h of ripening The maximum pH level in the S-TG samples was observed during the 6–12 h interval (5.87–5.93), and for the samples containing a complex of TG and BC (S-AD+TG and S-K+TG) at 12–18 h (5.97–6.02). Meiying *et al*. [2002] demonstrated that the pH of meat batter treated with TG was higher than that of meat without TG treatment. The pH value established in the samples of minced meat with TG was remote from the isoelectric point of muscle proteins, which significantly affected their functional properties, ensuring a high level of WBC, WHC, and low weight loss during heat treatment. Hughes *et al.* [2014] reported that loss of water from muscles and the degree of changes in their properties depends on the pH which muscle proteins are sensitive to.

Significant differences in the dynamics of WBC were noted depending on the composition of microorganisms in experimental samples with BC (Table 3). In sample S-K, WBC increased slightly and reached its maximum during 6–12 h of the ripening period. There was a rapid increase in WBC, which reached a maximum by 12–18 h of ripening, when a starter containing acidophilic and propionic acid cultures (samples S-AD and S-Pro) was added to the minced meat. It should be emphasized that the addition of BC Bifilact–Pro and Bifilact–AD significantly improved the water binding capacity of the meat system after 12 h of maturation to 51.8–52.1% ($p<0.05$) compared to the control. Cerning [1995] and Patel *et al.* [2012] proved that lactic acid bacteria and propionic bacteria are capable of producing EPS at low temperatures and pH levels close to 5.5. It can be assumed that the improvement in water binding capacity observed for S-Pro and S-AD samples is due to the water-absorbing properties of EPS, which accumulate during the vital activity of these types of bacteria. However, there was a considerable decrease in WBC of minced meat with bacterial starters to 39.9–46.3% ($p<0.05$) by the end of the ripening process (24 h), which was apparently due to the accumulation of lactic acid and the consequent decrease in pH to that close to the isoelectric point of muscle proteins. Mauriello *et al.* [2004] noted that WBC decrease is related to pH decrease; when pH is close to the isoelectric point of the protein, the functional properties of the meat proteins are reduced. Puolanne *et al.* [2013] researched the WHC of meat without added salt, which was found to have a distinct minimum at pH 5.0, which is the average isoelectric pH of meat structural proteins.

It should be emphasized that the combination of BC and TG has a synergistic effect on the functional and technological properties of minced systems. Thus, when TG and BC are added to minced meat, the protein matrix is rapidly formed during the first 6–12 h of exposure; the technological properties of meat are enhanced as a result of TG activity. This has been confirmed by the research results. The highest WBC was observed in the S-TG samples during the first 6–12 h of maturation, whereas in the S-Pro+TG, S-AD+TG, and S-K+TG samples – in 12–18 h of the maturation period. The data obtained correlate with an increase in pH of the meat system during the indicated periods. In addition, in the samples containing the complex of TG and bacterial cultures, the WBC increased considerably to 58.6–65.0% ($p<0.05$) as compared

TABLE 3. The functional and technological properties of minced meat samples.

Samples	0 h	6 h	12 h	18 h	24 h
			WBC (%)		
S-C	39.70 ± 0.014^{eH}	42.03 ± 0.062^{dF}	49.45 ± 0.017^{cG}	50.24 ± 0.057^{bE}	50.83 ± 0.03^{aE}
S-Pro	44.53 ± 0.014^{dC}	46.62 ± 0.015^{eD}	52.10 ± 0.013^{aE}	49.91 ± 0.015^{bF}	39.90 ± 0.016^{eH}
S-AD	41.29 ± 0.012^{eG}	42.80 ± 0.013^{dE}	51.75 ± 0.014^{aF}	50.50 ± 0.013^{bD}	46.35 ± 0.019^{cF}
S-K	44.61 ± 0.012^{cA}	46.60 ± 0.013^{aD}	45.11 ± 0.012^{bH}	44.34 ± 0.012^{dG}	43.93 ± 0.016^{eG}
S-TG	45.44 ± 0.011^{eB}	59.56 ± 0.015^{aA}	58.49 ± 0.063^{bD}	55.10 ± 0.017^{cB}	54.80 ± 0.015^{dC}
S-Pro+TG	41.52 ± 0.015^{eF}	46.66 ± 0.013^{dD}	61.58 ± 0.015^{aC}	57.04 ± 0.086^{bC}	53.46 ± 0.016^{cA}
S-AD+TG	43.17 ± 0.013^{eE}	48.42 ± 0.015^{dC}	64.68 ± 0.014^{aB}	58.66 ± 0.015^{bA}	55.91 ± 0.016^{cB}
S-K+TG	43.53 ± 0.018^{eD}	55.57 ± 0.012^{cB}	65.03 ± 0.023^{aA}	58.77 ± 0.013^{bA}	54.37 ± 0.014^{dD}
			Cooking loss (%)		
S-C	27.35 ± 0.14^{aBC}	26.08 ± 0.16^{bB}	25.40 ± 0.08^{cB}	24.34 ± 0.15^{dC}	24.03 ± 0.08^{dD}
S-Pro	27.91 ± 0.15^{aA}	27.18 ± 0.09^{bA}	24.96 ± 0.08^{dB}	25.58 ± 0.09^{cB}	28.09 ± 0.08^{aA}
S-AD	27.75 ± 0.14^{aAB}	26.20 ± 0.1^{bB}	23.54 ± 0.17^{dC}	23.05 ± 0.09^{dD}	24.94 ± 0.05^{cC}
S-K	28.02 ± 0.1^{aA}	26.76 ± 0.09^{cA}	26.17 ± 0.11^{dA}	27.75 ± 0.13^{abA}	27.32 ± 0.12^{bB}
S-TG	26.45 ± 0.05^{aE}	19.99 ± 0.14^{dE}	19.03 ± 0.07^{eE}	21.4 ± 0.03^{cE}	23.68 ± 0.04^{bE}
S-Pro+TG	27.62 ± 0.06^{aAB}	25.25 ± 0.05^{bC}	20.27 ± 0.2^{eD}	21.71 ± 0.1^{dE}	22.35 ± 0.05^{cF}
S-AD+TG	26.96 ± 0.05^{aCD}	24.78 ± 0.07^{bD}	17.91 ± 0.06^{eF}	18.61 ± 0.09^{dG}	20.92 ± 0.04^{cH}
S-K+TG	26.68 ± 0.04^{aDE}	24.33 ± 0.04^{bD}	18.96 ± 0.04^{eE}	19.58 ± 0.09^{dF}	21.60 ± 0.03^{cG}
			WHC (%)		
S-C	35.36 ± 0.179^{bA}	38.72 ± 0.168^{aD}	38.84 ± 0.201^{aC}	39.12 ± 0.252^{aD}	39.33 ± 0.256^{aD}
S-Pro	33.83 ± 0.195^{eB}	35.94 ± 0.246^{dE}	37.2 ± 0.259^{cD}	36.7 ± 0.214^{abE}	35.9 ± 0.272^{aE}
S-AD	35.2 ± 0.199^{eA}	36.48 ± 0.227^{dE}	37.86 ± 0.24^{cCD}	37.3 ± 0.259^{abE}	36.2 ± 0.251^{aE}
S-K	34.99 ± 0.156^{dA}	36.54 ± 0.242^{cE}	38.14 ± 0.22^{bCD}	37.9 ± 0.182^{abDE}	37.2 ± 0.283^{bcE}
S-TG	34.62 ± 0.23^{dAB}	48.12 ± 0.177^{abA}	48.62 ± 0.188^{aA}	47.34 ± 0.28^{bcC}	46.84 ± 0.298^{cC}
S-Pro+TG	34.87 ± 0.161^{dA}	43.24 ± 0.232^{cC}	45.9 ± 0.228^{bB}	48.16 ± 0.31^{aBC}	49.08 ± 0.334^{aB}
S-AD+TG	35.06 ± 0.171^{dA}	44.88 ± 0.159^{cB}	46.58 ± 0.304^{bB}	49.28 ± 0.36^{aAB}	49.84 ± 0.317^{aB}
S-K+TG	33.94 ± 0.144^{dB}	44.19 ± 0.25^{cBC}	46.34 ± 0.273^{bB}	50.16 ± 0.296^{aA}	51.23 ± 0.328^{aA}

Note: data are the mean values of five replicates ± standard deviation (n=5). Means in a row without a common superscript letter a, b, c – are statistically significantly different ($p<0.05$). Means in a column without a common superscript letter A, B, C – are statistically significantly different ($p<0.05$) as analyzed by one-way ANOVA and the TUKEY test.

S-C – control sample; S-Pro, S-AD, S-K – samples with the addition of 10% concentrate Bifilact–Pro, Bifilact–AD, Bifilact-Kefir; S-TG – samples with the addition of 0.2% transglutaminase; S-Pro+TG, S-AD+TG, S-K+TG – samples with the addition of 0.1% transglutaminase and 5% Bifilact–Pro, Bifilact–AD, Bifilact-Kefir; WBC – water binding capacity; WHC – water holding capacity.

with samples containing only the TG in 12–18 h of the ripening period. A slight decrease in WBC to 53.5–55.9% ($p<0.05$) was observed in these samples during the last exposure period, which can be associated with the rising proteolytic activity of the microorganisms and which leads to partial destruction of the protein matrix.

In the literature, there is contradictory information about the influence of TG on WBC and cooking loss, respectively. Some studies have found that TG does not significantly affect product loss [Uran *et al*., 2013], while others point to the negative impact of TG on cooking loss [Atilgan & Kilic, 2017]. Atilgan & Kilic [2017] demonstrated that the use of microbial TG had a negative effect on WBC and caused increased cooking loss in muscle-based foods. Aaslyng *et al.* [2003] noted the correlation between the WHC of raw pork muscle and cooking loss which was dependent on cooking temperature.

The results of our research showed that decreasing WHC and WBC was correlated to increased cooking loss. Significant differences in weight loss compared to the control sample

TABLE 4. Contents of major components of minced meat samples.

Samples	Fat (g/100 g)	Protein (g/100 g)	Moisture (g/100 g)
S-C	17.82±0.13^{a}	16.36±0.10^{ab}	65.16±0.26^{d}
S-Pro	16.56±0.07^{bc}	15.58±0.09^{cd}	67.08±0.29^{abc}
S-AD	16.50±0.17^{c}	15.18±0.11^{d}	67.40±0.26^{ab}
S-K	16.22±0.11^{c}	15.44±0.12^{d}	67.68±0.32^{a}
S-TG	17.22±0.27^{ab}	16.58±0.16^{a}	66.00±0.23^{cd}
S-Pro+TG	16.64±0.14^{bc}	16.20±0.07^{abc}	66.42±0.22^{ad}
S-AD+TG	16.90±0.17^{bc}	15.88±0.19^{ad}	66.26±0.18^{bd}
S-K+TG	16.90±0.12^{bc}	15.78±0.14^{bd}	66.56±0.13^{abc}

Note: data are the mean values of five replicates ± standard deviation (n=5). Means in a column without a common superscript letter a, b, c – are statistically significantly different ($p<0.05$) as analyzed by one-way ANOVA and the TUKEY test.

S-C – control sample; S-Pro, S-AD, S-K – samples with the addition of 10% concentrate Bifilact–Pro, Bifilact–AD, Bifilact-Kefir; S-TG – samples with the addition of 0.2% transglutaminase; S-Pro+TG, S-AD+TG, S-K+TG – samples with the addition of 0.1% transglutaminase and 5% Bifilact–Pro, Bifilact–AD, Bifilact-Kefir.

were found only in the S-TG and S-K+TG samples at the initial stage of maturation (0 h) (Table 3), while in the 18–24 h period, a considerable reduction in cooking loss in comparison to the control was established for all samples containing TG enzyme (0.1 and 0.2%). Thus, the greatest mass loss was observed in the samples without TG (S-C, S-Pro, S-AD, S-K), and this tendency was found to continue from 6 h until the end of the maturation process. The lowest cooking loss was observed in samples with high WBC and WHC. Thus, in the S-TG sample, the lowest cooking losses were established between 6–12 h and reached 19.03–19.99% ($p<0.05$), whilst in the S-AD+TG and S-K+TG samples the lowest weight loss was found in the 12–18 h period and amounted to 17.91––18.61% ($p<0.05$) and 18.96–19.58% ($p<0.05$), respectively.

Pietrasik *et al.* [2007] noted that TG increases the WHC of meat products and decreases cooking loss, which is in agreement with the results reported above. Zeng *et al.* [2017] determined that the degradation of myofibrils as a result of enzyme proteolytic activity led to improved water holding in myofibrils. The results obtained confirm that not only does the TG activity have a positive effect on WHC, but further that the microorganisms in BC possessing the ability to synthesize EPS, which are hydrocolloids, enhance the ability of the meat system to hydrate. Consequently, the highest values of WHC were detected in the samples with TG, *i.e.* 48.6% ($p<0.05$) in sample S-TG at 12 h of maturation, 51.2 and 49.8% ($p<0.05$) in samples S-K+TG and S-AD+TG, respectively at 24 h of maturation.

Accordingly, the results obtained confirm the synergistic effect of the activity of TG and BC on the development of the functional and technological properties of the meat system. Exposure for 12–18 h was found to be optimal for the development of the required technological qualities of minced meat with the addition of TG and BC.

Chemical composition

The results of determination of minced meat physicochemical indicators are presented in Table 4. The results obtained demonstrate that applying various biotechnological additives resulted in a significant decrease in the fat content in the experimental groups compared with the control group ($p<0.05$). Protein contents of the samples containing BCs (S-Pro, S-AD, S-K) were significantly reduced ($p<0.05$) in comparison to those in the control sample and samples with TG, which was probably due to the lower water binding capacity and water holding capacity of these samples and caused loss of nutrients. The moisture content of the samples with BCs (S-Pro, S-AD, S-K) was 67.1, 67.4, and 67.7 g/100 g, respectively, while the moisture content of the samples with TG (S-TG, S-Pro+TG, S-AD+TG, S-K+TG) ranged from 66.0 to 66.6 g/100 g. According to Atilgan & Kilic [2017], the use of TG did not cause significant differences in protein contents among groups of cooked ground beef. The protein content in our studies ranged from 15.2 to 16.6 g/100 g ($p<0.05$); no statistically significant differences were found in its values in the control and experimental groups with TG (0.1 and 0.2%). Similar results were obtained by Uran & Yilmaz [2017], who showed no statistically significant differences in the protein content in the control and other groups containing the enzyme in different concentrations (0.2, 0.6, 0.8, and 1%) during burger production.

Rheological properties

Minced meat has a plastic-elastic structure characterized by a complex of structural and mechanical properties. The deformation characteristics of the meat system depend on the moisture, fat content, and degree of grinding, and at the biochemical level – on the interaction forces between the functional groups of the molecules.

As a result of rheological studies, the effects of introducing TG and BC containing various types of microorganisms on the elastic-plastic properties of the meat system have been established. During the initial stages of ripening, relatively high rates of total and plastic deformation were established in minced meat containing both 5% and 10% BC (Table 5), which indicates the softening of the raw meat.

The most pronounced elastic properties were determined in samples S-TG, S-Pro+TG, S-AD+TG, and S-K+TG. Moreover, there was a tendency towards a progressive increase in these indicators with increasing exposure time of the minced meat. The gradient increase in elastic deformation over 24 h of maturation in the S-TG (from 2.25 to 3.26 mm) and S-Pro+TG (from 1.99 to 3.32 mm) samples was very clearly observed. The lowest total and plastic deformations in the 12–24 h period were established in the samples with 0.2% TG at 10.6–12.2 mm and 8.0–9.8 mm, respectively. In the samples that contained bacterial starters (S-Pro, S-AD, S-K), a "hopping" change in the elastic properties was observed, with the maximum elastic deformation detected in the 12–18 h period.

It should be noted that after 12 h of maturation, the maximum strengthening of the meat system structure occurred under the influence of both TG and microorganism enzymes. The total deformation decreased to 10.57–18.06 mm, plas-

TABLE 5. Deformation characteristics of minced meat samples.

Samples	0 h	6 h	12 h	18 h	24 h
			Total deformation (mm)		
S-C	14.56 ± 0.12^{abF}	15.00 ± 0.355^{aF}	14.07 ± 0.049^{bD}	13.96 ± 0.038^{bF}	13.77 ± 0.152^{bF}
S-Pro	23.10 ± 0.215^{aBC}	22.57 ± 0.474^{aBC}	16.05 ± 0.025^{dB}	19.98 ± 0.091^{cB}	21.44 ± 0.215^{bA}
S-AD	22.48 ± 0.167^{aCD}	21.73 ± 0.271^{aCD}	15.33 ± 0.380^{cBC}	19.12 ± 0.265^{bC}	18.65 ± 0.535^{bB}
S-K	26.28 ± 0.145^{aA}	25.63 ± 0.166^{aA}	18.06 ± 0.354^{cA}	22.01 ± 0.080^{bA}	21.06 ± 0.393^{bA}
S-TG	16.79 ± 0.040^{aE}	16.49 ± 0.173^{aE}	10.57 ± 0.240^{cF}	11.51 ± 0.205^{bG}	12.15 ± 0.101^{bE}
S-Pro+TG	21.90 ± 0.237^{aD}	20.98 ± 0.225^{bD}	14.00 ± 0.063^{eD}	16.69 ± 0.090^{dE}	18.75 ± 0.118^{cB}
S-AD+TG	23.47 ± 0.037^{aB}	23.77 ± 0.051^{aB}	12.93 ± 0.099^{dE}	17.16 ± 0.113^{cE}	18.07 ± 0.126^{bBC}
S-K+TG	22.37 ± 0.251^{aCD}	21.74 ± 0.333^{aCD}	14.83 ± 0.075^{cCD}	17.87 ± 0.079^{bD}	17.14 ± 0.396^{bC}
			Plastic deformation (mm)		
S-C	13.53 ± 0.232^{aC}	12.82 ± 0.353^{abA}	11.88 ± 0.349^{bcC}	12.03 ± 0.084^{bcD}	11.21 ± 0.199^{cD}
S-Pro	21.86 ± 0.223^{aA}	20.43 ± 0.368^{bBC}	12.71 ± 0.162^{eB}	16.95 ± 0.105^{dB}	18.51 ± 0.190^{cA}
S-AD	20.82 ± 0.132^{aAB}	19.58 ± 0.308^{aB}	12.28 ± 0.320^{cB}	14.22 ± 0.147^{bC}	15.58 ± 0.657^{bB}
S-K	24.75 ± 0.107^{aD}	23.92 ± 0.191^{aE}	15.66 ± 0.398^{cA}	19.42 ± 0.247^{bA}	18.60 ± 0.472^{bA}
S-TG	14.38 ± 0.215^{aC}	13.62 ± 0.192^{bD}	8.00 ± 0.127^{eE}	9.81 ± 0.073^{cE}	8.88 ± 0.041^{dE}
S-Pro+TG	19.64 ± 0.229^{aB}	18.56 ± 0.216^{bB}	10.96 ± 0.064^{eCD}	12.52 ± 0.065^{dD}	15.58 ± 0.087^{cB}
S-AD+TG	21.91 ± 0.030^{aA}	21.38 ± 0.024^{bC}	9.79 ± 0.084^{eD}	12.25 ± 0.195^{dD}	15.03 ± 0.142^{cB}
S-K+TG	19.49 ± 0.183^{aB}	19.56 ± 0.398^{aB}	12.02 ± 0.066^{cB}	14.97 ± 0.060^{bC}	13.92 ± 0.317^{bC}
			Elastic deformation (mm)		
S-C	1.986 ± 0.040^{cB}	2.188 ± 0.002^{bcB}	2.683 ± 0.146^{aBC}	2.369 ± 0.052^{abB}	2.652 ± 0.054^{aDE}
S-Pro	2.024 ± 0.048^{cAB}	2.142 ± 0.107^{cB}	3.339 ± 0.188^{aA}	3.118 ± 0.044^{abA}	2.796 ± 0.085^{bCD}
S-AD	1.930 ± 0.037^{bB}	2.156 ± 0.072^{bB}	3.050 ± 0.061^{aAB}	3.028 ± 0.062^{aA}	3.074 ± 0.123^{aAC}
S-K	1.583 ± 0.068^{bC}	1.708 ± 0.025^{bC}	2.400 ± 0.114^{aC}	2.194 ± 0.029^{aB}	2.459 ± 0.083^{aE}
S-TG	2.254 ± 0.043^{dA}	2.828 ± 0.020^{cA}	3.074 ± 0.047^{bAB}	3.089 ± 0.027^{bA}	3.260 ± 0.032^{aA}
S-Pro+TG	1.998 ± 0.068^{dB}	2.414 ± 0.008^{cB}	3.046 ± 0.061^{bAB}	3.007 ± 0.055^{bA}	3.318 ± 0.071^{aA}
S-AD+TG	2.115 ± 0.069^{cAB}	2.390 ± 0.075^{bB}	3.241 ± 0.052^{aA}	3.180 ± 0.047^{aC}	3.050 ± 0.016^{aAC}
S-K+TG	1.956 ± 0.029^{bB}	2.180 ± 0.065^{bB}	2.915 ± 0.159^{aAC}	3.008 ± 0.055^{aA}	2.928 ± 0.052^{aC}

Note: data are the mean values of five replicates ± standard deviation (n=5). Means in a row without a common superscript letter a, b, c – are statistically significantly different ($p<0.05$). Means in a column without a common superscript letter A, B, C – are statistically significantly different ($p<0.05$) as analyzed by one-way ANOVA and the TUKEY test.

S-C – control sample; S-Pro, S-AD, S-K – samples with the addition of 10% concentrate Bifilact–Pro, Bifilact–AD, Bifilact-Kefir; S-TG – samples with the addition of 0.2% transglutaminase; S-Pro+TG, S-AD+TG, S-K+TG – samples with the addition of 0.1% transglutaminase and 5% Bifilact–Pro, Bifilact–AD, Bifilact-Kefir.

tic deformation was up to 8.00–15.66 mm, and the extent of elastic deformation was 2.40–3.34 mm. Canto *et al.* [2014] reported that the increase in springiness and cohesiveness of meat systems containing TG might have been due to the enhanced protein cross-linking between particles of meat. A gradual decrease in total and plastic deformation and an increase in elastic deformation during the ripening period were observed in the control sample.

During the entire ripening period (24 h), it was noted that the S-K and S-Pro samples were characterized by the least elasticity and most pronounced plasticity. The observed changes were caused by the proteolytic activity of microorganisms in the composition of BC capable of polypeptides release, which leads to the relaxation of raw meat structure. Nevertheless, with the combined use of starter microorganisms and TG involved in the binding of food systems biopolymers, all samples of minced meat showed an improvement in both the plastic and elastic properties. As a result, the meat system with developed elastic-plastic properties can be easily moulded into a dense monolithic structure, subsequently

forming the elastic consistency of the meat product. Ionescu *et al.* [2008] showed that the increase in viscosity of myofibrillar protein with TG treatment is due to protein chain cross-linking catalyzed by TG. The cross-linkage between TG and protein can affect the viscosity of food system, making it stiffer and more rigid than the control. Lesiow *et al.* [2017] demonstrated that the presence of TG increased the hardness of control pork meat batters. The interactions of proteins and water have important effects on water holding and gelling in meat products, and consequently on their technological properties.

CONCLUSIONS

The results of the studies showed a unidirectional positive effect of TG and BC on the functional and technological properties of the meat system. It has been determined that a ripening time of 12–18 h is sufficient for the development of optimal WBC, WHC and rheological properties of minced meat with the addition of TG and BC. The results of this research indicate that the meat industry may achieve the benefits of improved technological properties and reduced cooking loss by using a combination of BC with TG in their formulations.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Original article
Section: Food Technology

Efficacy of an Aromatic Vinegar in Reducing Psychrotrophic Bacteria and Biogenic Amines in Salmon Fillets (*Salmo salar*) Stored in Modified Atmosphere Packaging

Jessica Di Toro[1], Raffaella Branciari[1,], Rossana Roila[1], Serena Altissimi[2], Haiyang Jang[3], Kang Zhou[3], Simona Perucci[4], Michaela Codini[5], David Ranucci[1]*

[1]*Department of Veterinary Medicine, University of Perugia, Via San Costanzo 4, 06123 Perugia, Italy*
[2]*Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Via G. Salvemini, 1, 06126, Perugia, Italy*
[3]*College of Food Science, Sichuan Agricultural University, 46 Xinkang Rd, Ya'an, Sichuan, China*
[4]*Freelance researcher, Perugia, Italy*
[5]*Department of Pharmaceutical Science, University of Perugia, Via San Costanzo 4, 06123 Perugia, Italy*

Key words: salmon, vinegar, spoilage bacteria, MAP, biogenic amines

Salmon flesh spoilage can be greatly reduced through the use of preservation methods, using natural products combined with low temperature and packaging. Microbiological and physicochemical characteristics of fresh salmon fillets (*Salmo salar)*, sprayed with an aromatic vinegar and stored in modified atmosphere packaging, were investigated. Fillets were kept at 4°C and sampled after 2 h and 3, 7 and 10 days. An untreated control group was used as well. Fish samples were analysed for microbiological (total viable count, *Enterobacteriaceae*, psychrotrophic microbial count, *Pseudomonas* spp.) and physicochemical (pH, colour, total volatile basic nitrogen, and biogenic amines) properties. Aromatic vinegar was found to beneficially contribute to the hygienic quality of the salmon, reducing microbial growth during storage and exerting a positive effect, mainly on psychrotrophic loads and *Pseudomonas* spp. The treatment had a positive effect on biogenic amine levels, showing lower values for histamine, putrescine, cadaverine, and tyramine.

INTRODUCTION

In the Western world, people are paying more attention to healthy eating; as a result of which seafood, including fish, crustaceans, molluscs and edible aquatic plants, is in high demand as an important source of omega 3 fatty acids [Branciari *et al*., 2017; Leisner & Gram, 2014; Tacon & Metian, 2013]. Aquaculture is the fastest growing food sector worldwide, and among seafood, farmed salmon is one of the most highly valued products [Briones *et al*., 2010; FAO, 2016], from both the nutritional and the economic perspectives [Sivertsvik *et al*., 2003]. Aquaculturists face many challenges to meet the global food demand while maintaining a high-quality product. Due to its high nutritional quality, fresh salmon is popular and is consumed throughout the world; however, it is also prone to spoilage [Wang *et al*., 2017a]. Food structure, together with chemical composition and storage conditions (temperature, time, packaging system, light, and antimicrobials), strongly affect the growth and proliferation of microorganisms [Corbo *et al*., 2009]. Indeed, fish is one of the most perishable food products [Fidalgo *et al*., 2018] due to the presence of non-protein nitrogenous substances, lipid composition, neutral pH and a high moisture content which are suitable conditions for microbial proliferation [Miraglia *et al*., 2016]. Microbial growth and activity are primarily responsible for the development of off-odours and off-flavours that make non-frozen fish products unacceptable or spoiled [Leisner & Gram, 2014] and can pose a significant threat to the health of consumers [Ozogul *et al*., 2017].

Market needs, added to consumer demand for fresh, refrigerated, minimally-processed and long-life food, have led to a considerable research effort aimed to define new technologies able to preserve texture, flavour and nutritional value and to ensure fresh fishery products' safety and quality [Fernández *et al*., 2009; Miks-Krajnik *et al*., 2016; Sallam, 2007]. Various methods of preservation have been assessed, investigating their effects on water activity, pH, low temperature or modified atmosphere packaging. These parameters have been shown to have a great effect on the microbial flora of fish and on the corresponding spoilage pattern [Leisner & Gram, 2014]. Green consumerism, that is the growing demand for fewer chemical preservatives, environmentally friendly antimicrobials and the sustainable use of molecules of natural origin [Corbo *et al*., 2009], together with the demand for high-quality fresh seafood, has intensified the search for technologies that favour fresh fish utilisation. Nevertheless, effects of modified atmosphere packaging (MAP) of fresh fish are controversial with author who report

* Corresponding Author: E-mail: raffaella.branciari@unipg.it (R. Branciari)

limited extension [Emborg *et al*., 2002] or an improvement of fish shelf life but in relation to fish species, initial microbial load, gas mixture, and temperature of storage [Emborg *et al*., 2002]. Among natural products, vinegar shows a significant biological activity, including sanitising characteristics and antioxidant effects [Lingham *et al*., 2012; Machado *et al*., 2011]. It is recognised as a natural antimicrobial and antioxidant product that may improve safety and enhance shelf life, provide acceptable sensory quality and reduce economic losses due to spoilage of fish products [Lingham *et al*., 2012].

In view of what has been said regarding the importance of fish from a nutritional, qualitative, and economic standpoint, the purpose of this study was to investigate the effects of surface treatment with an aromatic vinegar on some microbiological and physicochemical parameters related to the quality and safety of salmon fillets stored in MAP, and thus to reduce the bacterial populations, to determine changes in biogenic amines of the fish, which are beneficial for producer interest and consumer safety.

MATERIALS AND METHODS

Experimental design

Chilled fillets of salmon (*Salmo salar*) and aromatic vinegar (AV) obtained from sugar cane (Aromatic Vinegar GPI 6.2® – Lazzari Equipment & Packaging, Settimo di Pescantina, VR, Italy) were purchased from a local factory (Circeo Pesca S.r.l., San Mariano-Corciano, PG, Italy). Fillets were obtained seven days after harvesting from salmon weighing between 3 and 4 kg, stored in ice till filleting. The fillets were then divided in 150 g portions (12 g standard deviation) presenting both side (inner and outer part) and ventral and dorsal parts (surface of 90 cm^2 approximately). The skin was left on the outer side according to producer needs.

In order to perform analytical determinations of AV, on the first day of the trial, 100 mL of the product were sampled in triplicate from each of three vats containing 23 L of AV. Salmon fillets (150 g each) were treated by spraying homogeneously their inner surface with 1.5 mL AV/150 g of product by a hand nebulizer and subsequently packaged on polystyrene trays (Sirap-Gema S.p.A., Verolanuova, Italy) under MAP (Delta 2000; Ilapak Italia S.p.A. Foiano della Chiana, Italy) and covered with a stretch-film (Cryovac® BDFS100; thickness = 21 μm; density = 0.943 g/cm^3; Permeability: CO^2 = 100 cm^3/m^2, 24h; O^2 = 25 cm^3/m^2, 24h); they were referred to as the AV group. Untreated salmon fillets, stored at the same conditions as AV samples, were employed as a control group (C group). The gas content of MAP was measured using a CheckPoint Handheld Gas Analyser (PBI Dansensor, Ringsted, Denmark), and O_2 and CO_2 ranged from 13.46% and 28.22% on day 0 to 13.92% and 16.52% on day 10 of storage, respectively.

All fish samples were kept in the dark during storage under refrigeration (4±1°C) directly at factory level and were collected after 2 h (T1), 3 days (T3), 7 days (T7) (shelf life of the salmon fillets defined by the producer), and 10 days (T10). At each sampling point, a total of 20 samples were used, 10 each for both AV and C groups. All the fillets and AV samples were transported in refrigerated conditions (4±1°C) to the laboratory at the Department of Veterinary Medicine (University of Perugia, Italy) and promptly processed for microbiological and physicochemical analysis.

Physical-chemical determination of the aromatic vinegar

Vinegar samples were analysed for pH value, using a pH meter equipped with an insertion electrode (Crison pH25, Crison, Barcelona, Spain). Total polyphenols content (TPC) was determined using the Folin–Ciocalteu colorimetric method [Rashidinejad *et al*., 2013] using an Ultrospec 2100 pro UV/visible spectrometer (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 765 nm. The phenolic content was calculated based on a gallic acid calibration curve as reported in Miraglia *et al*. [2017] ($y = 0.001x - 0.0273$, $R^2 = 0.9943$). The content was expressed as mg gallic acid equivalents (GAE) per mL. No microbiological analyses were performed for vinegar, considering the characteristic of the products, and the product sheet reports (Lazzari Equipment & Packaging, Settimo di Pescantina, VR, Italy): total bacterial count < 5000 colony-forming units (CFU)/g; yeasts and moulds < 300 CFU/g; *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* absent in 25 g.

Microbiological analysis of salmon fillets

For sample preparation, a portion of 10 g (±0.1 g) from the upper left side of each salmon fillet was sampled using aseptic blades and forceps and was transferred into a sterile stomacher bag with 90 mL of sterile peptone water. After homogenisation (Stomacher 400 circulator; Seward Ltd, Norfolk, UK), the following microbial counts were determined: total viable count (TVC) according to ISO 4833–1:2013; *Enterobacteriaceae* count according to ISO 21528–2:2017; psychrotrophic microbial count according to ISO 17410:2001; and *Pseudomonas* spp. count by the spread method: 0.1 mL was incubated in Pseudomonas CFC Selective Agar Base (Biolife, Milan, Italy) plates, that were incubated at 25°C for 24 h in aerobic conditions. Results of microbial analyses are expressed as log CFU/g.

Salmonella spp. detection was performed according to ISO 6579–1:2017, and *L. monocytogenes* presence was investigated according to ISO 11290–1:2017.

Physical-chemical determinations of salmon fillets

Colour was evaluated on the cut surface of the fillet with a colorimeter (Minolta CR 400, Osaka, Japan) using the CIE L* a* b* system [CIE, 1986], and the measurements were performed in duplicate on the dorsal and ventral part of each fillet. Results are expressed as mean values of four measurements. The pH value of each salmon fillet was determined in the right dorsal part of the fillets in triplicate using a pH meter equipped with an insertion probe (Crison 25, Crison, Barcelona, Spain). Water activity (a_w) was determined using a HygroLab 3 hygrometer (Rotronic, Huntington, NY, USA) on three samples collected from the upper right part of the fillets. Total volatile basic nitrogen (TVB-N) content was determined in duplicate at the lower right part of the fillets according to Pearson [1991].

Content of biogenic amines (BA) was determined in eight samples for each treatment at each sampling point considered. BA extraction from the samples was carried out according to

the procedures developed by Zhai *et al*. [2012] with a little modification. Five grams of each sample was transferred into a centrifuge tube containing 5% (w/v) trichloroacetic acid (TCA). The mixture was vortexed and centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant was collected, and the residue was extracted again with the same volume of TCA. Both supernatants were filtered through Whatman paper No. 1 and combined. The final volume was adjusted to 25 mL with TCA. BA derivatization and quantification was carried out according to the procedures described by Zhai *et al*. [2012]; the quantification was performed using high--performance liquid chromatography (HPLC) analysis using a Shimadzu RF-20AXS instrument (Kyoto, Japan) consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, and a fluorescent detector. Histamine (HIS, 98%), tryptamine (TRP, 98%), cadaverine (CAD, 98%) and 2-phenylethylamine (2-PHE, 99.5%) were purchased as standards from Sigma Aldrich Italia (Milan, Italy); whereas spermine (SPM, 97%), tyramine (TYR, 99%), putrescine (PUT, 98%), and spermidine (SPD, 98%) were purchased as hydrochloride salts (Sigma Aldrich). Results were expressed as mg/kg.

Statistical analysis

The data of the different parameters considered were analysed by an ANOVA model (SAS Institute Inc., Cary, NC, USA) with treatment (AV, C) and time (T1, T3, T7, and T10) as fixed and variable factors, respectively, and their interactions. The ComBase software tool DMfit (Baranyi and Roberts model) was used to determine growth curves for all the microbial populations considered in AV and C samples. The results of the model, expressed as initial value, shoulder, μ max and final value, were analysed by a one-way ANOVA model (SAS Institute Inc., Cary, NC, USA). For all these parameters, the difference between the means was analysed with the Tukey test and considered significant at a p value <0.05.

RESULTS AND DISCUSSION

pH and TPC of aromatic vinegar

In order to assess the peculiar characteristics of the treatment, pH and TPC analyses were performed in the AV. The average pH value recorded was 6.20 (0.08 standard deviation), and the TPC value was 2.6 mg GAE/mL (0.2 standard deviation). The pH was exactly comparable to that reported in the product sheet, and relatively high compared to other vinegars reported in the literature as antimicrobials [Medina *et al*., 2007]. The TPC was comparable to that reported in other vinegars [Machado *et al*., 2011] but lower than those found in other natural compounds adopted in fish fillets and reported in the literature [Choulitoudi *et al*., 2016].

Salmon fillet microbiology

The microbial loads of fish fillets are shown in Figure 1. In order to assess whether the use of AV had any effect on bacterial growth in salmon fillets, microbiological data were analysed using ComBase, and the microbial responses are reported in Table 1. The parameters considered were initial value (log CFU/g), lag/shoulder (h), μ max (Log CFU/g/h), and final value (log CFU/g) [Baranyi & Tamplin, 2004].

Counts of all bacterial groups increased throughout the storage experiment at chilled temperature, showing initial counts between 10^2 and 10^4 CFU/g (Table 1). Salmon fillets were considered to be in good condition for human consumption before the storage experiment, as the initial contamination level for raw fish at time zero should be below 10^6 CFU/g of TVC as reported by Miks-Krajnik *et al*. [2016]. Initial TVC was between 4.35 and 4.59 Log CFU/g for the two groups, which was slightly lower than reported by Fidalgo *et al*. [2018] but in accordance with values reported by Chytiri *et al*. [2004]. The final TVC values were lower than those reported in the literature that reached 8 log CFU/g after 6 days of storage [Fidalgo *et al*., 2018; Miraglia *et al*., 2016]. No differences in the final TVC were recorded; therefore, it was shown that the treatment had no effect on TVC.

Initial *Enterobacteriaceae* loads varied between 2.37 and 2.50 log CFU/g, which is in accordance with Chytiri *et al*. [2004] and Fidalgo *et al*. [2018]. After 10 days, loads reached about 4.95 CFU/g in control samples, showing a similar pattern to those reported by Chytiri *et al*. [2004]. Even though the *Enterobacteriaceae* count showed consistent contamination in the two groups, and treated samples at T10 showed a slight decrease in bacterial loads, there was no significant difference due to the treatment. In addition,

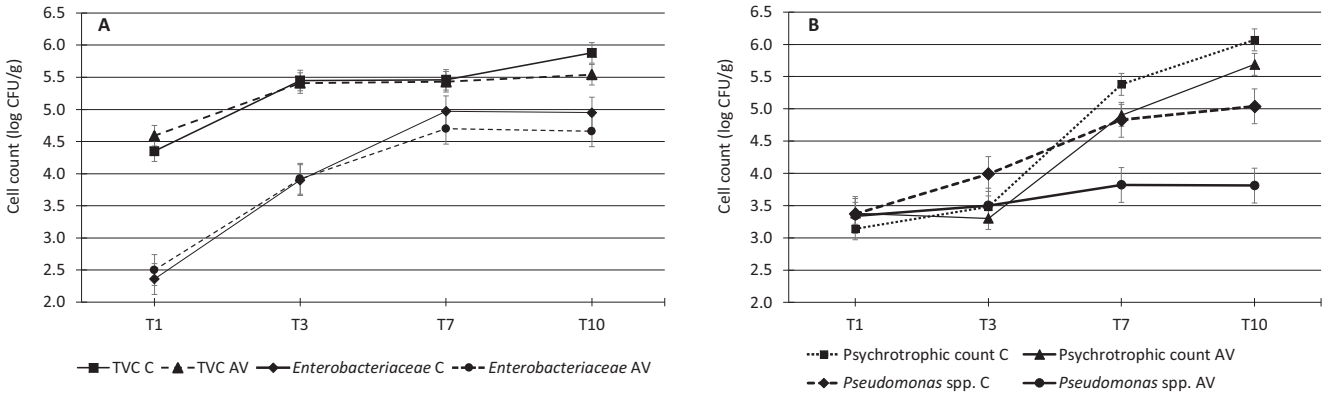

FIGURE 1. Total viable count (TVC), and *Enterobacteriaceae* (A), psychrotrophic and *Pseudomonas* spp. counts (B) of the salmon fillets throughout the storage time (T1 – 2h, T3 – 3 days, T7 – 7 days, T10 – 10 days). C – control group; AV – salmon fillets treated with aromatic vinegar.

TABLE 1. Effects of the aromatic vinegar addition to salmon fillets on kinetic parameters describing the growth of bacterial loads performed (following Baranyi–Roberts models).

Kinetic parameters of growth	Treatment	TVC	*Enterobacteriaceae*	Psychrophilic count	*Pseudomonas* spp.
Initial value (log CFU/g)	C	3.93	1.98	2.74	3.21
	AV	3.93	2.27	3.13	3.41
Lag/shoulder (h)	C	17.15	16.95	39.07^{a}	25.37
	AV	14.77	35.12	77.25^{b}	60.92
μ max (log CFU/g/h)	C	0.0294	0.0318	0.0372	0.0198^{b}
	AV	0.0264	0.0306	0.0198	0.0048^{a}
Final value (log CFU/g)	C	6.30	4.99	6.77^{b}	5.50^{b}
	AV	6.20	4.89	6.12^{a}	3.96^{a}

Within each microbial load, different superscript letters (a, b) indicate differences between treatments ($p \leq 0.05$). TVC – total viable count, C – control group, AV – salmon fillets with aromatic vinegar.

neither *Salmonella* spp. nor *L. monocytogenes* were detected in any of the samples tested.

The initial psychrotrophic load of control samples was 3.14 log CFU/g, similarly to that reported by Briones *et al*. [2010]. A limit of 10^5 CFU/g for psychrotrophic bacteria has been suggested for fresh fish [Pons-Sànchez-Cascado *et al*., 2006], and this limit was exceeded after 7 and 10 days of storage for control and treated samples, respectively. A significant difference between treated and control samples was detected at T3 (7 days), considered the end of commercial shelf life for this kind of product. As shown in Table 1, the addition of the aromatic vinegar seems to influence the growth dynamics of psychrotrophic bacteria through extension of the lag phase (λ) which also results in a lower final value. The initial *Pseudomonas* count showed values between 3.37 and 4.04 log CFU/g, *i.e.* slightly lower than those reported by Fidalgo *et al*. [2018] and in accordance with the loads reported by Chytiri *et al*. [2004] for filleted rainbow trout. However, in their study, a count of approximately 7 log CFU/g was reached for fillets after 10–11 days of storage, which was considerably higher than the maximum of 4.04 log CFU/g found in this work. Furthermore, *Pseudomonas* counts reported the most relevant differences as there was a significant difference between groups at both T3 and T10. The AV affected both their μ max and final values.

Although the specific mechanisms of action have not been completely clarified, it has already been reported in the literature that, within a specific matrix, natural compounds (such as R (+) limonene, oregano, thyme, and star anise essential oils) may act differently on different bacteria [Giarratana *et al*., 2016; Huang *et al*., 2018], mainly due to the specific microbial response. The effect of vinegars on microbial population are depending on both organic acids and polyphenols [Bakir *et al*., 2017; Chen *et al*., 2016]. As regards organic acids, the main mechanism of action proposed involves the acidification of the bacterial cytoplasm after the compounds penetrated the cell membranes, and is not merely a question of the pH value of the solution adopted [Bakir *et al*., 2017; Kundukad *et al*., 2017]. The lower pKa of the organic acids than the pH of the cytoplasm causes their dissociation into hydrogen ions, the rise of the acidity inside the cell and damages to both structural and functional proteins [Yagnik *et al*., 2018]. Furthermore, to counteract hydrogen ions concentration, a high quantity of energy is needed thus limiting the macromolecular synthesis and microbial growth [Van Immerseel *et al*., 2006]. Other proposed mechanisms of organic acids action are the increasing osmotic pressure and the production of antimicrobial peptides inside the cell [Chen *et al*., 2016]. Polyphenols either exert antimicrobial effects, depending on compounds structure and their concentration, through cell membrane disruption, interference with bacterial cell enzymes, and chelation of essential metals after they had penetrated the bacterial cell membrane [Chen *et al*., 2016; Daglia, 2012].

As mentioned before, for psychrotrophic bacteria, the AV inhibits the early steps of microbial growth and therefore influences the final concentration at the last sampling time, while for *Pseudomonas* spp., the effects are due to growth inhibition during the exponential phase.

Overall, the treatment had a positive effect, mainly on the inhibition of psychrotrophic bacteria and *Pseudomonas* spp. counts in salmon fillets, while no significant effect was observed on either TVC or *Enterobacteriaceae* count.

The microflora present in fish depends on the species, habitat, environment, harvesting method, and storage conditions and determines which bacteria are responsible for spoilage [Briones *et al*., 2010]. It is important to establish the count of *Pseudomonas* spp. as it represents the specific spoilage organism in freshwater fish [Gram & Dalgaard, 2002]. *Enterobacteriaceae* count is a hygiene indicator [Mexis *et al*., 2009], and different enteric species of histamine-producing bacteria have also been isolated from fish. Moreover, *Pseudomonas* spp. have also been reported as histamine producers [Hu *et al*., 2014; Wang *et al*., 2017b].

The definition of a threshold value for spoilage microbial growth depends on the product and the microorganism considered. For sea products, especially fish, some studies report that level of specific spoilage flora, such as *Pseudomonads* and *Enterobacteriaceae*, accounting for 10^7 CFU/g coincides with the appearance of irreversible alterations, therefore determining the end of the shelf-life [ICMSF, 1986; Koutsoumanis, 2001; Koutsoumanis & Nychas 2000]. In the present

study, the mentioned value was never reached in neither experimental group. Accordingly, the fillets remained at an acceptable standard for consumption throughout the storage.

Biogenic amines (Bas) in salmon fillets

BAs are non-volatile basic compounds formed by decarboxylation of the precursor amino acids as a result of metabolic processes of bacterial enzymes and are absent or present at very low levels in fresh fish [Chytiri *et al*., 2004; Pons-Sànchez-Cascado *et al*., 2006]. BAs are of importance due to food intoxication risk as they cause the most common food poisoning associated with fish consumption [Kim *et al*., 2009; Ozogul *et al*., 2017], and, although their formation in food does not necessarily correlate with the growth of spoilage organisms, they are useful chemical indicators of spoilage and thus of loss of freshness, hygienic quality of fish, and consumer acceptance [Kim *et al*., 2009; Pons-Sànchez-Cascado *et al*., 2006; Santos, 1996; Wunderlichová *et al*., 2014]. Although dominant microbial groups and concentrations in a fish species vary with temperature and storage conditions, affecting the formation of specific amines in the muscle [Chytiri *et al*., 2004], few studies have related bacterial and sensorial changes to BAs formation [Emborg *et al*., 2002]. Furthermore, *Salmonidae* have been regularly reported to cause histamine fish poisoning [Ozogul *et al*., 2017].

In this study, the presence of eight BAs was assessed but only seven were detected, namely 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine; while tryptamine was not detected. Histamine content was the highest, reaching a final value of 59 mg/kg in the control samples after 10 days, whereas it was not detected in the treated samples. Thus, the aromatic vinegar exerted a positive effect on histamine production (Table 2). The European Community established a value for histamine at 200 mg/kg in fish [Regulation (EC) No 1019/2013] and the Food and Drug Administration (FDA) allows a lower level (50 mg/kg) [FDA, 2011]. This value was surpassed after 10 days of storage in the control samples.

In the control samples, contents of putrescine, cadaverine, and histamine increased steadily between days 7 (T2) and 10 (T3). Sensorial and microbiological data showed that 13 mg/kg of putrescine is the upper limit for spoilage initiation in fresh rainbow trout fillets [Chytiri *et al*., 2004]. This value was achieved at T10 in the control samples, but not in the AV group. The elevated content of histamine along with those of putrescine and cadaverine depicts bacterial spoilage of fish tissue. Indeed, the count of certain microbial groups is relevant, such as enterobacteria and pseudomonads that have been described as active BA-producing microorganisms in fish [Pons-Sànchez-Cascado *et al*., 2006]. As it has been reported by Chytiri *et al*. [2004], *Pseudomonas* spp. can lead to the synthesis of putrescine and cadaverine, as they are responsible for the carboxylation of lysine and ornithine, respectively. Moreover, putrescine and cadaverine can interfere with the histamine detoxification system, enhancing the toxicity of histamine [Kim *et al*., 2009].

Moreover, there was a significant difference between the control and treatment groups in tyramine levels throughout the storage time, with values established for the treatment groups being much lower than these determined for the control (Table 2).

pH, a_w, colour and TVB-N in salmon fillets

The effect of the treatment and storage time on the pH value of salmon fillets is shown in Table 3. The initial pH value of the control and treated fillets was 6.13, which is in accordance with the mean cut-surface pH at time zero reported by Fletcher *et al*. [2002] and by Emborg *et al*. [2002]. During the study, pH remained stable, and only a slight decrease was reported after 3 days of storage, to 5.97 and 6.08 for AV and C groups, respectively. The pH values of salmon flesh decrease *post mortem* because of lactic acid formation, while increases are usually observed due to the production of basic nitrogen from bacterial growth [Briones *et al*., 2010]. Although there was an increase in the production of nitrogenous compounds during the storage period due to microbial growth, the pH did

TABLE 2. Biogenic amine content in salmon fillets, control and treated with aromatic vinegar (mg/kg), throughout the storage time.

Treatment	Time	2-PHE	PUT	CAD	HIS	TYR	SPD	SPM
C	T1	34.87±2.10	8.62±5.45	–	–	28.25±5.01^{b}	5.87±2.70	25.75±3.49
	T3	32.62±4.34	6.25±4.46	–	–	25.88±5.17^{b}	6.00±2.39	24.25±2.87
	T7	32.12±5.43	8.25±5.44	10.12±4.19^{bx}	8.25±3.79^{x}	24.13±4.05^{b}	5.75±2.82	23.38±2.96
	T10	34.62±5.04	12.87±8.63^{b}	45.13±14.94 ^{by}	58.63±13.56^{y}	26.00±4.17^{b}	5.62±2.13	20.37±3.42
AV	T1	32.87±2.42	5.37±3.85	–	–	7.38±4.27^{a}	5.12±2.23	22.12±2.70
	T3	30.62±5.15	4.37±4.00	2.95±0.07^{a}	–	5.39±2.50^{a}	5.37±1.92	23.58±2.77
	T7	26.37±6.52	5.25±4.27	3.75±1.98^{a}	–	8.75±3.58^{a}	6.25±1.98	20.39±3.29
	T10	32.62±4.14	5.37±3.81^{a}	4.25±1.67^{a}	–	9.25±3.33^{a}	5.38±2.26	25.63±4.57

Data are expressed as mean ± standard deviation.
n = 8 for each sampling point and each treatment; C – control group; AV – salmon fillets treated with aromatic vinegar; 2-PHE – 2-phenylethylamine; PUT – putrescine; CAD – cadaverine; HIS – histamine; TYR – tyramine; SPD – spermidine; SPM – spermine; T1 – 2 h; T3 – 3 days; T7 – 7 days; T10 – 10 days. Within each treatment, different superscript letters (x, y, z) indicate differences between storage periods ($p \leq 0.05$); within each storage period, different superscript letters (a, b) indicate differences between treatments ($p \leq 0.05$).

TABLE 3. Physical and chemical parameters (pH, colour and total volatile basic nitrogen values) of control and treated fillets throughout the storage experiment.

Attribute	Treatment	Storage time				SEM	P value		
		T1	T3	T7	T10		Tr	ST	Tr x ST
pH	C	6.13	6.08	6.17	6.10	0.0125	0.384	0.132	0.536
	AV	6.13	5.97	6.16	6.09				
L*	C	50.16	49.84	48.66	49.89	0.775	0.891	0.053	0.443
	AV	51.03	50.63	47.71	48.88				
a*	C	8.93^{w}	10.66^{x}	11.41^{x}	11.82^{x}	0.453	0.465	<0.001	0.156
	AV	10.33	10.87	11.40	11.16				
b*	C	14.02^{w}	16.10^{wx}	18.02^{x}	17.69^{x}	0.706	0.414	<0.001	0.265
	AV	15.71	17.18	17.57	17.01				
TVB-N (mg/100 g)	C	22.7^{w}	23.5^{w}	24.8^{x}	29.3	0.027	0.139	<0.001	0.110
	AV	21.9^{w}	23.8^{x}	24.8^{y}	28.7				

n = 10 samples for each treatment and sampling point; C – control group; AV – salmon fillets treated with aromatic vinegar; Tr – treatment; ST – Storage time; TVB-N – total volatile basic nitrogen; T1 – 2 h; T3 – 3 days; T7 – 7 days; T10 – 10 days. Within each storage period, different superscript letters (a, b) indicate differences between treatments ($p \leq 0.05$). Within each treatment, different superscript letters (w,x,y,z) indicate difference between storage times ($p \leq 0.05$).

not increase significantly during the experiment. Furthermore, the pH of the AV used was 6.2 and thus it was not able to alter the surface pH of the fish and induce protein denaturation.

The a_w values did not vary either for the treatment or during storage, ranging from 0.985 to 0.986 at T1 and from 0.983 to 0.982 at T3 for C and AV groups, respectively. The values were high enough to promote microbial growth and spoilage of the fillets.

Indeed, when selecting salmon fillets, surface colour and appearance are important indicators for consumer acceptance and product final price, since pigmentation intensity is considered a quality characteristic for the salmonids [Fidalgo *et al*., 2018; Lerfall *et al*., 2016; Wang *et al*., 2017a].

The initial values of L* (lightness), a* (redness), and b* (yellowness) were between 50.16 and 51.03, 8.93 and 10.33, and 14.02 and 15.71, respectively (Table 3). No significant differences were found in the L* values between the two groups throughout storage, and both groups showed a slight but not significant decrease in lightness. This finding was unexpected as generally the L* value increases during ice storage [Erikson & Misimi, 2008] but limited changes are reported in salmon fillets stored in MAP conditions [Gimenez *et al*., 2005].

The a* values increased during storage in the products but no difference was registered between the control and treated samples. These results are in accordance with those reported by Miraglia *et al*. [2016] in their study where *S. salar* raw fish was treated with a phenolic extract from olive vegetation water, and also by Fidalgo *et al*. [2018] for fresh Atlantic salmon during hyperbaric storage at room temperature. Moreover, the a* values were lower than those reported by Wang *et al*. [2017a] and by Fidalgo *et al*. [2018]. The b* value followed the same trend as reported for the a* value. The increasing presence of both a* and b* color components, reported also by other authors [Erikson & Misimi, 2008], could lead to a more brownish colour of the fillets, which could potentially affect their sensory quality. Despite no difference observed between the treated and untreated fillets, the AV seems to stabilize the a* and b* components, despite its pale–amber transparent colour not able to modify the original fillets colour.

However, the changes observed in salmon colour during storage may be caused mainly by microbial spoilage, enzymatic activity, oxidation processes, and different treatments applied to the samples, causing several structural changes to the muscle and variation of the colour parameters [Fidalgo *et al*., 2018].

TVB-N, which is constituted by trimethylamine (TMA-N), ammonia and other basic nitrogenous compounds, has been proposed as a quality indicator for spoilage in fresh and lightly preserved seafood [Dalgaard, 2000; Gram & Dalgaard, 2002; Pons-Sànchez-Cascado *et al*., 2006; Sallam, 2007] since it shows a close relationship with sensory score [Pons-Sànchez-Cascado *et al*., 2006]. High values of TVB-N are not desirable since they indicate the existence of nitrogenous compounds deriving from the degradation, operated by proteolytic bacteria, of molecules containing nitrogenous compounds, such as proteins and nucleic acids [Wang *et al*., 2017a]. In the current study, the initial TVB-N values (mg N/100 g of product) ranged from 21.9 in the treated samples to 22.7 in the control samples (Table 3). Increases in the TVB-N values were detected throughout the storage period of the experiment, reaching values of 29.3 and 28.3 mg N/100 g by day 10 in the control and treated samples, respectively. The samples analysed during the experiment were all below the maximum value of 35 mg N/100 g flesh stipulated by the EC guidelines for different species of raw fish [European Commission Decision 95/149/EC, 1995]. The initial content of TVB-N was higher than that reported by both

Zaragozà *et al*. [2014] and Sallam [2007] who found initial TVB-N values in control samples of Pacific salmon below 10 mg N/100 g; and values of 22.7 mg/100 g muscle were reached only on day 9. TVB-N increases are related to storage conditions, the activity of spoilage bacteria, and hygienic practices [Zaragozà *et al*., 2014], and TVB-N values are also affected by the species, season, harvesting area, age and sex of fish [Sallam, 2007]. The TVB-N content increased in all samples, which demonstrates their successive deterioration throughout the study.

CONCLUSIONS

The spoilage process in salmon fillets was monitored in this study *via* microbiological and physicochemical parameters in order to find some natural treatments that can influence microorganisms growth and their ability to produce BA, and therefore to obtain safe products. The hygienic level of the production was mostly high, as fillets showed a TVC always below 7 log CFU/g, even if a trend of increasing counts of *Enterobacteriaceae* and other spoiling bacteria was highlighted.

This preliminary study shows that the use of AV can really improve the overall hygienic quality of fresh salmon fillets stored in MAP, controlling the microbial populations in fish, such as *Pseudomonas* spp. and psychrotrophic loads. In particular, a significant effect was found on BAs production, with lower contents determined in the treated fillets for putrescine, cadaverine, tyramine, and histamine.

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Original article
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Survival of Wild Strains of Lactobacilli During Kombucha Fermentation and Their Contribution to Functional Characteristics of Beverage

Dragoljub Cvetković[1], Aleksandra Ranitović[1], Dragiša Savić[2], Nataša Joković[3], Ana Vidaković[1], Lato Pezo[4], Siniša Markov[1]*

[1]*Department of Biotechnology and Pharmaceutical Engineering, Faculty of Technology Novi Sad, University of Novi Sad, 1 Bulevar cara Lazara, 21000 Novi Sad, Serbia*
[2]*Department of Biotechnology, Faculty of Technology, University of Niš, 124 Bulevar Oslobođenja, 16000 Leskovac, Serbia*
[3]*Department of Molecular Biology and Phisiology, Faculty of Science and Mathematics, University of Niš, 33 Višegradska, 18106 Niš, Serbia*
[4]*Institute of General and Physical Chemistry, University of Belgrade, 12/V Studentski trg, 11000 Belgrade, Serbia*

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Kombucha is a fermented tea beverage, which is traditionally prepared by fermenting sweetened black tea with tea fungus, which is a consortium of yeasts and acetic acid bacteria. In this paper, viability of selected wild strains of lactobacilli during Kombucha fermentation, their interaction with tea fungus and their contribution in obtaining a beverage of increased functional characteristics were tested. Five wild strains which were isolated from traditionally fermented foods were separately added on the second day of Kombucha fermentation. Count of yeasts, acetic acid bacteria and lactobacilli, as well as pH, titratable acidity, and content of L- and D-lactic acid during Kombucha fermentation were determined. Wild strains of lactobacilli demonstrated a differentiated survivability and the counts in fermentation broth (*i.e.* Kombucha beverages) depending on the strain applied. The addition of wild *Lactobacillus* spp. during Kombucha fermentation had no effect on the physiological activity of tea fungus, but they contributed to a significant increase in lactic acid content in the beverage. The highest lactic acid content during Kombucha fermentation was produced by the strain of *Lactobacillus plantarum* isolated from 40-day-old cream, while the strain of *Lactobacillus hilgardii* (from sour dough) showed the highest viability.

INTRODUCTION

Kombucha is a traditional fermented beverage originating from the northeast China (former Manchuria region) and later spread to Russia and the rest of the world. Nowadays, Kombucha has become very popular in the Western world (North America and Europe) and is often claimed to exhibit healthful properties, which include anti-microbial, anti-oxidant, anti-carcinogenic [Jayabalan *et al*., 2011], anti-diabetic [Hiremath *et al.*, 2002; Aloulou *et al.*, 2012] effects, as well as treatment for gastric ulcers [Banerjee *et al.*, 2010] and high cholesterol [Yang *et al.*, 2009]. It has also been shown to impact the immune response [Ram *et al.*, 2000] and liver detoxification [Lončar *et al.*, 2000].

Kombucha is typically prepared by fermenting sweetened black tea inoculated with tea fungus pellicle or previously fermented broth (*i.e.* Kombucha) at a level of 100–200 mL/L. The substrate is incubated statically under aerobic conditions at 20–28°C [Sievers *et al.*, 1995]. Duration of fermentation could differ, even up to 60 days, and in that case, the obtained beverage has a mild vinegar taste [Chen & Liu, 2000]. In order to obtain a pleasantly sour, sweetened beverage, fermentation should be terminated when titratable acidity (TA) of fermentation broth reaches 4–4.5 g acetic acid/L, that is confirmed by Kombucha consumers [Cvetković *et al.*, 2008]. The tea fungus is a consortium of acetic acid bacteria (AAB) (*Gluconacetobacter xylinum,* previously known as *Acetobacter xylinum*, which is the primary and best studied bacteria in Kombucha) and yeasts (species of the genera *Saccharomyces*, *Torulopsis*, *Pichia*, *Brettanomyces*, *Zygosaccharomyces*, *Candida* and *Saccharomycoides*) [Yamada *et al.*, 1997; Greenwalt *et al.*, 2000; Teoh *et al.*, 2004; Chu & Chen, 2006]. It is well known that the microbial community may vary between different Kombucha cultures across the globe depending upon the source of the inoculum used. The role of yeasts in the Kombucha fermentation is to hydrolyze sucrose from cultivation medium to glucose and fructose and metabolize these monosaccharides to ethanol, which is further oxidized to acetic acid by AAB. AAB cannot uptake sucrose alone, because of the lack of enzymes for the extracellular hydrolysis of sucrose or its transport into the cell. Also, AAB use yeast-derived glucose to synthesize gluconic acid and bacterial cellulose in the form of pellicle which is commonly described as the "fungus"

* Corresponding Author: +381214853730;
E-mail: sanja@tf.uns.ac.rs (A. Ranitović)

[Greenwalt *et al.*, 2000; Kurtzman *et al.*, 2001]. Acetic acid, ethanol and gluconic acid are the main tea fungus products [Blanc, 1996]. Other components present in Kombucha are fructose, glucose, ethyl-gluconate, oxalic acid, saccharic acid, keto-gluconic acids, lactic acid as well as tea components (catehins, theaflavins, flavonols *etc.*), and enzymes (invertase, amylase, other oxidative enzymes *etc.*) [Reiss, 1994; Greenwalt *et al.*, 2000]. Although the type of sugar (sucrose, lactose, glucose or fructose) may have a distinct influence on the formation of ethanol and lactic acid, the concentration of individual sugars has no pronounced effect [Reiss, 1994]. When lactose was used as a carbon source, the final products of fermentation were acetic and lactic acid as well as ethanol [Belloso-Morales & Hernández-Sánchez, 2003]. Lactic acid appears in Kombucha in its most potent L-form [Malbaša *et al.*, 2002]. L-Lactic acid is the biologically important isomer that assists circulation of the blood and prevents decay in the bowels and constipation. Also, it maintains the balance between acids and alkalines, as well as stimulates secretion of stomach enzymes and detoxification process [Kaufman, 1995].

It is well known that lactic acid is the major product during sugar fermentation in lactic acid bacteria (LAB), but these bacteria were not detected in the Kombucha according to Sievers *et al.* [1995]. Generally, there are insufficient data about the presence of LAB in Kombucha, *i.e.* tea fungus culture and their number during fermentation of sweetened tea. One of the reasons is that the majority of microbiological analyses of tea fungus have been based on culture techniques [Chakravorty *et al.*, 2016]. Recently, Marsh *et al.* [2014] have performed sequence-based analysis of the bacterial and yeast populations of Kombucha. They were identified *Lactobacillus* spp. as prominent population (up to 30%), with a number of sub-dominant genera. According to Marsh *et al.* [2014], lactobacilli have been isolated in only two previous Kombucha studies, both of which focused on Chinese Kombuchas. Velićanski [2012] did not detect LAB (streptococci, lactobacilli and bifidobacteria) in tea fungus which was used in her study as a starter culture. In the absence of LAB, the lactic acid during tea fungus cultivation was formed by AAB from ethanol and acetic acid [Reiss, 1994]. Yeasts lack the lactate dehydrogenase enzyme, and are unable to process during glycolysis and produce lactic acid from pyruvate [Gao *et al.*, 2009].

Previous results of Velićanski [2012] also have shown very low viability of starter commercial LAB cultures during Kombucha fermentation. Taking into account beneficial health effects of LAB (especially as probiotics), as well as the absence of literature data, the author tested the possibility to produce a Kombucha with improved functional (probiotic) characteristics using commercial cultures of LAB. Probiotic foods which contain viable cells (least 10^6 cfu/mL) of probiotic cultures such as lactobacilli and bifidobaterium provide several health benefits, as they help in maintaining a good balance and composition of the intestinal microbiota, and increase the resistance against invasion of pathogens [Tripathi & Giri, 2014].

The aim of this study was therefore to test viability of selected wild strains of lactobacilli during Kombucha fermentation, their interaction with tea fungus and their contribution in obtaining a beverage of increased functional characteristics compared with the traditional one.

MATERIALS AND METHODS

Tea fungus

Fermentation was performed by using the local tea fungus culture, for which previous studies showed that it contained at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* spp., and *Zygosaccharomyces* spp.) and two bacterial strains of the *Acetobacter* genera [Markov *et al.*, 2001; Velićanski, 2012].

Tea fungus cultivation

The substrate for tea fungus cultivation was prepared by adding 70 g of sucrose into 1 L of tap water. The medium was boiled and 3 g/L of black tea ("Fructus", Bačka Palanka, Republic of Serbia) was added. After 15 min, tea leaves were removed by filtration. After cooling it to the room temperature, the tea was inoculated with 100 mL/L of the fermentation broth from the previous fermentation obtained under the same conditions. Sterile glass jars (volume 0.72 L, diameter 8 cm) were filled in with 0.33 L of inocula. The glass jars were covered with a cheesecloth, and the fermentation at 28±0.2°C was monitored to obtain a beverage of optimal acidity (TA=4–4.5 g/L). All fermentations were performed in triplicate.

Preparation of lactobacilli suspensions for inoculation

Suspensions of lactobacilli for inoculation were added into the fermentation broth two days after the beginning of fermentation. Wild *Lactobacillus* strains (Lb-1–Lb-5) included in this study were isolated from traditional fermented food from the territory of the Republic of Serbia (Table 1). Identification of strains was performed by the molecular identification by using $(GTG)_5$-PCR and sequence analysis of 16S rRNA gene. Strains were stored at -80°C in Lactobacillus MRS Agar (Himedia, Mumbai, India), supplemented with 200 g/L of glycerol in the Laboratory of Microbiology, Faculty of Technology, University of Novi Sad, Serbia. Wild *Lactobacillus* strains from the stock were subcultured twice in MRS broth (Himedia, Mumbai, India) at 28°C for 24 h. Subcultured wild strains were centrifuged at 2504×*g* (LC-320 centrifuge, Tehtnica, Železniki, Slovenia) for 15 min. The sediment was resuspended twice in 8.5 g/L NaCl and centrifuged under the same conditions.

Each suspension was separately added into glass jar after 48 h from the start of Kombucha fermentation in the quantity

TABLE 1. Wild *Lactobacillus* strains applied in Kombucha fermentation.

Strain designation	Name of the strain	Origin of the strain	Type of sugar metabolism
Lb-1	*L. hilgardii*	sour dough	heterofermentative
Lb-2	*L. fermentum*	1-month old cheese	heterofermentative
Lb-3	*L. plantarum*	2-month old cheese	homofermentative
Lb-4	*L. plantarum*	40-day old cream	homofermentative
Lb-5	*L. plantarum*	7-month old cream	homofermentative

of 10% (v/v). The expected number of lactobacilli at the start point of fermentations was not lower than 10^7 cfu/mL, while the exact number was determined by the pour plate method one hour after inoculation and during the following two days.

Sampling

Sampling of fermentation broth during tea fungus cultivation was performed periodically for further analysis. Each glass jar was sampled only once in order to avoid a potential contamination. From each jar, 1 mL of fermentation broth was taken under sterile conditions for the microbiological analysis and 20 mL for measuring the pH value and TA. The control sample (Control Kombucha) was sweetened black tea which was not inoculated with lactobacilli. After four days of fermentation, samples of Kombucha inoculated with *Lactobacillus* strains were bottled and stored at +4°C for testing bacteria viability during storage. During 10-day storage, samples were taken to determine pH, TA and number of lactobacilli.

Methods of analysis

The pH values were measured by using an electronic pH meter (HI 9321, HANNA Instruments, Limena, Italy) calibrated at pH 4.0 and 7.0. The TA was determined according to Jacobson [2006]. After removing CO_2 from the fermentation broth, a 20-mL aliquot was taken and titrated with 0.1 mol/L of NaOH. The TA was expressed in grams of acetic acid per liter of the sample. D-lactic acid and L-lactic acid content was determined by using D-/L-lactic acid kit (Megazyme, Co. Wicklow, Ireland, K-DLATE 06/08). Number of yeasts, AAB, lactococci, lactobacilli, and bifidobacteria in the fermentation broth was determined by the plate counting method. The medium for enumeration of yeasts was Sabouraud-4% Maltose Agar (Merck, Darmstadt, Germany) with the addition of 50 mg/L of chloramfenicol (Sigma-Aldrich, St. Louis, USA); plates were incubated at 28°C for 72 h. The medium for the determination of the number of AAB was Yeast Peptone Mannitol Agar (Difco, Detroit, USA), containing 500 mg/L of cycloheximide (Sigma-Aldrich, St. Louis, USA). The incubation at 28°C took 5–7 days. Number of lactobacilli was determined using Lactobacillus MRS Agar (Himedia, Mumbai, India) by adding 500 mg/L of cycloheximide. Streptococci was determined by using M17 Agar Base (Himedia, Mumbai, India) under aerobic conditions at 28°C for 72 h. Number of bifidobacteria were determined by using Wilkins Chalgren Anaerobic Agar Base (Himedia, Mumbai, India) and TOS MUP Medium (Merck, Darmstadt, Germany). The incubation was performed under anaerobic conditions by using Anaerocult A® (Merck, Darmstadt, Germany) at 28°C for 72 h.

Statistical analysis

All experiments were performed in triplicate, under the same conditions, while each quantity was measured three times. The obtained values used for further processing are the averages of all the measurements presented as a mean ± standard deviation. Analysis of variance (ANOVA) and Tukey's HSD test for comparison of sample means were used to analyze variations in the observed parameters among the samples. The data were processed statistically using the software package STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Chemical and microbial changes during Kombucha fermentations

Results of determination of lactobacilli, streptococci, and bifidobacteria (LAB) numbers in the fermentation broth prior to inoculation and during fermentation of Control Kombucha showed absence of these bacteria (Table 2). Wild strains of lactobacilli Lb-1–Lb-5 (Table 1) were separately added the second day of tea fungus cultivation after chemical (pH and TA) and microbiological (number of yeasts and AAB) analysis of the fermentation broth. One of the reasons why lactobacilli were not added at the beginning of cultivation was to avoid the osmotic stress, which cells would be exposed to due to a high concentration of sucrose (70 g/L) as the only carbon source in the cultivation medium [Panesar *et al*., 2007]. The other reason is a more favorable profile of sugar as a carbon source for lactobacilli in the cultivation medium after two days of fermentation. Namely, the previous results have shown that after two days of cultivation of tea fungus, the greatest portion of sucrose in the broth was hydrolyzed and that quantities of glucose and fructose in the fermentation broth were less than 20 g/L [Cvetković *et al.*, 2008]. Such conditions in the cultivation broth are much more favorable for lactobacilli, both in terms of concentration and type of the carbon source, bearing in mind that LAB easily uptake glucose and fructose [Nauth, 2006]. In addition to the nutrient type and concentration, LAB viability and fermentation ability are also affected by cultivation temperature and pH of the environment. Temperature for tea fungus cultivation of 28°C is not a limiting factor for growth of LAB and proliferation as, according to the data, the optimal temperature for growth of LAB varies across genera from 20 to 45°C [Kandler & Weiss, 1984]. Depending on the optimal temperatures, most LAB come under the mesophilic category [Panesar *et al.*, 2007]. A pH of a fermentation broth has a more significant impact on the survival and physiological activity of LAB in the course of Kombucha fermentation. According to the data of Panesar *et al.* [2007], an optimal pH range for rapid and complete carbohydrate fermentation by LAB is 5.5–6.0. Fermentation is strongly inhibited at a lower pH and ceases at pH values below 4.5. As a matter of fact, a significant reduction in the number of commercial cultures (*Lactobacillus acidophilus* LAFTI®L10, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus casei* LAFTI® L26 (DSM Food Specialties, Australia)) used in the previous study of Velićanski [2012], can be explained by unfavorable values of environment pH. Also, these cultures of lactobacilli were intended for milk fermentation and their selection was made on the basis of conditions existing during milk fermentation, which are different from those during Kombucha fermentation. According to the results presented in Table 2, statistically significant differences ($p<0.05$) between the means in the number of lactobacilli were observed for most of the samples after the inoculation (the second fermentation day). Significant differences were also noticed in most of the samples between means in the content of D-lactic acid and L-lactic acid .

The pH value of the sweetened black tea before inoculation was 6.94 and it declined to 4.29–4.52 after the inoculation with

TABLE 2. Number of lactobacilli (log cfu/mL) and content of lactic acids (mg/L) in Control Kombucha and Kombucha inoculated with wild strains of lactobacilli (Kombucha+Lb-1 – Kombucha+Lb-5).

Sample	Fermentation time (days)	Number of lactobacilli	D-Lactic acid	L-Lactic acid
Control Kombucha*	0	<1	0.3 ± 0.1^{p}	3.3 ± 0.5^{s}
	1	<1	1.7 ± 0.1^{n}	12.5 ± 0.1^{j}
	2	<1	3.8 ± 0.6^{l}	10.2 ± 0.8^{m}
	3	<1	4.2 ± 0.1^{k}	12.9 ± 0.9^{j}
	4	<1	7.7 ± 0.1^{j}	38.4 ± 0.1^{g}
Kombucha + Lb-1	0	<1	0.3 ± 0.1^{p}	3.0 ± 0.6^{su}
	1	<1	1.8 ± 0.1^{m}	13.0 ± 0.2^{j}
	2	8.16 ± 0.15^{a}	3.7 ± 0.9^{l}	9.8 ± 0.3^{m}
	3	7.76 ± 0.10^{b}	150.7 ± 1.5^{d}	44.9 ± 0.1^{e}
	4	7.60 ± 0.04^{c}	189.1 ± 4.2^{c}	51.9 ± 0.1^{d}
Kombucha + Lb-2	0	<1	0.5 ± 0.1^{o}	3.8 ± 0.4^{r}
	1	<1	1.9 ± 0.1^{m}	14.5 ± 0.3^{i}
	2	7.18 ± 0.01^{f}	3.9 ± 0.1^{l}	6.6 ± 0.1^{p}
	3	6.10 ± 0.11^{h}	43.2 ± 1.4^{g}	31.4 ± 0.1^{h}
	4	<1	58.2 ± 0.7^{f}	37.6 ± 0.4^{g}
Kombucha + Lb-3	0	<1	0.5 ± 0.1^{o}	3.8 ± 0.5^{r}
	1	<1	1.9 ± 0.5^{m}	11.5 ± 0.1^{k}
	2	7.38 ± 0.03^{e}	3.5 ± 0.0^{l}	7.0 ± 0.1^{o}
	3	2.02 ± 0.09^{l}	14.4 ± 0.1^{h}	30.0 ± 1.1^{h}
	4	<1	11.1 ± 0.3^{i}	53.6 ± 0.5^{c}
Kombucha + Lb-4	0	<1	0.3 ± 0.1^{p}	3.3 ± 0.5^{s}
	1	<1	2.1 ± 0.3^{m}	10.5 ± 0.3^{l}
	2	7.54 ± 0.02^{d}	3.8 ± 0.2^{l}	8.5 ± 0.2^{n}
	3	3.61 ± 0.41^{i}	203.7 ± 1.4^{b}	71.0 ± 1.0^{b}
	4	2.95 ± 0.12^{j}	247.6 ± 0.5^{a}	95.5 ± 0.5^{a}
Kombucha + Lb-5	0	<1	0.6 ± 0.2^{o}	3.0 ± 0.3^{u}
	1	<1	1.7 ± 0.1^{n}	12.5 ± 0.1^{j}
	2	7.13 ± 0.02^{g}	3.3 ± 0.1^{l}	5.1 ± 0.5^{q}
	3	2.47 ± 0.32^{k}	94.6 ± 0.5^{e}	37.8 ± 0.3^{g}
	4	1.90 ± 0.01^{m}	95.0 ± 0.2^{e}	40.5 ± 0.5^{f}

*The obtained results for Control Kombucha refer also to the number of bifidobacteria and streptococci. Different letters in superscript in columns indicate a significant difference at $p<0.05$ according to Tukey's HSD test. Lb-1−Lb-5: see Table 1.

the fermentation broth from the previous cultivation (Table 3). After two days of fermentation, the pH decreased by about 1 unit and in the next two days the decrease was only by 0.3––0.4 units. At the end of the process, the pH of fermentation broths reached values of 3.03–3.16. Changes in the pH during tea fungus cultivation were similar for both, Control Kombucha as well as medium inoculated with *Lactobacillus* strains. The TA (Table 3) increased constantly from the beginning till the end of the fermentation for all samples. TA of cultivation medium after inoculation was around 0.3 g/L and after two days of fermentation, it reached 1.62–2.16 g/L. In the following two days, some differences were noted between the acidity of samples. The highest TA at the end of fermentations was determined for Kombucha with strain Lb-3 (7.01 g/L), which was higher by 0.78–1.55 g/L compared with the other beverages. The TA of the Control Kombucha after four days of fermentation was 6.23 g/L. During Kombucha fermentations, the value of pH decreased significantly ($p<0.05$) in the first

TABLE 3. The change of pH, titratable acidity (TA) (g/L), and numbers of acetic acid bacteria and yeasts (log cfu/mL) during Kombucha fermentations.

Sample	Fermentation time (days)	pH	TA	Number of acetic acid bacteria	Number of yeasts
Control	0	4.43 ± 0.00^{b}	0.28 ± 0.00^{r}	4.30 ± 0.10^{g}	5.78 ± 0.03^{c}
	1	4.19 ± 0.06^{d}	0.51 ± 0.03^{o}	5.58 ± 0.12^{cd}	6.71 ± 0.09^{ab}
	2	3.57 ± 0.02^{f}	1.62 ± 0.05^{l}	6.12 ± 0.02^{b}	6.87 ± 0.04^{a}
	3	3.21 ± 0.03^{kl}	4.32 ± 0.05^{e}	6.34 ± 0.14^{a}	6.87 ± 0.01^{a}
	4	3.05 ± 0.01^{m}	6.23 ± 0.07^{b}	5.46 ± 0.11^{d}	6.73 ± 0.09^{ab}
Lb-1*	0	4.43 ± 0.00^{b}	0.28 ± 0.00^{r}	4.92 ± 0.10^{e}	5.78 ± 0.03^{c}
	1	4.18 ± 0.06^{d}	0.50 ± 0.00^{o}	5.73 ± 0.10^{c}	6.86 ± 0.04^{a}
	2	3.52 ± 0.02^{g}	1.83 ± 0.03^{k}	6.30 ± 0.04^{a}	6.89 ± 0.03^{a}
	3	3.39 ± 0.02^{h}	2.85 ± 0.04^{h}	5.70 ± 0.11^{c}	6.75 ± 0.04^{ab}
	4	3.16 ± 0.01^{l}	5.86 ± 0.02^{c}	5.69 ± 0.15^{c}	6.80 ± 0.04^{ab}
Lb-2	0	4.43 ± 0.00^{b}	0.28 ± 0.00^{r}	4.16 ± 0.01^{h}	5.78 ± 0.03^{c}
	1	4.05 ± 0.02^{e}	0.63 ± 0.03^{m}	4.76 ± 0.12^{f}	6.66 ± 0.16^{ab}
	2	3.41 ± 0.03^{h}	2.16 ± 0.02^{i}	6.10 ± 0.02^{b}	6.76 ± 0.11^{ab}
	3	3.30 ± 0.01^{j}	3.23 ± 0.05^{g}	5.82 ± 0.02^{c}	6.60 ± 0.18^{b}
	4	3.05 ± 0.04^{m}	5.46 ± 0.04^{d}	5.75 ± 0.15^{c}	6.65 ± 0.06^{ab}
Lb-3	0	4.52 ± 0.00^{a}	0.38 ± 0.00^{p}	4.25 ± 0.10^{g}	5.50 ± 0.16^{d}
	1	4.32 ± 0.04^{c}	0.50 ± 0.03^{o}	5.65 ± 0.07^{c}	6.61 ± 0.03^{b}
	2	3.44 ± 0.08^{gh}	1.88 ± 0.05^{k}	6.24 ± 0.05^{a}	6.61 ± 0.13^{ab}
	3	3.25 ± 0.02^{k}	3.83 ± 0.05^{f}	6.33 ± 0.09^{a}	6.88 ± 0.10^{a}
	4	3.01 ± 0.01^{n}	7.01 ± 0.01^{a}	6.38 ± 0.04^{a}	6.72 ± 0.10^{ab}
Lb-4	0	4.30 ± 0.00^{c}	0.25 ± 0.00^{s}	4.35 ± 0.10^{g}	5.66 ± 0.02^{d}
	1	4.06 ± 0.03^{e}	0.56 ± 0.02^{n}	5.25 ± 0.14^{d}	6.68 ± 0.13^{ab}
	2	3.33 ± 0.03^{i}	2.06 ± 0.04^{j}	5.89 ± 0.14^{bc}	6.78 ± 0.08^{a}
	3	3.04 ± 0.02^{m}	3.85 ± 0.05^{f}	6.11 ± 0.06^{b}	6.91 ± 0.12^{a}
	4	3.03 ± 0.01^{m}	6.01 ± 0.03^{c}	5.73 ± 0.10^{c}	6.68 ± 0.06^{ab}
Lb-5	0	4.29 ± 0.00^{c}	0.32 ± 0.00^{q}	4.25 ± 0.10^{g}	5.67 ± 0.01^{d}
	1	4.00 ± 0.05^{e}	0.57 ± 0.03^{n}	5.60 ± 0.11^{cd}	6.80 ± 0.09^{ab}
	2	3.34 ± 0.01^{i}	2.14 ± 0.02^{i}	6.17 ± 0.08^{ab}	6.76 ± 0.06^{ab}
	3	3.14 ± 0.06^{l}	3.29 ± 0.08^{g}	5.65 ± 0.20^{cd}	6.67 ± 0.07^{ab}
	4	3.06 ± 0.02^{m}	5.99 ± 0.14^{c}	5.69 ± 0.12^{c}	6.68 ± 0.16^{ab}

Different letters in superscript in columns indicate a significant difference at $p<0.05$ according to Tukey's HSD test. Lb-1–Lb-5: see Table 1.

two days as a consequence of the physiological activity of the tea fungus and production of organic acids (primarily acetic acid). After this period, the pH changed much slower despite the continued production of organic acids (Table 2), which could be explained by the buffer capacity of the fermentation broth. Namely, during the Kombucha fermentation, carbon dioxide is released as a result of metabolic activity of tea fungus yeasts. The water solution of CO_2 dissociates and produces the amphiprotic hydrocarbonate anion (HCO_3^-), which easily reacts with hydrogen ions (H^+) from the present organic acids, preventing further changes in pH, thus contributing to a buffer character of the system [Cvetković *et al.*, 2008]. This typical trend of pH changes during Kombucha fermentation was observed by other authors who used similar cultivation conditions [Blanc, 1996; Sreeramulu *et al.*, 2000; Belloso-Morales & Hernández-Sánchez, 2003]. Considering changes of basic chemical parameters during Kombucha fermentation, TA of fermentation broth should be used as

a critical parameter instead of pH which determines the end of fermentation. Beverages with optimal consumption acidity (TA=4–4.5 g/L) in this paper were obtained in less than four days of fermentation.

The number of yeasts after inoculation was in the range from 5.50 to 5.78 log cfu/mL (Table 3). In the following 24 h, this number increased by up to one log unit. After that, the number of yeasts in the fermentation broth was uniform until the end of the process, for most of the samples (6.65––6.80 log units at the end), which has been confirmed by Tukey's HSD test (Table 3). The number of AAB at the start of fermentation was in the range from 4.16 to 4.92 log cfu/mL (Table 3). The highest number of AAB at the end of fermentation was detected for Kombucha+Lb-3 (6.38 log cfu/mL) while Control Kombucha had the lowest AAB number (5.46 log cfu/mL). The number of AAB in fermentation broths reached the maximum after two days of fermentation for samples Kombucha+Lb-1 (6.30 cfu/mL), Kombucha+Lb-2 (6.10 cfu/mL), and Kombucha+Lb-5 (6.17 cfu/mL); after three days for Control Kombucha (6.34 cfu/mL) and Kombucha+Lb-4 (6.11 cfu/mL); and after four days for Kombucha+Lb-3 (6.38 cfu/mL). Afterwards, the number of cells was reduced by 0.35–0.89 log units until the end of fermentation. Numbers of yeasts and AAB in fermentation broths during tea fungus cultivation obtained in this study are comparable with the results reported by other authors. Teoh *et al.* [2004] found that the count of individual yeast species in fermentation broth was 4 log units at the beginning of the process and reached the maximum of 7 log units on the sixth day. During fermentation, the viable population of yeasts in Kombucha beverage followed a standard growth curve pattern, in which yeast grew exponentially for up to 8–10 days, dying off as nutrients depleted and pH decreased. Sreeramulu *et al.* [2000] studied the growth patterns of AAB in Kombucha fermentation broth and found that the count of AAB increased rapidly in 4 days of fermentation (from 4 log units at the start of fermentation to more than 7 log units after 4 days), declined drastically in 6 days of fermentation, and thereafter continued to decrease. The decreased count of AAB during tea fungus cultivation was probably caused by an acid shock. Production of acetic acid during tea fungus cultivation is directly related to the physiological activity and the count of AAB which enzymatically oxidize ethanol into acetic acid. However, the physiological activity of AAB during tea fungus cultivation cannot be considered separately from the role of yeasts in this consortium, it being the carbon source (glucose, fructose, and ethanol) needed by AAB. However, in all fermentations in this study, the number of yeasts and AAB during cultivation was not below 6 log cfu/mL, *i.e.* 5 log cfu/mL (Table 3) which has provided efficient biotransformation of sweetened teas according to the TA results. These data on the number of yeasts and AAB are significant in the development of a concept of using isolated strains (pure culture) of yeasts and AAB as a starter culture for Kombucha fermentation.

The results of determinations of pH, TA, number of yeasts and AAB during Kombucha fermentation clearly demonstrated that the addition of wild strains of lactobacilli on the second day of fermentation affected the growth pattern, *i.e.* number of cells and physiological activity of tea fungus.

The cell number of wild *Lactobacillus* strains in fermentation broth determined after inoculation was between 7.13 (Lb-5) and 8.16 log cfu/mL (Lb-1) (Table 2). This number was maintained until the end of the process only for strain Lb-1 (*L. hilgardii*). At the end of fermentation, cells of Lb-2 and Lb-3 were not detected in Kombucha beverages. At the same time, the initial number of strains Lb-4 and Lb-5 during fermentation decreased by 5 log units. In the case of strains Lb-3, Lb-4, and Lb-5 a significant reduction in their cell number was detected 24 h after inoculation (5.36, 3.93, and 4.66 log unit, respectively), which confirms an assumption that Kombucha fermentation broth is not the most favorable environment for growth and proliferation of lactobacilli. Lee *et al.* [2016] have also examined the influence of different initial pH values at 30°C on the growth rate of LAB. According to their results, all strains of *L. plantarum* did not grow at the initial pH of 2.0, while their slow growth was observed at the initial pH of 3.0. On the other hand, the species *L. hilgardii* seem to be more tolerant to lower pH, as well as to high concentrations of ethanol and the environments with minor nutrient concentrations [Reis *et al.*, 2018]. Cell number of *Lactobacillus hilgardii* from the moment of inoculation until the end of fermentation decreased only by 0.5 log cfu/mL. However, despite the largest number of viable cells during Kombucha fermentation, *Lactobacillus hilgardii* did not produce the largest quantity of lactic acids (Table 2).

The highest content of lactic acids (247.6 mg/L and 95.5 mg/L of D- and L-lactic acid, respectively) at the end of the process was produced by strain Lb-4 (*Lactobacillus plantarum* from 40-day old cream) (Table 2) in spite of a significant reduction ($p<0.05$) in the number of cells during fermentation (4.59 log units) (Table 3). In turn, the lowest content of d- and l-lactic acids was produced by strains Lb-2 (58.2 and 37.6 mg/L, respectively) and Lb-3 (11.1 and 53.6 mg/L, respectively). The contents of L- and D-lactic acids in the Control Kombucha after 4 days of fermentation were 38.4 and 7.7 mg/L, respectively. These results point to the fact that viability of lactobacilli does not necessarily have to influence the production of lactic acids. That is confirmed by Mathara *et al.* [2008] who reported about situations in which cell viability was not required for the physiological activity of LAB. In these cases, health beneficial effects have been linked to non-viable cells or to cell components, enzyme activities or fermentation products. However, wild strains of lactobacilli significantly affected the content and profile of lactic acid in fermentation broths and beverages (Table 2). In all fermentation broths, 24 h after inoculation with wild strains (the third day of cultivation), a significant ($p<0.05$) increase was detected in the content of L- and D- lactic acid, compared with Control Kombucha. In the following 24 h, the increase continued except for strain Lb-3 where d-lactic acid content was by 3.3 mg/L lower than on the previous day. Hence, while L-lactic acid predominates in the Control Kombucha, D-lactic acid prevailed in beverages obtained by the addition of wild strains. The content of D-lactic acid in the fermentation broth was higher than that of L-lactic acid for all wild strains with the exception of strain Lb-3 (*Lactobacillus plantarum* from 2-month old cheese). Malbaša *et al.* [2008] found that L-lactic acid was dominant in Kombucha and its final concentration af-

TABLE 4. Value of pH, titratable acidity (TA) (g/L), and number of cells (cfu/mL) of wild lactobacilli (LB-1, LB-4 and LB-5) during storage of Kombucha at +4 °C.

Storage duration (days)	Lb-1			Lb-4			Lb-5		
	pH	TA	Number of cells	pH	TA	Number of cells	pH	TA	Number of cells
0	3.16±0.01^{b}	5.86±0.02^{a}	7.60±0.04^{a}	3.03±0.03^{b}	6.00±0.03^{b}	2.95±0.12	3.06±0.02^{b}	6.04±0.16^{a}	1.90±0.01
2	3.16±0.01^{b}	5.19±0.10^{b}	7.55±0.03^{a}	3.04±0.01^{b}	6.09±0.09^{ab}	<1	3.08±0.01^{b}	6.08±0.08^{a}	<1
4	3.16±0.02^{b}	5.18±0.11^{b}	7.37±0.05^{b}	3.03±0.03^{b}	6.08±0.05^{ab}	<1	3.06±0.03^{b}	6.10±0.05^{a}	<1
6	3.25±0.03^{a}	5.31±0.03^{b}	7.15±0.03^{c}	3.15±0.04^{a}	6.15±0.01^{a}	<1	3.15±0.02^{a}	6.15±0.10^{a}	<1
10	3.26±0.01^{a}	5.20±0.02^{b}	7.18±0.00^{c}	3.17±0.02^{a}	6.16±0.05^{a}	<1	3.16±0.03^{a}	6.18±0.02^{a}	<1

Different letters in superscript in columns indicate a significant difference at $p<0.05$ according to Tukey's HSD test. Lb-1, Lb-4 and Lb-5: see Table 1.

ter 14 days of fermentation on sucrose was 53 mg/L. Belloso-Morales & Hernández-Sánchez [2003] found that production of lactic acid in the black tea Kombucha reached a maximum (1.15 g/L) after 96 h of cultivation. Generally, some differences in chemical parameters (such as lactic acid content), process duration, and cell counts in Kombucha beverages obtained in different studies are expected because of using inoculums (tea fungus culture) from different locations. The variations could be due to geographic, climatic, and cultural conditions as well as local species of wild yeasts and bacteria or, possibly, due to cross-contamination between cultures [Mayser *et al.*, 1995; Teoh *et al.*, 2004].

Survival of wild strains of lactobacilli in Kombucha during storage

After fermentation, the beverages containing living cells of wild strains of lactobacilli (Lb-1, Lb-4, and Lb-5) were bottled and stored at +4°C. Changes of pH and TA, as well as the number of cells during storage for a period of 10 days are presented in Table 4. After 2 days of storage, cells of strains Lb-4 and Lb-5 were not detected in the bottled samples. During 10 days, the chemical characteristics (pH and TA) of each of three beverages changed statistically significantly ($p<0.05$), except TA for beverage with strain Lb-5. At the same time, the number of *Lactobacillus hilgardii* (Lb-1) in the beverage decreased by 0.37 log cfu/mL. According to the Tukey's HSD test, statistically significant differences ($p<0.05$) were noticed between mean numbers of lactobacilli during the storage period.

Strain Lb-1 (*Lactobacillus hilgardii*) showed an outstanding viability during both Kombucha fermentation (Table 2) and storage (Table 4). Resistance of *Lactobacillus hilgardii* to ecological conditions during fermentation and storage can be brought into correlation with the origin of this wild strain, which was the only one isolated from the sourdough. Sourdough is a medium which represents association of LAB and yeasts, and *Lactobacillus hilgardii* is a LAB species generally associated with sourdough fermentation [Corsetti & Settanni, 2007]. It is well known that strains isolated from traditionally fermented food have enhanced metabolic activities in comparison to microorganisms that are used as industrial starter cultures [Klijn *et al.*, 1995]. These include differences in growth rate and competitive growth behavior in mixed cultures, adaptation to a particular substrate or raw material, antimicrobial properties, and flavor, aroma, and quality attributes [Leroy & De Vuyst, 2004].

CONCLUSIONS

Wild strains of *Lactobacillus* spp. have a potential to adapt to the environment such as Kombucha fermentation broth. Addition of these strains during Kombucha fermentation had no effect on the physiological activity of tea fungus, but contributed to a significant increase in lactic acids content in the beverage. The survival ability of wild lactobacilli, along with their ability to produce greater quantities of primarily L-lactic acid and possible probiotic properties, are key factors in the selection of strains which could be used in Kombucha fermentation. All the mentioned should result in producing a beverage of increased functional properties which is to be confirmed by further study.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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Original article
Section: Food Quality and Functionality

Design of Bacterial Cultures in Fermented Functional Maize Product Formulation

Zuzana Matejčeková[1], Anna Mikulajová[1], Eva Vlková[2], Denisa Liptáková[3], Silvia Mošovská[1], Eva Hybenová[1], Ľubomír Valík[1]*

[1]Department of Nutrition and Food Quality Assessment, Faculty of Chemical and Food Technology, Slovak University of Technology Bratislava, Radlinského 9, 812 37 Bratislava, Slovak Republic
[2]Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague 6, Czech Republic
[3]State Institute for Drug Control, Bratislava, Kvetná 11, 825 08 Bratislava, Slovak Republic

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In this work, the effect of single fermentation of maize mashes with Fresco DVS 1010 culture (*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus*) was studied for 8 h (37°C) followed by a storage period of 21 days (6°C). Although milk is a typical growth medium for lactic acid cocci of the Fresco culture, fermentation increased its counts by about 3 log CFU/mL after 8 h (37°C), whereas pH of 4.54 and 4.69 was achieved after the fermentation in products with sucrose and caramel, respectively. Fermentation process was observed to significantly increase ($p<0.05$) the total phenolics content (143.9–206.1%) and the antioxidant activities (53.7–107.4%) of the samples. Potentially probiotic *Lactobacillus plantarum* HM1, *Bifidobacterium choerinum* K1/1, *Bifidobacterium pseudolongum* K4/4, *Bifidobacterium animalis* ssp. *animalis* J3II, and *Bifidobacterium thermophilum* DSM 20212 inoculated after the fermentation process were well maintained (>5 log CFU/mL) in combination with the mixed Fresco culture in the prepared maize products within 21 days of storage. Based on the overall sensory acceptance, caramel mashes after 12 days of storage period were evaluated as satisfactory (2.4 to 3.1 from 4.0), while 21-day stored products achieved good acceptability scores (3.0 to 3.6 from 4.0), hence the tendency of the positive effect of prolonged shelf-life was noted.

INTRODUCTION

Increased awareness of consumers over health and a steady increase in life expectancy have resulted in the demand for food products that can improve consumer well-being [Ogunremi *et al*., 2015]. Therefore, the industry is directing development of new products towards the area of functional foods. Research in this area has moved progressively towards introducing the concept of synbiotics, a combination of pro- and prebiotics in a single product that may affect gut microbial composition. Cereals have a significant role in human nutrition in most parts of the world and are grown at over 73% of the total world harvest area [Charalampopoulos *et al.*, 2002]. Cereal grains represent an important nutritive component both in developed and in developing countries. They are considered as one of the most important sources of carbohydrates (starch and fiber), vitamins (group B), minerals, and phenolic compounds with many proven health effects. They also have the potential to offer consumer prebiotic and whole grain benefits [Lamsal & Faubion, 2009]. While lysine represents a limiting amino acid in cereals, a combination of such substrates with milk or dairy products constitutes one of the most appropriate ways of improving the nutritional value of final products. Although a variety of technologies (*e.g*., cooking, sprouting, and milling) are used for cereal processing, fermentation still remains the most appropriate choice to improve the nutritional, sensorial, and shelf-life properties [Coda *et al*., 2011]. This is the main reason why a large proportion of cereals is processed into foods and beverages by fermentation prior to consumption [Nout, 2009]. Indeed, lactic acid bacteria (LAB) (*Lactobacillus*, *Pediococcus* spp.), yeasts (*Candida*, *Debaryomyces*, *Hansenula*, *Pichia*, and *Saccharomyces* spp.), and filamentous fungi (*Amylomyces*, *Aspergillus*, *Mucor*, and *Rhizopus* spp.) are mainly involved in the manufacture of cereal-based beverages [Blandino *et al.*, 2003]. There are also few reports on the suitability of cereals as a carrier of probiotic LAB. An oat-based synbiotic drink made by fermenting an oat substrate with *Lactobacillus plantarum* B28 was developed in a study of Prado *et al*. [2008]. Pelikánová *et al*. [2015] proved the potential of maize flour for the production of fermented products containing lactobacilli that were well maintained above the suggested minimum level (6 log CFU/mL) during 21 days of storage at 6°C. While selecting a preferable probiotic strain, several aspects of functionality have to be considered. Bifidobacteria have long been recognized as bacteria possessing probiotic,

* Corresponding Author: E-mail: matejcekovaz@gmail.com (Z. Matejčeková)

nutritive, and therapeutic properties [Picard *et al*., 2005]. Various health-promoting functions of bifidobacteria have poised them to be an ideal dietary supplement. There have been some studies describing incorporation of bifidobacteria in several kinds of dairy products, *e.g.*, fermented dairy milks [Kongo *et al*., 2006] or cheese [Dinakar & Mistry, 1994]. Traditional fermented foods prepared from common types of cereals (rice, wheat, maize) are well known mainly in Asia and Africa. As the European market for probiotic products is expected to grow continuously, research on the development of food products containing probiotics and the isolation of new strains with potential probiotic properties is important. A considerable potential at manufacturing new products towards the area of functional foods led us to prepare functional food products with the probiotic potential for celiac patients based on gluten-free cereal. Thus, this study aimed at manufacturing and characterizing the chemical and microbiological properties of prepared fermented maize products as well as at determining the survival of selected bacteria of *Bifidobacterium* and *Lactobacillus* spp. genera. The next part of our study involved detailed sensory evaluation of final products, where sucrose was adjusted by caramel flavor to make products more attractive to consumers.

MATERIALS AND METHODS

Microorganism

A Fresco DVS 1010 starter culture consisting of *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus* is a commercial culture from Christian and Hansen (Hørsholm, Denmark) and was kindly provided by Rajo a. s. (Bratislava, Slovakia). The Fresco culture was kept in a deep freezer. The starter culture was obtained aerobically overnight at 30±0.5°C in M17 broth (Biokar Diagnostics, Beauvais, France). A potentially probiotic isolate *L. plantarum* HM1 was isolated from breast milk and identified by Liptáková *et al*. [2016]. The isolate was first sub-cultured three times for 24 h at 37±0.5°C (5% CO_2) in de Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) from the frozen stock containing MRS broth and 25% of glycerol before using it as an inoculum (stored at -30°C). The following potentially probiotic bifidobacterial strains were provided by prof. Vlková (Czech University of Life Sciences, Prague, Czech Republic): *Bifidobacterium choerinum* K1/1 and *Bifidobacterium pseudolongum* K4/4 were isolated from goat feces, *Bifidobacterium animalis* ssp. *animalis* J3II was isolated from lamb faeces, and *Bifidobacterium thermophilum* DSM 20212 is a collection strain (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Göttingen, Germany (DSMZ)). The sub-cultivation of bifidobacteria was performed in serum bottles in Wilkins-Chalgren (WCH) broth (Oxoid, Brno, Czech Republic) supplemented with 0.5% (w/v) of soya peptone (Oxoid, Brno, Czech Republic), Tween (0.1% (v/v); Biolife, Milan, Italy) and cysteine-hydrochloride (0.05% (w/v) Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) was added to act as an oxygen scavenger to provide a low redox potential. Bifidobacterial strains were stored in WCH broth added with glycerol (20% v/v) before using it as an inoculum (stored at -20°C). Pure cultures of the studied bacteria were centrifuged (3461×*g* for 5 min, Centrifuge EBA 20, Hettichlab, Tuttlingen, Germany); the cell pellet was washed in 10 mL of sterile distilled water and centrifuged again under the same conditions. After centrifugation, supernatant was removed and pellets were re-suspended in distilled water to its original volume in compliance with the procedure of Matejčeková *et al*. [2018].

Enumeration of bacteria

The presumptive numbers of the Fresco culture were enumerated on M17 agar plates (Biokar Diagnostics, Beauvais, France) according to the STN ISO 15214. Inoculated Petri dishes were cultivated at aerobic conditions for 24 h (30±0.5°C). Presumptive numbers of *L. plantarum* were estimated using Vegiton MRS agar (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Inoculated Petri dishes were cultivated at anaerobic conditions at 37±0.5°C (5% CO_2). Bifidobacteria were cultivated and enumerated on selective transgalactosylated oligosaccharide mixture propionate agar with added mupirocin (TOS-MUP) (Merck, Darmstadt, Germany). Petri dishes were cultivated at 37±0.5°C for 72 h under anaerobic conditions using anaerobic jars and Anaerocult A system (Merck, Darmstadt, Germany).

Evaluation of growth and metabolic parameters

Growth and metabolic parameters (specific growth rate, rate constant for decrease of counts, rate constant for decrease of pH) of the studied LAB in maize mashes were fitted and calculated using the mechanistic model DMFit by Baranyi & Roberts [1995]. Growth and metabolic parameters were calculated from each growth curve. Specific growth rates μ (1/h) were recalculated from the $\log_{10}$ based growth rates (G_R) according to the equation $\mu = \ln 10 \times G_R$.

The pH values were measured during fermentation and storage using a pH meter with a penetration electrode (Knick Portamess, Berlin, Germany).

Preparation of maize substrate

The maize mash used as a substrate was prepared from maize flour (8% (w/v)) (Solčanka, Solčany, Slovak Republic), 2% (w/v) of sucrose, and ultra-pasteurized (UHT) milk (fat content 1.5%). Mashes were heated at 100°C for 20 min while stirred, and then sterilized for 20 min at 121°C. The mashes were subsequently cooled. In part of the samples, sucrose was replaced with a commercial caramel component (35% caramel syrup, 10% glucose fructose syrup, 28.5% sucrose, modified maize starch, caramelized sugar, water) (Agrana, Vienna, Austria), to achieve better sensory characteristics. In the flavored mashes, the caramel component was added after sterilization.

Fermentation process

For static fermentation, maize mashes were inoculated with 5% (v/v) of Fresco DVS 1010 culture (Danisco, Copenhangen, Denmark) to achieve inoculation levels of approximately 6 log CFU/mL. Static fermentation was performed for 8 h at 37±0.5°C (5% CO_2). The samples for microbiological analyses of counts and pH values were taken every 2 h. Potential probiotic strains of LAB (n=5) were inoculated into the substrates after the fermentation process in concen-

trations of approximately 8–9 log CFU/mL. Subsequently, the mashes remained for another 21 days at 6±0.5°C with periodical determination of pH values and viability of the studied LAB. The experiments were carried out in duplicate.

Sensory evaluation

The procedure of sensory evaluation was conducted in accordance with ISO 13299 in the Laboratory of Sensory Analysis of the Slovak University of Technology in Bratislava, Slovak Republic. The detailed sensory assessment of the samples was performed with implementation of the Quantitative Descriptive Analysis (QDA) according to the procedure described by Stone *et al*. [2012]. The individual samples of each maize mash with caramel products (weighing approximately 15 g) were placed in transparent, odorless, plastic boxes (125 mL) covered with lids. The samples were evaluated at room temperature (20–22°C). Still mineral water was used for rinsing palates between samples as a neutralizer. The sample sets for each evaluator were coded individually with three-digit numbers to avoid the carry over effect. The samples were presented randomly. Finally, 10 trained assessors (9 women and 1 men) between the ages of 25 and 43 (mean = 32.6) were chosen from sensory experts trained in accordance with STN EN ISO standard 8586–2, having broad experience in sensory evaluation of different food products.

Sensory analysis included characteristics of color, consistence, texture, overall acceptability as well as individual descriptors of aroma and taste. Overall sensory quality was evaluated using a hedonic scale for each attribute (0 representing "dislike extremely" and 4 representing "like extremely"). Fresh fermented mashes (37±0.5°C/8 h) and stored products (6±0.5°C for 12 and 21 days) were evaluated at the same time.

Sample extract preparation

Samples were freeze-dried and extracted using 65% (v/v) ethanol for 1 h at 80°C, three times [Mikulajová *et al*., 2007a]. Phenolic compounds were subsequently reextracted with ethyl acetate (Centralchem, Bratislava, Slovak Republic), concentrated to dryness, and dissolved in 96% (v/v) ethanol. The ethanolic extracts obtained were used for determination of total phenolics content, total flavonoids content, and antioxidant activity that was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay and by ferric reducing antioxidant power (FRAP) assay.

Analysis of total phenolics content (TPC)

The total phenolics content was determined using the Folin–Ciocalteu's reagent (Sigma-Aldrich, Steinheim, Germany) according to Yu *et al*. [2002]. Gallic acid (Sigma-Aldrich, Steinheim, Germany) was used as a standard. Gallic acid base solution in 96% (v/v) ethanol (1.0 mg/mL) was diluted to obtain 0.01–0.80 mg/mL concentrations. Results were expressed in mg of gallic acid equivalent per gram of sample (mg GAE/g).

Analysis of total flavonoids content (TFC)

The total flavonoids content was determined using the $AlCl_3$ method described by Kumar *et al.* [2011]. Quercetin (Sigma-Aldrich, Steinheim, Germany) was used as a standard. Quercetin base solution in 96% (v/v) ethanol (1.0 mg/mL) was diluted to obtain 0.01–0.40 mg/mL concentrations. Results were expressed in mg of quercetin equivalent per gram of sample (mg QE/g).

Determination of antiradical activity

The antiradical activity was determined by using stable free radicals ($DPPH^{\bullet}$) (Sigma-Aldrich, Steinheim, Germany) as described by Yen & Chen [1995]. Absorbance of the samples was measured at 517 nm after 10 min of reaction. Results were expressed as the amount of scavenged DPPH radicals per gram of sample (mg DPPH/g).

Analysis of ferric reducing antioxidant power (FRAP)

FRAP was measured according to Pohanka *et al*. [2009]. Absorbance of the samples was measured at 730 nm after 30 min of reaction. 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma-Aldrich, Steinheim, Germany) was used as a standard and results were expressed in mg of Trolox equivalent per gram of sample (mg Trolox/g).

Statistical analysis

All the results are expressed as means ± standard deviation (SD). Each fermentation experiment was carried out in duplicate and each analysis was performed in three separate trials. Statistical analyses were carried out using Microsoft Excel 2013 (Microsoft, Redmond, U.S.A.). Data were analyzed by Student's *t*-test with a least significant difference of 95% and by correlation analysis. Correlation coefficients were determined according to Asuero *et al*. [2006].

RESULTS AND DISCUSSION

In this study, maize flour was selected as a substrate for lactic acid fermentation and vehicles for probiotics. To protect probiotic bacteria, especially bifidobacteria from oxygen in yoghurt, the incorporation of high oxygen consuming strains of *S. thermophilus* has been proposed. *S. thermophilus* alone or as a part of mesophilic cultures has been suggested as a solution for reduction of fermentation time with increasing growth rates; absence of certain sensory and textural defects and further improvement of nutritional value of 'bifidus' products [Gomes & Malcata, 1999]. Thus, based on our previous research [Matejčeková *et al*., 2018], and the above facts, a mixed Fresco DVS 1010 culture was selected for lactic acid fermentation of the prepared maize mashes.

Microbial counts and stability during cold storage period

Within 5% (v/v) concentration of the mixed Fresco culture at 8 h fermentation the levels reached populations of 9.15±0.24 log CFU/mL representing 3 log units increase compared to the initial state. Short fermentation time (8 h) was preferable in order to minimize the risk of contamination. Pelikánová *et al*. [2015] also proved the potential of maize substrates for lactic acid fermentation within 10 h when growth rates of lactobacilli ranged from 0.155 to 0.811 log CFU/(mL×h). In our study, Fresco culture entered immediately the exponential phase of growth with almost the same

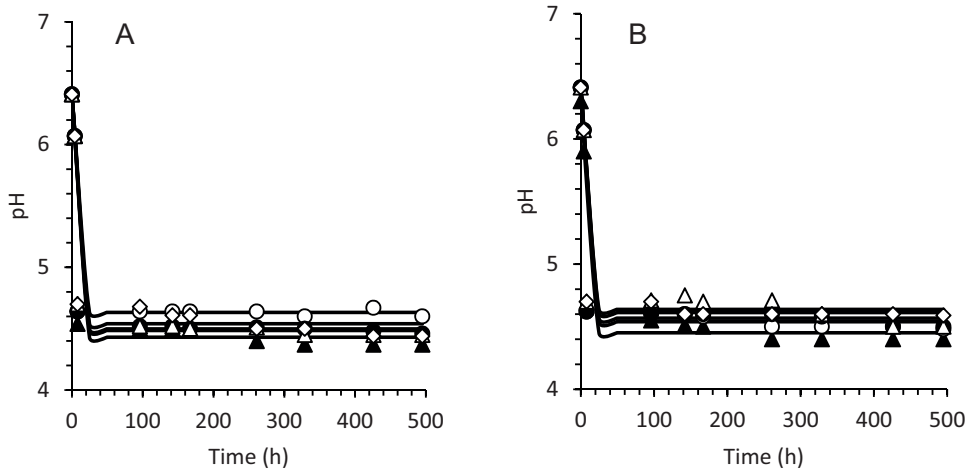

FIGURE 1. Changes in pH as a function of incubation time of milk-based maize mashes with sucrose (A) and caramel (B) inoculated with Fresco DVS 1010 and next (after 8 h) with *L. plantarum* HM1 (▲), *B. thermophilum* DSM 20212 (○), *B. animalis* ssp. *animalis* J3II (●), *B. pseudolongum* K4/4 (Δ), and *B. choerinum* K1/1 (◊).

specific growth rates in milk-based maize mash with sucrose (μ_{suc} = 1.34 1/h) and caramel (μ_{car} = 1.33 1/h) (equivalent to a generation time of 31 min; data not shown). In our previous study, addition of chocolate flavour in buckwheat substrates increased the specific growth rates of the cocci of the Fresco culture by about 1.4–30.3% compared to those with sucrose only [Matejčeková *et al*., 2017]. Due to the accumulation of lactic acid produced as a result of metabolic activity, a rapid drop in pH was observed in single fermentation process (Figure 1). The pH values measured after 8 h of fermentation were 4.54 and 4.69 in maize products with sucrose and caramel, respectively. In our previous study, efficient acidification profiles (pH below 4.5) were generally achieved during co-culture fermentation of Fresco culture with *L. plantarum* [Matejčeková *et al*., 2018]. Salmerón *et al*. [2014] noted a decrease of pH below 3.7 after 10 h in cereal beverages (oat, barley, and malt substrates), whereas Rathore *et al*. [2012] recorded pH below 3.5 for all cereal fermentations both with *L. acidophilus* and *L. plantarum* strains.

Good viability of the microbial starter culture is the first and the most important criterion to ensure good quality and health-promoting properties of the product. To achieve health benefits, the minimum level of 6 log CFU/mL, concerning the population of probiotic microorganism at the end of the product shelf life, is usually considered as acceptable [Georgieva *et al*., 2009]. Thus, the changes in cell counts of potentially probiotic bacteria were evaluated. In general, the bacterial counts in samples decreased by about 0.3–1.7 log units at 6°C during the period of 21 days and were well maintained above the limit of 6 log CFU/mL except *B. choerinum* K 1/1 (Figure 2). Despite the decline in levels of *B. choerinum* K1/1 strain in the product with sucrose by about 3.8 log units, the counts remained above 5 log CFU/mL, *i.e.* at the minimum level of probiotics suggested by some authors [Gueimonde *et al*., 2004]. Hence, a beneficial relationship occurred between cocci of the Fresco culture and *Bifidobacterium* spp. (*L. plantarum* HM1) in the prepared products. Concentrations of Fresco culture were not significantly affected ($p>0.05$) by the potentially probiotic strains tested, whereas average counts varied within 9.08±0.36 log CFU/mL after 21 days (data not shown). Gueimonde *et al*. [2004] evaluated counts of LAB in commercial fermented milks, whereas counts of streptococci were present at levels ranging from 7 to 9 log CFU/mL during cold storage (4°C), while bifidobacteria showed a decrease between 0.17 and 1.10 log units. Co-culture fermentation of milk by *B. lactis* and *L. acidophilus* (1:1 inoculum ratio) led to the enhanced growth rates and acidification profiles when compared with single strain, suggesting some degree of symbiosis [Gomes & Malcata, 1999].

Over 21 days, *L. plantarum* and *Bifidobacterium* spp. strains continued metabolism of saccharides and accumulation of organic acids resulting in pH decrease to 4.37–4.60 (Table 1), representing a decline of about 1.8–2.0 units. Survival of bifidobacterial strains is dependent on pH of the environment. While low pH decreases their survival, tolerance of *Bifidobacterium* spp. to acidic conditions has been reported to be strain specific [Kailasapathy & Chin, 2000]. Despite this fact, in our study, all tested bifidobacterial strains of different origin were well maintained in the final products as reported above. Nowadays, the origin of probiotics from the human gastrointestinal tract intended for human consumption is not an essential criterion. Zielińska & Kolozyn-Krajewska [2018] showed that several microorganisms found in consumed food products did not originate from human hosts, including *e.g. B. animalis* ssp. *lactis* and *Saccharomyces cerevisiae* var. *boulardii*.

Determination of contents of total phenolics and total flavonoids

An increase in TPC (Table 2) after the fermentation process (8 h) from 0.093 to 0.226 mg GAE/g and from 0.130 to 0.398 mg GAE/g was observed in maize mashes with sucrose and caramel, respectively. Inoculation with LAB strains and storage for 21 days (6°C) resulted in an increase of TPC by about 10.1–33.7% in the samples with sucrose, and in a decrease by about 11.7–17.9% in caramel mashes except the product with *B. pseudolongum* K4/4 strain, where TPC did not change significantly ($p>0.05$). Lactic acid fermentation of the prepared samples resulted in significantly higher

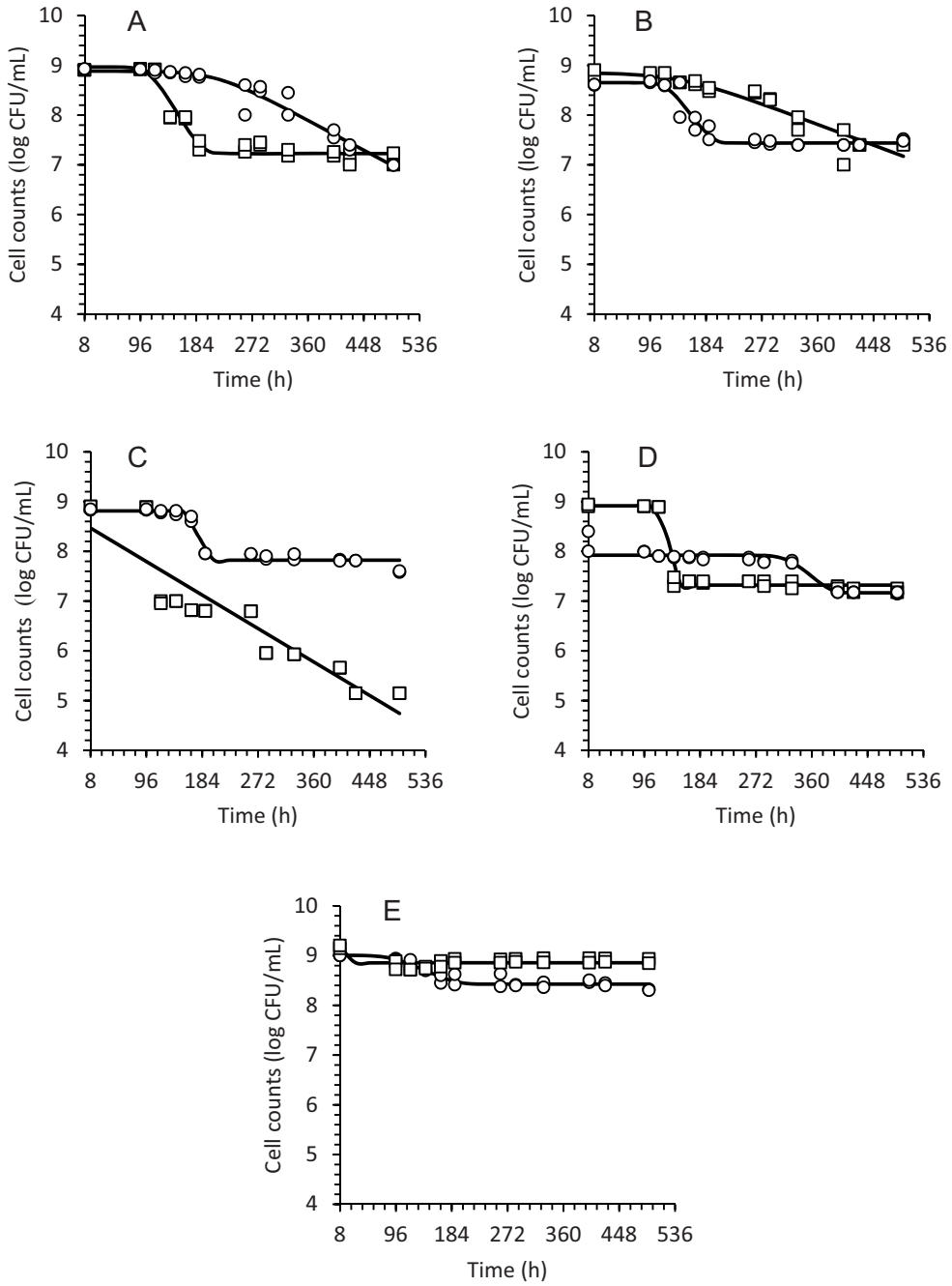

FIGURE 2. Survival of *B. thermophilum* DSM 20212 (A), *B. animalis* ssp. *animalis* J3II (B), *B. choerinum* K 1/1 (C), *B. pseudolongum* K 4/4 (D), and *L. plantarum* HM1 (E) strains during cold storage of fermented (8 h, Fresco DVS 1010) milk-based maize mashes with sucrose (□) and caramel (○).

levels of phenolic compounds (151.4–226.1%) in comparison to the non-fermented ones. Katina *et al*. [2007] observed an increase in TPC by about 17% in rye fermented by *L. plantarum* at 30°C (20 h) compared to the non-fermented sample.

Fermentation process also significantly ($p<0.05$) increased TFC by about 50.8 and 92.1% in the product with sucrose and caramel, respectively. In fermented and 21-day stored with LAB strain products, TFC was significantly higher compared to the non-fermented samples (17.6–136.7%).

Our results showed that even short fermentation time (8 h) was able to increase phenolic compounds levels. Several mechanisms of increasing phenolics content are reported. Microbial enzymes together with those derived from cereals (amylase, xylanase, and protease) may disrupt cell walls of grains resulting in the release of components bound in the cell structures. By this means, an increase is observed in the amount of phenolic compounds occurring in the bounded insoluble form of covalent bonds linked to the cell wall components [Katina *et al*., 2007; Hur *et al*., 2014]. Furthermore, *Bacillus subtilis* and *L. plantarum* strains showed a glucosidase activity [Wang *et al*., 2014], leading to the release of phenolic compounds from their soluble conjugated forms (bond to the carbohydrate components). Moreover, lactic acid produced by microbiota can help release bounded phenolics to their free forms. Increasing substrate acidity may have various effects on the stability of releasing phenolic compounds, and it can result in their dif-

TABLE 1. Parameters evaluating behaviour of lactic acid bacteria after they had been added to fermented (8 h, Fresco DVS 1010) milk-based maize mashes with sucrose and caramel and stored for 21 days.

Maize mash product	k_{d1} (log CFU/(mL×h))	N_{end1} (log CFU/mL)	k_{pH1} (1/h)	pH_{end1}	k_{d2} (log CFU/(mL×h))	N_{end2} (log CFU/mL)	k_{pH2} (1/h)	pH_{end2}
	Sucrose				Caramel			
Fresco + *L. plantarum* HM1	-0.183	8.9[a]	-0.440	4.37	-0.005	8.3[a]	-0.326	4.40
Fresco + *B. pseudolongum* K 4/4	-0.088	7.2[c]	-0.374	4.45	-0.011	7.2[d]	-0.409	4.50
Fresco + *B. animalis* subsp. *animalis* J3II	-0.004	7.4[b]	-0.401	4.46	-0.016	7.5[c]	-0.439	4.50
Fresco + *B. thermophilum* DSM 20212	-0.021	7.2[c]	-0.511	4.60	-0.007	6.9[e]	-0.424	4.48
Fresco + *B. choerinum* K 1/1	-0.008	5.1[d]	-0.381	4.44	-0.035	7.6[b]	-0.396	4.59

k_d – rate constant for decrease of counts, N_{end} – counts after storage period, k_{pH} – rate constant for decrease of pH, pH_{end} – pH value after storage period, [a-e] Means within a column with different superscript letters differ significantly ($p<0.05$).

TABLE 2. Content of total phenolics and total flavonoids in fermented products of milk-based maize mashes with sucrose and caramel.

Maize mash product	Storage	Total phenolics content (mg GAE/g)		Total flavonoids content (mg QE/g)	
		Sucrose	Caramel	Sucrose	Caramel
After sterilization (non-fermented)		0.093±0.001[e,y]	0.130±0.002[d,x]	0.396±0.012[f,x]	0.326±0.015[e,y]
8 h after fermentation (Fresco DVS 1010)	F	0.223±0.005[d,y]	0.398±0.008[a,x]	0.597±0.012[d,y]	0.627±0.006[d,x]
Fresco + *L. plantarum* HM1	21 D	0.303±0.008[a,y]	0.327±0.002[c,x]	0.697±0.003[b,y]	0.748±0.030[ab,x]
Fresco + *B. pseudolongum* K 4/4	21 D	0.249±0.006[c,y]	0.410±0.003[a,x]	0.577±0.013[d,y]	0.650±0.009[c,x]
Fresco + *B. animalis* ssp. *animalis* J3II	21 D	0.253±0.003[c,y]	0.345±0.011[c,b,x]	0.466±0.005[e,y]	0.723±0.017[b,x]
Fresco + *B. thermophilum* DSM 20212	21 D	0.295±0.00[4a,]y	0.351±0.010[b,x]	0.632±0.008[c,y]	0.773±0.019[a,x]
Fresco + *B. choerinum* K 1/1	21 D	0.265±0.003[b,y]	0.339±0.001[b,x]	0.818±0.018[a,x]	0.664±0.008[c,y]

F – fermented product; 21 D – fermented stored product after 21 days of storage; [a-e] Means within a column with different superscript letters differ significantly ($p<0.05$), [x-y] Means within a line with different superscript letters differ significantly ($p<0.05$).

ferent extractability. According to Schmidt *et al*. [2014], ferulic and gallic acid showed the most substantial increase during fermentation of rice bran with *Rhizopus oryzae*.

Determination of antioxidant activity

The antioxidant activity of maize mash products was evaluated as antiradical activity against DPPH radicals and as FRAP. These methods are widely used to evaluate the antioxidant capacity [Rufino *et al*., 2010]. The antioxidant activity of maize mashes determined by both methods increased within 8 h of fermentation (like TPC and TFC). Compared to the non-fermented product, the antiradical activity was higher about 80.5 and 53.7% for the samples with sucrose and caramel, respectively, FRAP increased about 80.2 and 107.4%, respectively (Table 3). After 21 days of storage, an increase was observed in DPPH radical scavenging activity and FRAP of maize mash with sucrose and *L. plantarum* HM1 (Table 3). Products containing sucrose and *Bifidobacterium* strains showed a lower antioxidant activity at the end of storage compared to the fermented product (8 h). DPPH$^{\bullet}$ scavenging activity of all products with caramel decreased during 21 days of storage. FRAP decreased only in products with *B. animalis* J3II and *B. pseudolongum* K 4/4. Different studies demonstrated that phenolics content and antioxidant activity firstly increased significantly to the certain time, and then increased slightly [Schmidt *et al*., 2014] or decreased [Salar *et al*., 2012] during fermentation. Fermentation of maize by *Thamnidium elegans* CCF-1456 at 25°C in a study of Salar *et al*. [2012] proved the maximum TPC content and the highest antiradical activity on the 5th day of incubation, with similar change tendency noted in the activity of carbohydrate hydrolysing enzymes (α-amylase, β-glucosidase, and xylanase) during fermentation. In cereal matrixes, antioxidant activity was highly correlated to the phenolics content [Mikulajová *et al*., 2007b, 2015]. Results of the present study also supported this finding, where moderate, high and/or very high correlations were found between TPC and results of DPPH assay (correlation coefficient (r)=0.628 and r=0.795 for products with sucrose and caramel, respectively), between TPC and FRAP (r=0.877 and r=0.918, respectively), as well as between TFC and FRAP (r=0.770 and r=0.889, respectively). This fact indicates that phenolic compounds are involved in the antioxidant properties of final products. These results are in agreement with our previous findings [Mikula-

TABLE 3. Antioxidant activity of fermented products of milk-based maize mashes with sucrose and caramel determined by DPPH and FRAP assays.

Maize mash product	Storage	DPPH scavenging activity (mg DPPH/g)		FRAP (mg Trolox/g)	
		Sucrose	Caramel	Sucrose	Caramel
After sterilization (non-fermented)		$0.525\pm0.010^{e,y}$	$0.569\pm0.003^{d,x}$	$0.172\pm0.002^{e,y}$	$0.215\pm0.004^{c,x}$
8 h after fermentation (Fresco DVS 1010)	F	$0.948\pm0.011^{b,x}$	$0.874\pm0.008^{a,y}$	$0.309\pm0.001^{b,y}$	$0.446\pm0.012^{a,x}$
Fresco + *L. plantarum* HM1	21 D	$0.983\pm0.012^{a,x}$	$0.670\pm0.021^{c,y}$	$0.344\pm0.007^{a,y}$	$0.424\pm0.017^{ab,x}$
Fresco + *B. pseudolongum* K 4/4	21 D	$0.778\pm0.021^{c,x}$	$0.764\pm0.024^{b,y}$	$0.297\pm0.007^{bc,y}$	$0.406\pm0.004^{b,x}$
Fresco + *B. animalis* ssp. *animalis* J3II	21 D	$0.775\pm0.010^{c,x}$	$0.664\pm0.018^{c,y}$	$0.260\pm0.009^{d,y}$	$0.407\pm0.006^{b,x}$
Fresco + *B. thermophilum* DSM 20212	21 D	$0.790\pm0.022^{c,x}$	$0.706\pm0.020^{bc,y}$	$0.284\pm0.003^{c,y}$	$0.423\pm0.008^{ab,x}$
Fresco + *B. choerinum* K 1/1	21 D	$0.602\pm0.015^{d,y}$	$0.664\pm0.012^{c,x}$	$0.301\pm0.005^{b,y}$	$0.446\pm0.002^{a,x}$

F – fermented product; 21 D – fermented stored product after 21 days of storage; $^{a-f}$ Means within a column with different superscript letters differ significantly ($p<0.05$), $^{x-y}$ Means within a line with different superscript letters differ significantly ($p<0.05$).

TABLE 4. Evaluation of sensory attributes of fermented products of milk-based maize mashes with caramel.

Maize mash product	Storage	Colour	Consistency	Texture	Overall acceptability
8 h of fermentation (Fresco DVS 1010)	F	3.7 ± 0.5^{a}	1.5 ± 0.5^{c}	1.3 ± 0.5^{c}	1.7 ± 0.5^{f}
Fresco + *L. plantarum* HM1	12 D	2.9 ± 0.5^{d}	2.0 ± 0.5^{b}	2.5 ± 0.5^{b}	2.9 ± 0.6^{bc}
	21 D	3.3 ± 0.4^{bc}	2.3 ± 0.4^{b}	2.8 ± 0.4^{b}	3.3 ± 0.4^{b}
Fresco + *B. pseudolongum* K 4/4	12 D	3.3 ± 0.6^{abd}	2.4 ± 0.5^{ab}	2.8 ± 0.7^{ab}	3.0 ± 0.6^{b}
	21 D	3.4 ± 0.5^{ab}	2.3 ± 0.4^{ab}	2.8 ± 0.7^{ab}	3.2 ± 0.4^{b}
Fresco + *B. animalis* ssp. *animalis* J3II	12 D	3.1 ± 0.5^{bd}	2.5 ± 0.5^{a}	2.9 ± 0.5^{ab}	3.1 ± 0.8^{abd}
	21 D	3.3 ± 0.5^{abd}	2.1 ± 0.3^{b}	2.5 ± 0.5^{b}	3.6 ± 0.5^{a}
Fresco + *B. thermophilum* DSM 20212	12 D	3.5 ± 0.5^{ab}	2.4 ± 0.5^{ab}	2.8 ± 0.4^{b}	2.8 ± 0.4^{cd}
	21 D	3.1 ± 0.4^{cd}	2.3 ± 0.7^{ab}	2.8 ± 0.4^{b}	3.1 ± 0.5^{bd}
Fresco + *B. choerinum* K 1/1	12 D	3.3 ± 0.8^{abd}	2.4 ± 0.5^{ab}	2.6 ± 0.5^{b}	2.4 ± 0.5^{e}
	21 D	3.4 ± 0.6^{ab}	2.4 ± 0.5^{ab}	3.3 ± 0.6^{a}	3.3 ± 0.7^{ab}

F – fermented product; 12 D – fermented stored product after 12 days; 21 D – fermented stored product after 21 days; 0 – worst possible value, 4 – best possible value; the results are means±standard deviation of ten evaluations, $^{a-f}$ Means within a column with different superscript letters differ significantly ($p<0.05$).

jová *et al*., 2007b, 2015], where correlations with r ranging from 0.794 to 0.984 were noted depending on types of cereals. In baked round rolls fermented with *Saccharomyces cerevisiae*, a very high correlation (r=0.987) was recorded compared to fermented milk maize products. This fact could be attributed to the interactions of raw material used, *e.g.* formation of complexes of phenolic compounds with milk components [Muniandy *et al*., 2016]. Within fermentation process, changes in component structure are observed with synthesis of new components, which differ in their features and availability for extraction and analytical determinations.

Sensory analysis

Detailed sensory evaluation of final products with caramel flavour was assessed in this section. Caramel flavour was added to make products more attractive and sensory acceptable to consumers, thus only caramel mashes were sensory analysed. Fresh fermented products and products stored for 12 and 21 days were evaluated, to determine the differences within the storage period.

According to the employed method of profile assessment, the following significant quality characteristics were determined for maize mash products with caramel: evaluation of colour, consistency, texture and overall acceptability (Table 4), as well as taste and aroma as individual descriptors (Figure 3). Scores given for colour to the milk-based maize mashes ranged from 2.9 to 3.7, indicating very good, almost excellent colour of the prepared products. In our previous study [Matejčeková *et al*., 2018], storage period did not significantly ($p>0.05$) affect the evaluation of colour, which was almost the same compared to the products right after single fermentation process. The attributes of taste and aroma were

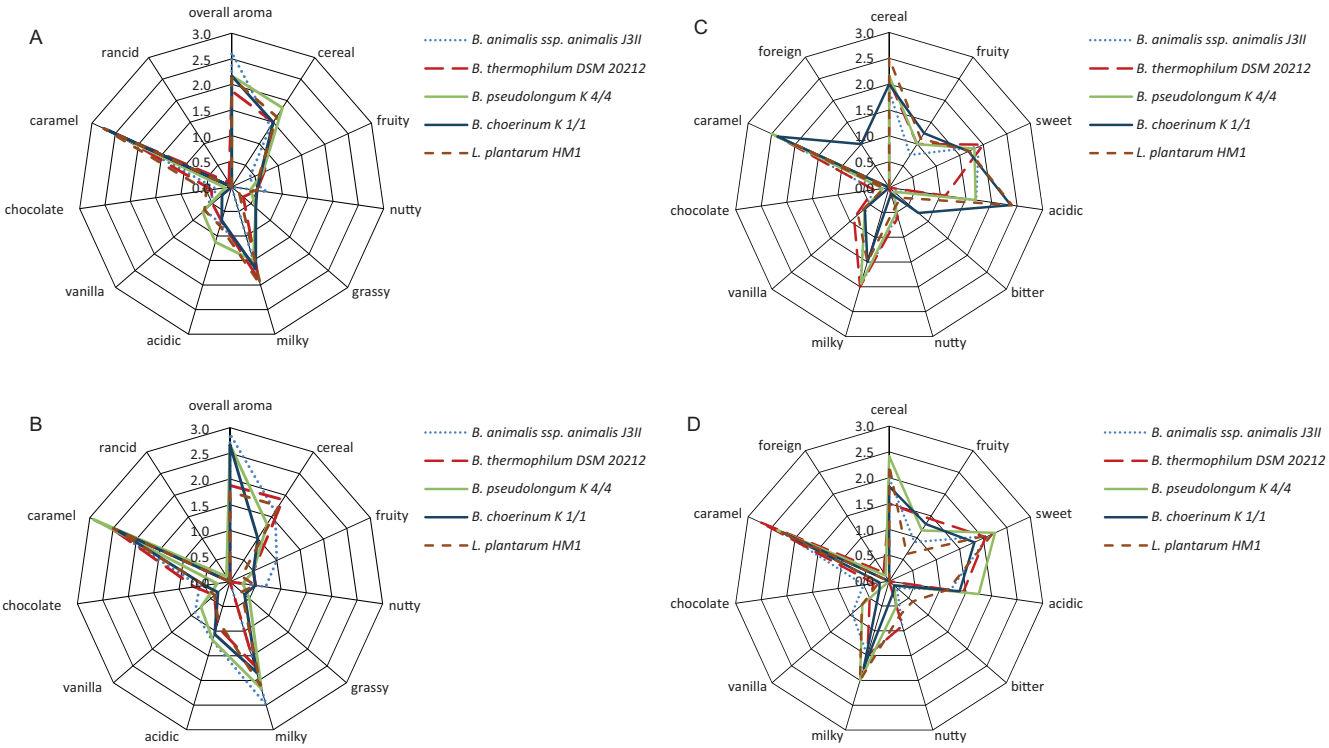

FIGURE 3. The qualitative sensory evaluation of milk-based maize mashes with caramel after fermentation (8 h, Fresco DVS 1010) and cold storage with lactic acid bacteria strains. A – aroma after 12 days of storage; B – aroma after 21 days of storage; C – taste after 12 days of storage; D – taste after 21 days of storage.

divided by the descriptors that could have positive and negative effects on the overall sensory value of final products. For better interpretation, the results are presented in spider web graphics (Figure 3). In the final flavoured products, the main purpose was to preserve the primary descriptors – caramel, cereal, and milky aroma/taste. In addition, the evaluation of unacceptable aroma/taste (*e.g.*, rancid, foreign), was an important step in the assessment. Several species or subspecies of LAB may provide different sensory and physicochemical characteristics to the products. The final metabolic compounds of some cultures, either pure or mixed, may provide unpleasant sensory characteristics. Moreover, bifidobacteria are able to produce acetic and lactic acids in a proportion of 3:2 during fermentation, hence, the growth and survival of these bacteria may enhance the acetic flavour of final products, which undermines their sensory acceptance [Cruz *et al.*, 2010]. In the prepared mashes, intensity of the overall aroma ranged from 1.8 to 2.9 (Figure 3), representing moderate to strong intensity. In the stored products, cereal aroma was evaluated as moderate, representing an average value of 2.3, while in the product right after the fermentation (8 h) it was evaluated as the strongest (2.9). The same results were reported in our previous research [Matejčeková *et al.*, 2018], when cereal aroma was evaluated as the strongest (2.3) in buckwheat lactose-free products after the fermentation (8 h). In our study, several assessors identified nutty, fruity, vanilla or chocolate aroma, but they were rated as with low intensity. Unacceptable foreign aroma (0.02) was also noted in the samples but with almost unnoticeable intensity. As for the evaluation of taste (Figure 3), the descriptor denoting cereal taste prevailed in the mashes with *L. plantarum* HM1 and *B. pseudolongum* K4/4 strains (average value of 2.5). In other products, cereal taste was scored from 1.5–2.2, indicating moderately strong cereal taste. Unacceptable bitter and foreign taste were evaluated as slight (0.2).

Final evaluation of the overall acceptability (Table 4) of fermented milk-based mashes with caramel was one of the most important steps pointing to the total sensory acceptability of the final products. Caramel mashes after 21 days of storage received higher scores (3.0–3.6), which indicated good acceptability when compared to the fermented product (1.7), thus the positive effect of storage period was noted. In our previous study, we had reported an increase of the overall acceptability over a storage period in buckwheat chocolate and caramel products [Matejčeková *et al.*, 2017]. On the other hand, Kocková & Valík [2014] noted a negative effect of 21-day storage period on the overall acceptability of buckwheat product with salt fermented by *L. rhamnosus* GG which decreased from 3.31 to 2.44 points.

CONCLUSION

Ensuring high quality and safety of fermented products requires a deep understanding of the fermentation process, as well as types and roles of microbes in final products characteristics. In the present study, we assessed the use of potentially probiotic microorganisms with emphasis on *L. plantarum* and *Bifidobacterium* spp. as live supplements in the prepared fermented (8h) milk-based maize mashes. *Bifidobacterium* spp. strains and *L. plantarum* survived adequately (>5 log CFU/mL) to confer potentially probiotic properties of the fermented and stored mashes. The high levels of LAB

and the production of organic acids did not cause undesirable changes in the overall acceptability of final caramel products. Our results showed that even short fermentation time (8 h) was able to increase levels of phenolic compounds and antiradical activity of fermented samples compared to the non-fermented product. Thus, if the right amount of cereal substrate and promoting synergy between selected LAB within storage is observed, good bioactive properties and probiotic potential are formulated. Therefore, inclusion of probiotic bacteria in fermented products enhances their value as therapeutic functional foods.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Instruction for Authors
Section: Editorial News

INSTRUCTIONS FOR AUTHORS

SUBMISSION. Original contributions relevant to food and nutrition sciences are accepted on the understanding that the material has not been, nor is being, considered for publication elsewhere. **All papers should be submitted and will be processed electronically via Editorial Manager system (available from PJFNS web site: http://journal.pan.olsztyn.pl). On submission, a corresponding author will be asked to provide: Cover letter; Files with Manuscripts, Tables, Figures/Photos; and Names of two potential reviewers (one from the author's homeland – but outside author's Institution, and the other from abroad).** All papers which have been qualified as relevant with the scope of our Journal are reviewed. All contributions, except the invited reviews are charged. Proofs will be sent to the corresponding author or to the first author and should be returned within one week since receipt. No new material may be inserted in the text at proof stage. It is the author's duty to proofread proofs for errors.

Authors should very carefully consider the preparation of papers to ensure that they communicate efficiently, because it permits the reader to gain the greatest return for the time invested in reading. Thus, we are more likely to accept those that are carefully designed and conform the instruction. Otherwise, papers will be rejected and removed from the on-line submission system.

SCOPE. The Polish Journal of Food and Nutrition Sciences publishes original, basic and applied papers, and reviews on fundamental and applied food research, preferably these based on a research hypothesis, in the following Sections:

Food Technology:
– Innovative technology of food development including biotechnological and microbiological aspects
– Effects of processing on food composition and nutritional value

Food Chemistry:
– Bioactive constituents of foods
– Chemistry relating to major and minor components of food
– Analytical methods

Food Quality and Functionality:
– Sensory methodologies
– Functional properties of food
– Food physics
– Quality, storage and safety of food

Nutritional Research Section:
– Nutritional studies relating to major and minor components of food (excluding works related to questionnaire surveys)

"News" section:
– Announcements of congresses
– Miscellanea

OUT OF THE SCOPE OF THE JOURNAL ARE:
– Works which do not have a substantial impact on food and nutrition sciences
– Works which are of only local significance i.e. concern indigenous foods, without wider applicability or exceptional nutritional or health related properties
– Works which comprise merely data collections, based on the use of routine analytical or bacteriological methods (i.e. standard methods, determination of mineral content or proximate analysis)
– Works concerning biological activities of foods but do not provide the chemical characteristics of compounds responsible for these properties
– Nutritional questionnaire surveys
– Works related to the characteristics of foods purchased at local markets
– Works related to food law
– Works emphasizing effects of farming / agricultural conditions / weather conditions on the quality of food constituents
– Works which address plants for non-food uses (i.e. plants exhibiting therapeutic and/or medicinal effects)

TYPES OF CONTRIBUTIONS. *Reviews:* (at least: 30 pages and 70 references) these are critical and conclusive accounts on trends in food and nutrition sciences; *Original papers:* (maximally: 30 pages and 40 references) these are reports of substantial research; *Reports on post and forthcoming scientific events, and letters to the Editor* (all up to three pages) are also invited (free of charge).

REVIEW PROCESS. All scientific contributions will be peer-reviewed on the criteria of originality and quality. Submitted manuscripts will be pre-evaluated by Editor-in-Chief and Statistical Editor (except for review articles), and when meeting PJFNS' scope and formal requirements, they will be sent to a section Editor who upon positive pre-evaluation will assign at least two reviewers from Advisory Board Members, reviewers suggested by the author or other experts in the field. Based on the reviews achieved, Section Editor and Editor-in-Chief will make a decision on whether a manuscript will be accepted for publication, sent back to the corresponding author for revision, or rejected. Once a manuscript is sent back to the corresponding author for revision, all points of the reviews should be answered or rebuttal should be provided in the Explanation letter. The revised manuscripts will be checked by Section Editor and by the original reviewers (if necessary), and a final decision will be made on acceptance or rejection by both Section Editor and Editor-in-Chief.

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A manuscript will not be published once the signed form has not been submitted to the Editor with the manuscript revised after positive reviews.

ETHICAL APPROVAL OF STUDIES AND INFORMED CONSENT. For all manuscripts reporting data from studies involving human participants or animals, formal approval by an appropriate institutional review board or ethics committee is required and should be described in the Methods section. For those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be followed. For investigations of humans, state in the Methods section the manner in which informed consent was obtained from the study participants (i.e., oral or written). Editors may request that authors provide documentation of the formal review and recommendation from the institutional review board or ethics committee responsible for oversight of the study.

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Every paper should be divided under the following headings in this order: a **Title** (possibly below 150 spaces), **Running title** (up to 50 spaces, submitted under the Title); the Names(s) of the author(s) in full. In paper with more than one author, the asterisk indicates the name of the author to whom correspondence and inquiries should be addressed, otherwise the first author is considered for the correspondence. Current full postal address of the indicated corresponding author or the first author must be given in a footnote on the title page; the Place(s) where the work was done including the institution name, city, country if not Poland. In papers originated from several institutions the names of the authors should be marked with respective superscripts; the **Key words** (up to 6 words or phrases) for the main topics of the paper; an **Abstract** (up to 250 words for regular papers and reviews and 100 words for Short Reports) summarizing briefly main results of the paper, no literature references; an **Introduction** giving essential background by saying why the research is important, how it relates to previous works and stating clearly the objectives at the end; **Materials and Methods** with sufficient experimental details permitting to repeat or extend the experiments. Literature references to the methods, sources of material, company names and location (city, country) for specific instruments must be given. Describe how the data were evaluated, including selection criteria used; **Results and Discussion** presented together (in one chapter). Results should be presented concisely and organized to supplement, but not repeat, data in tables and figures. Do not display the data in both tabular and graphic form. Use narrative form to present the data for which tables or figures are unnecessary. Discussion should cover the implications and consequences, not merely recapitulating the results, and it must be accomplished with concise **Conclusions**; **Acknowledgements** should be made to persons who do not fill the authorship criteria (see: Authorship forms); **Research funding** should include financial and material support; and **References** as shown below.

REFERENCES each must be listed alphabetically at the end of the paper (each should have an Arabic number in the list) in the form as follows: **Periodicals** – names and initials of all the authors, year of publication, title of the paper, journal title as in Chemical Abstracts, year of publication, volume, issue, inclusive page numbers; **Books** – names and initials of all the authors, names of editors, chapter title, year of publication, publishing company, place of publication, inclusive page numbers; **Patents** – the name of the application, the title, the country, patent number or application number, the year of publication.

For papers published in language other than English, manuscript title should be provided in English, whereas a note on the original language and English abstract should be given in parentheses at the end.

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Examples:

Article in a journal:

Słonimski, B.A., Campbell, L.D., Batista, E., Howard B. (2008). Gas chromatographic determination of indole glucosinolates. *Journal of Science and Food Agriculture*, *40*(5), 131–143.

Book:

Weber, W., Ashton, L., Milton, C. (2012). *Antioxidants – Friends or Foes?* 2nd edition. PBD Publishing, Birmingham, UK. pp. 218–223.

Chapter in a book:

Uden, C., Gambino, A., Lamar, K. (2016). Gas chromatography. In M. Queresi, W. Bolton (Eds.), *CRC Handbook of Chromatography*, CRC Press Inc., Boca Raton, Florida, USA, pp. 44–46.

ABBREVIATIONS AND UNITS. Abbreviations should only be used when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. Metric SI units should be used. The capital letter L should be used for liters. Avoid the use of percentages (% g/g,% w/w; Mol-%; vol-%), ppm, ppb. Instead, the expression such as g/kg, g/L, mg/kg, mg/mL should be used. A space must be left between a number and a symbol (e.g. 50 mL not 50mL). A small x must be used as multiplication sign between numeric values (e.g. 5×10^2 g/mL). Statistics and measurements should be given in figures, except when the number begins a sentence. Chemical formulae and solutions must specify the form used. Chemical abbreviations, unless they are internationally known, Greek symbols and unusual symbols for the first time should be defined by name. Common species names should be followed by the Latin at the first mention, with contracting it to a single letter or word for subsequent use.

FIGURES should be submitted in separate files. Each must have an Arabic number and a caption. Captions of all Figures should be provided on a separate page "Figure Captions". Figures should be comprehensible without reference to the text. Self-explanatory legend to all figures should be provided under the heading "Legends to figures"; all abbreviations appearing on figures should be explained in figure footnotes. Three-dimensional graphs should only be used to illustrate real 3-D relationships. Start the scale of axes and bars or columns at zero, do not interrupt them or omit missing data on them. Figures must be cited in Arabic numbers in the text.

TABLES should be submitted in separate files. They should be as few in number and as simple as possible (like figures, they are expensive and space consuming), and include only essential data with appropriate statistical values. Each must have an Arabic number and a caption. Captions of all Tables should be provided on a separate page "Table Captions". Tables should be self-explanatory; all abbreviations appearing in tables should be explained in table footnotes. Tables must be cited in Arabic numbers in the text.

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Ms. Joanna Molga
Editorial Office of Pol. J. Food Nutr. Sci.
Institute of Animal Reproduction and Food Research
Tuwima 10 Str., 10–748 Olsztyn 5, Poland
phone (48 89) 523–46–70, fax (48 89) 523–46–70;
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