ISSN (1230-0322)

2 0 1 9, Vol. 69, No. 1

Food

Polish Journal of Food and Nutrition Sciences

formerly Acta Alimentaria Polonica

Published

by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn

Published since 1957 as
Roczniki Chemii i Technologii Żywności and Acta Alimentaria Polonica (1975–1991)

SCOPE: The Journal covers fundamental and applied research in food area and nutrition sciences with a stress on interdisciplinary studies in the areas of food, nutrition and related subjects.

POLICY: Editors select submitted manuscripts in relation to their relevance to the scope. Referees are selected from the Advisory Board and from Polish and international scientific centres. Identity of referees is kept confidential.

AUTHORSHIP FORMS referring to Authorship Responsibility, Conflict of Interest and Financial Disclosure, Copyright Transfer and Acknowledgement, and Ethical Approval of Studies are required for all authors.

FREQUENCY: Quarterly – one volume in four issues (March, June, September, December).

COVERED by Web of Science, Current Contents/Agriculture, Biology & Environmental Sciences, Journal Citation Reports and Science Citation Index Expanded, BIOSIS (Biological Abstracts), SCOPUS, FSTA (formerly: Food Science and Technology Abstracts), CAS (Chemical Abstracts), AGRICOLA, AGRO-LIBREX data base, EBSCO, FOODLINE, Leatherhead FOOD RA data base FROSTI, AGRIS and Index Copernicus data bases, Polish Scientific Journals Contents (PSJC) Life Science data base available at http://psjc.icm.edu.pl and any www browser; ProQuest: The Summon, Bacteriology Abstracts, Immunology Abstracts.

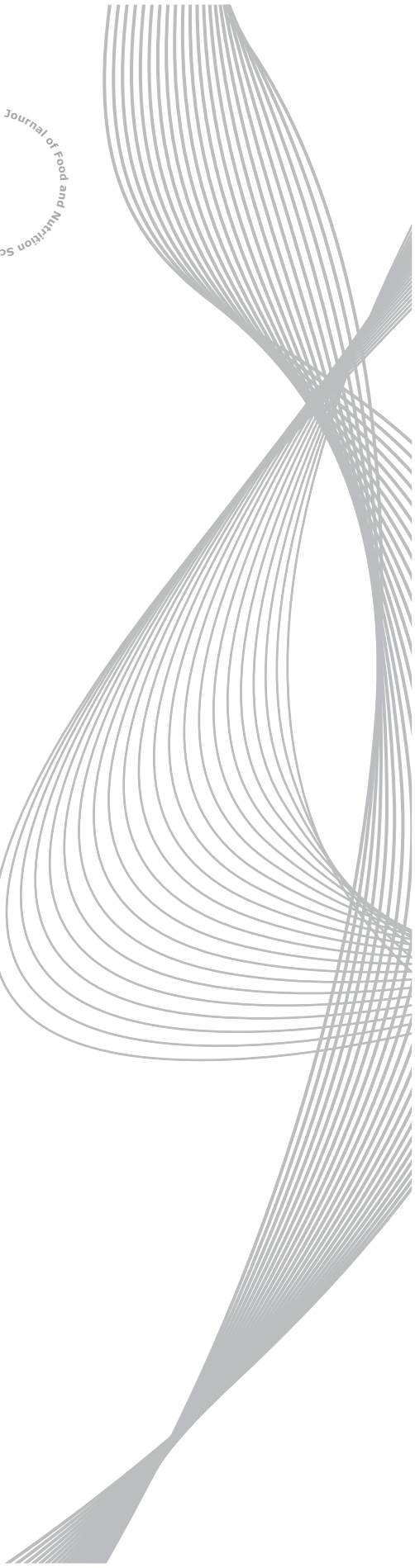
EDITORIAL AND BUSINESS CORRESPONDENCE: Submit contributions (see Instructions to Authors) and address all communications regarding subscriptions, changes of address, etc. to:

CORRESPONDENCE TO: Ms. Joanna Molga
Polish Journal of Food and Nutrition Sciences
Institute of Animal Reproduction and Food Research
of Polish Academy of Sciences
ul. Tuwima 10, 10-747 Olsztyn, Poland
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

ISSN (1230–0322)
2 0 1 9, Vol. 69, No. 1

Polish Journal of Food and Nutrition Sciences

formerly Acta Alimentaria Polonica



Published

by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn

Pol. J. Food Nutr. Sci. 2019, Vol. 69, No. 1

Advisory Board of PJFNS 2019–2022

Pol. J. Food Nutr. Sci. 2019, Vol. 69, No. 1

POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
COVERED BY CURRENT CONTENTS/AGRICULTURE, BIOLOGY & ENVIRONMENTAL SCIENCES, JOURNAL CITATION REPORTS AND SCIENCE CITATION INDEX EXPANDED, BIOSIS, SCOPUS, FSTA (FORMERLY FOOD SCIENCE AND TECHNOLOGY ABSTRACTS) AND CAS ABSTRACTS, AND AGRO-LIBREX, FROSTI, PSJC, AGRIS AND INDEX COPERNICUS DATA BASES
2019, VOL. 69, NO. 1

Subscription

2019 – One volume, four issues per volume. Annual subscription rates are: Poland 150 PLN, all other countries 80 EUR. Prices are subject to exchange rate fluctuation. Subscription payments should be made by direct bank transfer to Bank Gospodarki Żywnościowej, Olsztyn, Poland, account No 17203000451110000000452110 SWIFT code: GOPZPLWOLA with corresponding banks preferably. Subscription and advertising offices at the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, ul. J. Tuwima 10, 10-747 Olsztyn, Poland, tel./fax (48 89) 5234670, fax (48 89) 5240124, e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

Zamówienia prenumeraty: Joanna Molga (e-mail: pjfns@pan.olsztyn.pl)

Wersja pierwotna (referencyjna) kwartalnika PJFNS: wersja papierowa (ISSN 1230–0322)
Nakład: 100 egz.; Ark. wyd. 15,5; Ark. druk. 15,5
Skład i druk: Mercurius, www.mercurius.com.pl

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 5–21
DOI: 10.31883/pjfns-2019-0005
http://journal.pan.olsztyn.pl

Review article
Section: Food Technology

Technological and Nutritional Challenges, and Novelty in Gluten-Free Breadmaking: a Review

*Paola Conte[1], Costantino Fadda[1], Natalia Drabińska[2], Urszula Krupa-Kozak[2]**

[1]Dipartimento di Agraria, Università degli Studi di Sassari, Sassari, Italy; pconte@uniss.it;
[2]Department of Chemistry and Biodynamics of Food, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland

Key words: gluten-free breadmaking, gluten-related disorders, texture characteristics, nutritional value, sensory properties, review

The presence of gluten is considered fundamental for successful breadmaking. However, the ingestion of gluten by susceptible individuals has been associated with the development of gluten-related disorders such as celiac disease, wheat allergy, and non-celiac gluten sensitivity. The elimination of gluten from cereal-based baked products has a detrimental effect on the breadmaking process and sensory properties, and raises technological challenges in terms of making good quality leavened bread. The use of non-gluten raw materials changes the rheological behaviour of the gluten-free dough, which may result in different processing performance and associated post-baking quality of the obtained bread. Gluten-free bread tends to have a poor visual texture characteristics, a low nutritional value, reduced mouthfeel and flavour, as well as a shorter shelf-life. The aim of this review is to present the main problems related to gluten-free breadmaking technology and to summarise recent findings in the improvement of the technological, nutritional, and sensory properties of gluten-free bread. A great deal of this review focuses on the development of novel and healthy gluten-free breads formulated with flours, starches, hydrocolloids, and alternative nutrient-dense raw materials, which should fulfil all quality requirements for bakery products as well as meet the needs of celiac consumers.

INTRODUCTION

Bread is considered a staple food worldwide [Simićet *et al.,* 2018; Wandersleben *et al.*, 2018]. Gluten proteins or, specifically, the gliadin fraction of wheat and the prolamins from other cereals such as barley (hordeins) and rye (secalins) are responsible for the viscoelastic properties of the dough and the bread-baking unique asset [Peña-Bautista *et al.*, 2017]. However, for individuals who suffer from celiac disease (CD) or other gluten-related disorders (such as wheat allergy (WA) and non-celiac gluten sensitivity (NCGS)) even the ingestion of a small amount of gluten can lead to deleterious and serious health risks [Scherf *et al.,* 2016; Bathrellou *et al.*, 2018], which are graphically depicted in Figure 1. A strict and life-long gluten-free diet (GFD) is the only available therapy for patients suffering from CD, and research results showed improvements in both symptomatology and small bowel histology [Haines *et al.*, 2008]. Patients with NCGS and WA also benefit from a GFD, although individuals with WA do not need to restrict rye, barley, and oats from their diet [Pietzak, 2012].

In reality, a complete avoidance of gluten intake is very difficult, due to gluten ubiquity in human foods, hidden gluten sources, and food contamination. Due to the on-going need for gluten-free products, the gluten-free market is growing fast. While a strict adherence to GFD may lead to nutritional deficiencies in the longer term, the absence of gluten has a detrimental effect on breadmaking process and raises technological challenges in making good quality leavened bread [Horstmann *et al.,* 2018]. In fact, the technological and sensory characteristics of gluten-free bread (GFB) are worse than those of conventional gluten-containing bread [Primo-Martín *et al.*, 2006]. In general, gluten-free baked products are characterized by a crumbling texture, pale crust colour as well as a faster staling rate [Gallagher *et al.,* 2003]. Considerable efforts have been made to improve GFB quality: nutrient-dense ingredients, additives with gluten-imitating function and diverse technologies have been studied in the hopes of narrowing the gap between gluten-free and gluten-containing bread [Capriles & Arêas, 2014; Drabińska *et al.,* 2016]. The aim of this review is to provide recent findings in improving the technological, nutritional and sensory properties of GFB.

CONVENTIONAL *VS.* GLUTEN-FREE BREADMAKING

Conventional and gluten-free breadmaking are processes that differ substantially in terms of the complexity of formulations used (including main ingredients and amount of water), dough rheological behaviour, and overall quality of the final product [Conte *et al.,* 2016; Morreale *et al.,* 2018a]. In traditional breadmaking, the term "bread" usually refers to a yeast-leavened product or sourdough bread specifically made with wheat flour. The reason why bread is usually made with wheat flour derives from the unique properties of the in-

* Corresponding Author: Tel.: +48 89 523 46 18; Fax: +48 89 523 46 01; E-mail: u.krupa-kozak@pan.olsztyn.pl (U. Krupa-Kozak)

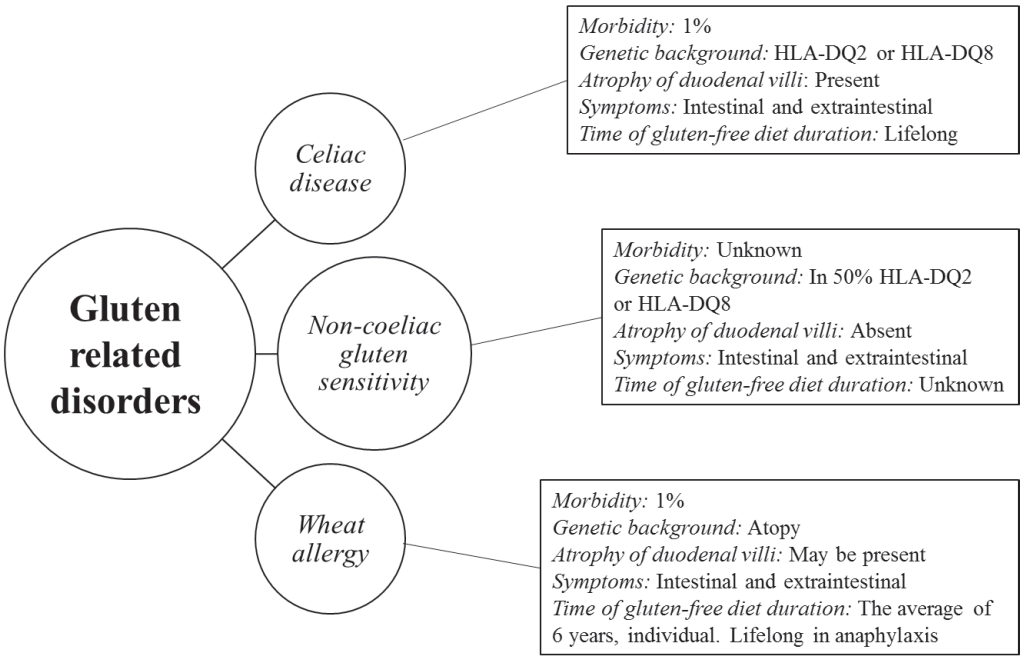


FIGURE 1. Short characteristics of gluten-related disorders.

soluble fraction of wheat protein (gliadin and glutenin) to form, when flour is hydrated and in the presence of mechanical work input, a cohesive viscoelastic mass called a gluten network. Thus, in wheat bread, gluten is considered the main structure-forming complex with an exceptional ability to form a tenacious, extensible, and elastic dough capable of entrapping gas during both proofing and the early stage of baking as well as enclosing starch granules and fibre fragments [Scanlon & Zghal, 2001; Gallagher *et al.*, 2004]. These characteristics of the dough lead to a final bread with a good loaf volume and a typical crumb structure that results in a sponge-like texture, which is highly desirable in the leavened baked goods.

In gluten-free breadmaking, in which the elimination of gluten becomes a necessity, the absence of this continuous three-dimensional protein-starch matrix significantly affects the rheology of the doughs, the production process, and the overall quality of the final breads [Ronda *et al.,* 2017]. Gluten-free doughs, which cannot develop a network similar to traditional breads due to differences in their protein properties, are less elastic and cohesive, and stickier and more difficult to handle than their gluten-containing counterparts [Ronda *et al.*, 2017]. In order to obtain doughs with acceptable consistency and better behaviour during the mixing phase, gluten-free flours/starches require higher amounts of water than wheat flour does. Thus, gluten-free doughs, which have a viscosity more like that of cake batters, are often called batters instead of doughs. The final products, in turn, tend to show some quality defects such as lower specific volume; lighter crumb and crust colour; rough, dry and crumbly texture; and a shorter shelf life [Gallagher *et al.*, 2004; Houben *et al.*, 2012; Jnawali *et al.*, 2016]. Therefore, the production of GFBs, unlike their wheat-containing counterparts, requires different technological solutions. The development of GFB has generally involved the use of complex formulations consisting of a combination of different ingredients and additives able to imitate the viscoelastic properties of gluten and, consequently, the appearance, quality, and sensory properties of the bread-like products. To this end, due to their negligible structure-building capability, the most common flours and/or starches from different origins (such as rice, corn, potato, and cassava) usually included in GFB formulations, were often combined with binding agents, such as hydrocolloids and protein [Capriles & Arêas, 2014]. Certainly, the most suitable basic ingredient for GFB preparation is rice (*Oryza sativa*) flour. This is probably due to its neutral flavour, white colour, hypoallergenic properties, high amount of easily digested carbohydrates, and its low sodium content [Rosell & Marco, 2008]. However, the use of rice flour in gluten-free breadmaking is also associated with some technological disadvantages. In fact, due to the poor functional properties of its proteins and the low level of prolamins, it is unable to form viscoelastic doughs required to retain the carbon dioxide produced during proofing, leading to a product with low specific volume and a compact crumb [Rosell & Marco, 2008]. However, despite some discrepancies reported in the literature, it seems that not all varieties of rice and their related amylose contents influence the quality of GFB in the same way [Cornejo & Rosell, 2015]. As reported by Kadan *et al.* [2001], the partial replacement (10%) of a long-grain rice variety with a short grain rice resulted in breads with softer crumb and slower retrogradation than those produced with 100% long-grain rice. de la Hera *et al.* [2013] obtained short-grain GFB with higher specific volume and lower hardness values than those of breads made of long rice varieties. On the contrary, Cornejo & Rosell [2015] analysing the breadmaking potential of six long-grain rice varieties, obtained GFBs with characteristics similar to those previously reported for breads made of short-grain varieties. They concluded that the breadmaking properties of rice flour might be more affected by the synergic effect of other factors (particle size,

protein conformation, starch structure) than by the length of the rice grain. Apart from rice flour, corn meal (*Zea mays*) is the second basic ingredient most often used in gluten-free products. In particular, the yellow corn varieties, due to their characteristic yellow colour and distinctive flavour, are more suitable to produce pasta, whereas, the white maize varieties are the flour/starch sources most often used in gluten-free breadmaking [Hager *et al.*, 2012].

Not only starch-containing flours, but also starches from different origins are the main players in providing structure and texture of the manufactured breads [Jnawali *et al.*, 2016]. During the bread baking process, starch is able to bind water and form a gas-permeable structure influencing, in turn, water retention and dough rheology [Houben *et al.*, 2012; Witczak *et al.,* 2016]. In fact, starch granules gelatinize (trough granules swelling and partial solubilisation of starch) and form a starch paste able to trap air bubbles [Zannini *et al.*, 2012]. As a result, the addition of starch in GFB formulae (either as a single starch or a composite starch mixture) leads to a product with a higher volume, and a softer, more cohesive and compact crumb structure [Gómez & Sciarini, 2015]. However, it is worth highlighting that not all kinds of starches behave in the same way, as their functional characteristics depend on their origin, particle size, amylose/amylopectin content, water swelling and solubility, pasting and gelling, starch treatment, and interaction with other ingredients [Witczak *et al.,* 2016; Zhang *et al.,* 2017]. However, during food processing, despite the numerous advantages of starch, its native forms show a limited resistance to physical conditions, such as high tendency to retrogradation and syneresis, loss of viscosity (especially at low pH conditions), low thermal stability, and inappropriate rheological characteristics of pastes and gels. For this reason, modifications of starches by chemical reactions or physical methods have been proposed to overcome some of these shortcomings [Witczak *et al.*, 2012; Yousif *et al.,* 2012]. Korus *et al.* [2009] observed that the partial replacement of corn starch with increasing percentages of tapioca and corn resistant starch preparations led to gluten-free doughs with increased elastic behaviour (increase of both storage and loss moduli, and $G' > G''$) and rheological properties typical of a week gel ($\tan \delta > 0.1$). The resulting GFBs showed diminished crumb hardness values with the increasing amounts of resistant starch preparations applied. These authors also found that the addition of resistant starch raised total dietary fibre, by up to 89%, as compared to the control samples.

The relatively poor functional properties of the above-mentioned basic ingredients, as well as the necessity to mimic the functionalities of gluten, and the inclusion of other polymeric substances such as hydrocolloids and non-gluten protein, are critical factors in a starch-based system like GFB. Hydrocolloids, also known as food gums, are substances consisting of hydrophilic, long-chain, high molecular weight molecules, including different polysaccharides but also some proteins, such as gelatin [Hoefler, 2004]. When they are incorporated in a water-based system, they exhibit a range of functions, including the primary gelling and thickening, but also emulsifying and encapsulating ones [Hoefler, 2004]. Moreover, the hydrocolloids classified as soluble fibres serve important physiological functions [Matos & Rosell, 2015]. The addition of hydrocolloids to GFB formulae could have several effects on both intermediate and end products. (a) At the dough level, due to their ability to act as water binders, they raise the viscosity of the system, improve the viscoelastic properties of the dough and increase, in turn, its gas-holding capacity. Furthermore, they affect swelling and gelatinization of the starch granules. (b) At the bread level, due to their ability to reduce moisture loss, they slow down starch retrogradation and extend the shelf life of the products, preserving their overall quality over time. Additionally, they influence other bread quality attributes, such as specific volume, crumb structure, texture, and sensory properties [Lazaridou *et al.,* 2007; Jnawali *et al.*, 2016]. Different types of hydrocolloids of both natural (agar-agar, carrageen, pectin and β-glucan, gum arabic, locust bean gum, guar gum and psyllium fibre) and synthetic origin (synthesized cellulose derivatives: hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC), and methyl cellulose (MC); microbial biosynthetic: xanthan gum) have been proposed as gluten replacers in GFBs [Lazaridou *et al.,* 2007; Schober *et al.*, 2008; Demirkesen *et al.*, 2014; Mohammadi *et al.*, 2014; Naji-Tabasi & Mohebbi, 2014]. However, not all kinds of hydrocolloids behave in the same way. In fact, their interaction with other food polymers, such as starch and protein included in the formulation, the specific hydrocolloid used and its supplementation level (usually up to 2%), as well as the parameter of the process, could change the type and extent of influence exerted on both dough and bread properties [Lazaridou *et al.,* 2007; Rosell & Marco, 2008; Capriles & Arêas, 2014; Jnawali *et al.*, 2016].

GLUTEN-FREE BATTER/DOUGH CHARACTERISTICS

The quality of GFBs is greatly influenced by the selection and combination of structural ingredients (mainly polysaccharides) that are able to provide stability to the system (by increasing viscosity), as well as prevent an excessive weakening of the protein/starch/hydrocolloids coherent matrix [Scanlon & Zghal, 2001]. For this reason, in the gluten-free breadmaking process, the development of processable doughs able to stretch in response to the expansion of gases released during fermentation, as well as the formation of dough films able to stretch without rupturing and with sufficient strength to prevent the collapse of the structure, are crucial prerequisites to obtain high quality yeast-leavened products [Singh & MacRitchie, 2001; Mir *et al.,* 2016]. From a rheological point of view, due to the large amount of water usually added in the complex recipes and also the absence of gluten, gluten-free doughs resemble a semiliquid system which greatly varies in terms of consistency, viscoelasticity, and structural networking [Gallagher *et al.,* 2003; Hager *et al.,* 2012]. Findings from selected recent research related to the rheological properties of dough and bread are summarized in Table 1. Różyło *et al.* [2015] demonstrated that, depending on the type of flour used (corn, rice, and buckwheat flour), the addition of different amounts of water (from 80% to 120%) to the dough can affect the quality parameters (such as specific volume and crumb hardness) of different types of GFBs. In particu-

TABLE 1. Different types of flour/starch and amount of water used in gluten-free breadmaking.

Basic ingredients	Amount of water	Dough properties	Bread properties	References
Corn flour Corn starch Potato starch Rice flour Buckwheat flour	90%, 102%	Dough yield Rheological properties (Rheometer) Leavening properties (Rheofermentometer)	Baking yield Specific volume Sensory evaluation	[Pruska-Kędzior *et al.,* 2008]
Rice flour Oat flour Quinoa flour Sorghum flour Teff flour Buckwheat flour	120% 95% 90% 90% 90% 85%	Microstructural properties Leavening properties (Rheofermentometer)	Bake loss Specific volume Crumb texture Crumb structure Shelf-life Sensory evaluation	[Hager *et al.,* 2012]
Short-grain rice flour Long-grain rice flour	80% 110%	Microstructural properties Leavening properties (Rheofermentometer)	Weight loss Specific volume Crumb texture	[de la Hera *et al.,* 2013]
Corn starch Rice starch Rice flour	44.4%, 44.7%, and 52.7%	Mixing properties (Farinograph, Water absorption) Leavening properties (Rheofermentometer, Image anlaysis)	Specific volume Crumb structure Colour	[Cappa *et al.,* 2013]
Rice flour Corn flour Corn starch Quinoa flour	80%		Specific volume Crumb texture Sensory evaluation	[Elgeti *et al.,* 2014]
Rice flour	150%	Rheological properties (Rheometer)	Specific volume Hardness Sensory evaluation	[Demirkesen *et al.,* 2014]
Rice flour	70–110%		Weight loss Specific volume Crumb texture Starch digestibility and glycaemic index	[de la Hera *et al.,* 2014]
Corn flour Rice flour Buckwheat flour	80–120%		Specific volume Hardness Crumb structure Sensory evaluation	[Różyło *et al.,* 2015]
Rice flour	90–110%	Rheological properties (Rheometer)	Specific volume Hardness	[Mancebo *et al.,* 2015]
Rice flour	78–141%	Dough extrusion (Texture Analyzer) Rheological properties (Rheometer)	Weight loss Crumb texture Crumb structure Colour Shelf-life	[Ronda *et al.,* 2015]
Long grain rice flour	110 %		Specific volume Crumb texture Crumb colour	[Cornejo & Rosell, 2015]
Rice flour Potato starch Quinoa flour Buckwheat flour	87%	Rheological properties (Rheometer)	Bake loss Crumb texture Crumb microstructural properties Colour Sensory evaluation	[Turkut *et al.,* 2016]
Maize flour Rice flour Maize starch Wheat starch Potato starch	100%	Microstructural properties Rheological properties (Rheometer)	Crumb microstructural properties Specific volume Crumb texture	[Martínez & Gómez, 2017]
White rice flour	90–110%	Rheological properties (Mixolab®)	Specific volume Crumb texture Crumb structure Colour	[Morreale *et al.,* 2018b]

lar, they observed that GFBs made with corn flour required the largest amount of water (120%), while, those made with rice flour required the lowest (80%). The importance of hydration levels in determining the viscoelastic behaviour of gluten-free dough/batter and influencing the rheological characteristics of the resulting bread was also recently confirmed by Morreale *et al.* [2018b]. In the same study, besides the dough hydration levels, the authors underlined the role

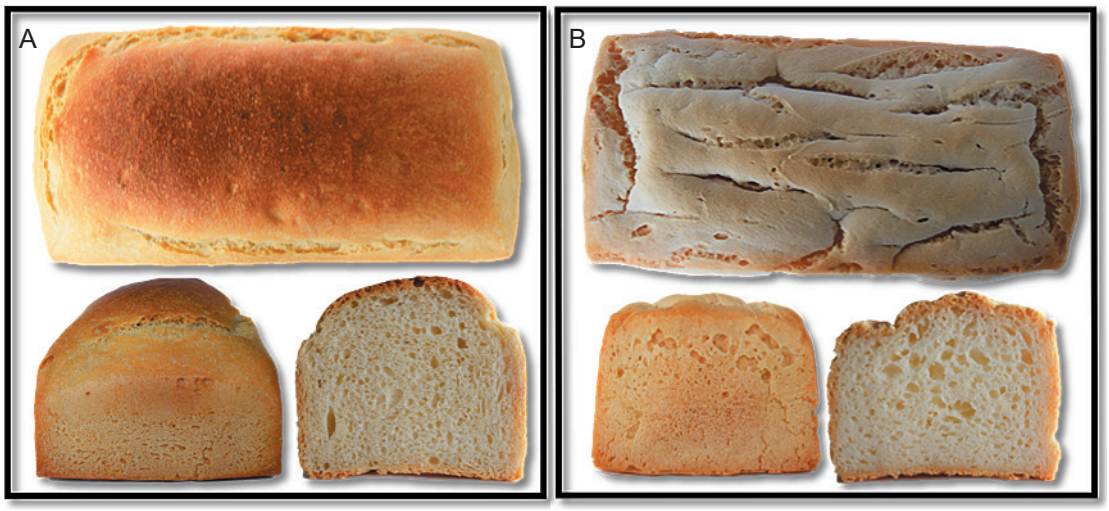


FIGURE 2. Visual appearance of gluten-containing (A) and gluten-free bread (B).

of viscosity (100, 4000, and 15,000 mPa×s) and levels of inclusion (1, 2, 3%) of the hydrocolloid HPMC to improve both batter consistency and some technological parameters of the final products (crumb hardness, cohesiveness, and resilience). The feasibility of improving the rheological properties of gluten-free doughs through the use of different types of hydrocolloids and amounts of water was also explored by many other authors [Cappa *et al.,* 2013; Mancebo *et al.,* 2015; Ronda *et al.,* 2015]. As reported by Pruska-Kędzior *et al.* [2008], the distinct rheological behaviour of gluten-free doughs may also be due to differences in the endogenous protein content and the fractional compositions of the basic ingredients included in the formulation (maize starch and flour, potato starch, rice flour and buckwheat flour). According to the authors, a complex approach involving rheological and fermentographic methods in future studies on gluten-free dough technology could be useful in the development of GFBs with improved sensory profile and prolonged shelf-life. However, although a number of studies have been conducted to characterize the dough/batter rheological properties using both fundamental and empirical analyses, finding reliable predictors able to correlate gluten-free dough rheological properties with the quality of the resulting breads, still remains an important task [Matos & Rosell, 2015]. Matos & Rosell [2013], in an attempt to determine such quality indicators at dough levels, tested seven different gluten-free complex formulations (one based on corn starch, and the other six on rice flour) and evaluated the rheological properties of the dough and the technological and sensory characteristics of the resulting breads. They reported that dough Mixolab parameters showed high correlation coefficients with the physical quality of fresh breads, but relatively low correlations with their sensory characteristics. In particular, a strong relationship was observed between bread crumb hardness and dough consistency during mixing and after cooling. Martínez & Gómez [2017], using the most common types of flours (rice and maize) and starches (maize, wheat and potato) usually included in gluten-free formulae, studied the mechanistic relations among the development of the starch/flour structure, dough rheology, and bread quality. These authors reported that the structure and morphology of the starch granules and flour particles were the major determinants of dough changes produced during the fermentation and baking phases. The large and compact particles of the flours produced dough with high consistency and breads with volume and textural properties lower than those obtained with starches. In particular, among the starch-based formulations, the small wheat starch granules, which also exhibited the lowest pasting temperature, formed a continuous and uniform starch-hydrocolloid matrix, leading to doughs with a lower consistency but higher holding capacity and, consequently, breads with higher specific volume and better textural properties.

QUALITY OF GLUTEN-FREE BREAD

Gluten-free bread appearance, texture, and shelf-life

Despite the considerable advances made in improving gluten-free bakery products, some major problems related to their technological and sensory quality must to be overcome. Due to the need of using non-gluten raw materials, the changes observed in the technological properties of gluten-free doughs may result in different processing performance and associated post-baking quality defects of the resulting breads, which tend to have unattractive appearance, in particular pale crust colour with irregular surface, poor mouthfeel and flavour, and a shorter shelf-life [Houben *et al.*, 2012; Jnawali *et al.*, 2016; Conte *et al.,* 2018]. Visual texture is defined as the appearance of the product, ranging from the crust colour to the loaf volume to the crumb grain structure of each single slice of bread [Wang *et al.,* 2013]. The colour of both crust and crumb is known to be one of the most important visual characteristics, which strongly influence consumer choice [Gallagher *et al.*, 2003; Conte *et al.,* 2018]. GFBs, unlike their wheat-containing counterparts, show unappealing and often too white coloration (Figure 2). It may be due to the natural colour of the conventional raw materials included in the formulations [Gallagher *et al.,* 2003; Różyło *et al.,* 2015]. Furthermore, a small amount of proteins and high water contents hamper the browning reaction, which is the main responsible for the formation of brown pigments [Mohammadi *et al.,* 2014].

The specific volume is one of the most important technological parameters of bread quality. However, due to the multiplicity of bread varieties, each of which has its own particular characteristics, the specific volume cannot be considered a quality factor itself. For instance, in the case of breads baked in pans, high values of specific volume, being usually associated with a proper aeration of the bread loaves, are required to obtain products able to satisfy the widest cross-section of consumers. A suitable gas bubble entrapment and a subsequent stabilisation of the foam structure are also fundamental to achieve a fine alveolate structure, in which the resulting pores should be small, regular and spread evenly across the crumb [Elgeti *et al.*, 2014]. It is during baking that the further growth of the gas bubbles previously incorporated during mixing determines dough expansion and, consequently, the final volume and appearance of breads [Scanlon & Zghal, 2001]. Naji-Tabasi & Mohebbi [2015] reported that in formulations based on rice flour, corn flour, and corn starch, the addition of cress seed gum and xanthan gum as gluten substitutes led to breads with higher specific volumes and improved cell area fraction. They also reported that hydrocolloids, by forming thick layer, influenced the stability of the gas cells, leading to more regular and solid pores, especially in breads containing cress seed gum. Furthermore, hydrocolloids (by limiting the diffusion of water) had a positive effect on crumb colour during storage.

Apart from visual texture defects, GFBs tend to have high crumb hardness, low cohesiveness and elasticity and, as a consequence, high brittleness with a pronounced tendency to fracture or crumble [Gallagher *et al.,* 2003]. It is well established that such a mechanical behaviour of GFBs, which is usually described by the above-mentioned physical texture parameters, is strongly influenced by product density and porosity. This implies that, in dough processing, all the aspects that change bread volume and cell structure are also major determinants of the bread texture. Demirkesen *et al.* [2014] studied the effects of the addition of different gums (xanthan, guar, locust bean, agar, MC, CMC, and HPMC) and gum blends (xanthan-guar and xanthan-locust bean) on crumb structure and texture parameters of rice-based GFBs. The authors found that the use of both gum blends and single xanthan, CMC, and HPMC led to breads with homogenous and finer crumb structure in terms of lower porosity and average area of pores, and higher number of pores. They also reported that the hardness, cohesiveness and springiness values were correlated with the internal structure of the crumb. In particular, it was observed that GFBs with softer, more cohesive and elastic crumb, also showed the lower values of porosity, lower average size of pores and higher number of pores. Similarly, de la Hera *et al.* [2014], studying the impact of dough hydration levels (70, 90, and 110%) and particle size distribution (fine and coarse) on the quality of rice-based GFBs, found a strong correlation ($r=-0.8931$; $P<0.001$) between hardness values and specific volume. In spite of a slightly detrimental effect in the nutritional aspect, better results were obtained with coarse flour and large amounts of water.

Another major disadvantage normally associated with GFBs is the impossibility to keep breads fresh for a longer time. In fact, during storage, baked products undergo a number of physicochemical changes, ranging from the crumb hardening to the loss of crust crispiness and organoleptic freshness to the gradual decrease in consumer acceptance [Fadda *et al.,* 2014]. These alterations are commonly referred to as staling, which is a complex, but not-well understood process that has been associated primarily with moisture redistribution, starch retrogradation, polymers reorganization, and starch-protein interactions [Fadda *et al.,* 2014]. If, on the one hand, the transfer of moisture from the internal to the external region of the loaf is commonly recognized as mainly responsible for the staling of the crust, on the other hand, staling of the crumb is a more complex phenomenon that involves multiple factors [Gray & Bemiller, 2003]. Certainly, the most widely accepted mechanism involved in the firming of the crumb is the recrystallization of amylopectin. However, also the moisture content and its redistribution among the different constituents of the crumb seems to play a critical role [Gray & Bemiller, 2003; Ronda *et al.,* 2011]. It should be noted that most GFBs, which are often based on pure starches and require an extra amount of water, are more prone to stale than their gluten-containing counterparts. In fact, owing to the absence of gluten, the transfer of moisture may increase, leading to a product with softer crust and firmer crumb [Gallagher *et al.,* 2003; Sciarini *et al.,* 2010]. In an attempt to delay the staling of GFBs, several authors reported different promising strategies, including: (a) the use of enzymes such as α-amylase and cyclodextrin glycosyltransferase, which are able to partially degrade amylopectin, thus hindering its recrystallization, as well as to modify both protein-starch and protein-protein interactions, respectively [Gujral *et al.,* 2003; Haghighat-Kharazi *et al.,* 2018]; (b) the use of some hydrocolloids such as xanthan, CMC, and HPMC, which are able to decrease the loss of moisture content during storage and, consequently, retard both starch retrogradation and crumb hardening [Mohammadi *et al.,* 2014; Mir *et al.,* 2016]; and (c) the use of sourdough technology [Rinaldi *et al.,* 2017].

Nutritional quality of gluten-free bread

Another concern regarding GFB is related to its nutritional value. Gluten-free cereal-based products, including bread, are mainly made of starches from different sources or refined flours, they lack several macro- and micronutrients and provide smaller amounts of valuable nutrients needed in a healthy and balanced diet [Gallagher *et al.,* 2004; Martin *et al.,* 2013]. Compared to their gluten-containing counterparts, commercial gluten-free products have a lower protein content, inadequate amount of B-group vitamins [Thompson, 1999; Yazynina *et al.,* 2008], and minerals (including iron, zinc, calcium) [Wronkowska *et al.,* 2008; Saturni *et al.,* 2010]. Besides, gluten-free flours are not enriched or fortified like the refined wheat-based flour or products are on a voluntary or mandatory basis [Department of Health, 1998; Akhtar *et al.,* 2011]. Meanwhile, the enrichment of gluten-free flours in minerals and/or vitamins could increase the nutritional status of CD patients and increase the amount of minerals to the level sufficient for prophylactic or therapeutic use.

Nutritional deficiencies are frequently detected in newly diagnosed and untreated CD patients due to chronic inflam-

mation and malabsorption problem [Wierdsma *et al.,* 2013]. Besides, a GFD which is the only recommended CD treatment may not be nutritionally adequate and well-balanced thus may generate deficiencies that persist in CD patients following this eliminative diet [Saturni *et al.,* 2010; Penagini *et al.,* 2013]. Iron deficiency anaemia [Theethira *et al.,* 2014] and lower bone mineral density [Meyer *et al.,* 2001; Krupa-Kozak, 2014] were frequently determined in CD patients at time of diagnosis. Hallert *et al.* [2002] found a poor vitamins status, in particular lower contents of folic acid and vit. B6 in CD patients on a GFD for over 10 years. In turn, Sdepanian *et al.* [2003] revealed that the mean bone mineral density in adolescents with CD was significantly lower than in the control groups, and that they showed high percentages of magnesium and calcium as well as phosphorous deficiencies.

Recently, a growing number of studies related to gluten-free bakery products besides the focus on the better sensory quality and prolonged shelf-life, pay special attention on the development of products characterised by an improved nutritional quality [Capriles & Arêas, 2014]. Inclusion of microelements into a GFB formulation is one of the promising methods for the improvement of the nutrition value of GFB without compromising its sensory quality. A GFB enriched in calcium was obtained by Krupa-Kozak *et al.* [2011a] who evaluated the effect of the individual and combined addition of calcium caseinate and calcium citrate to GFB formulations. Compared with unfortified control GFB, in breads containing calcium citrate alone (2%) or containing a mixture of calcium citrate and calcium caseinate (1.3% and 0.7%, respectively), a significant increase of calcium levels was determined, which allowed considering the obtained GFBs as good sources of calcium, providing approx. 100–140 mg of calcium per serving. Moreover, the technological and sensory characteristics of calcium-enriched GFBs were favourably modified. In a subsequent study, Krupa-Kozak *et al.* [2012] assessed the suitability of different organic or inorganic calcium sources (calcium lactate, calcium citrate, calcium chloride, calcium carbonate) in GFB formulations with inulin. The best results were achieved with the addition of calcium carbonate, which additionally raised the overall consumer acceptability of GFB. Kiskini *et al.* [2012] compared the effects of iron supplements (ferric pyrophosphate, ferric pyrophosphate with emulsifiers, sodium iron EDTA, microencapsulated ferrous sulfate, and elemental iron) on the sensory and physical quality of iron-fortified GFB (40 mg/kg solid compound). They observed that the electrolytic iron was stable during thermal treatment, and thus its addition caused no adverse changes to GFB. In contrast, authors indicated the limitations of other iron supplements administered into GFB, in particular observed that the addition of ferric pyrophosphate and NaFeEDTA caused undesirable changes in bread, in particular darkening of crust and crumb and a metallic taste.

More often, however, the use of highly nutritious naturally gluten-free ingredients such as pseudocereals, minor cereals, legumes, and protein from various sources has been suggested as an important and dietary method for the improvement of the nutritional value of GFB. However, while the incorporation of such raw materials in gluten-free formulae may have advantages due to their high nutritional value, there are also disadvantages due to their technological limitations, which can change the appearance, colour, texture, aroma, and taste of GFB [Capriles *et al.,* 2016]. Several excellent reviews summarized the nutritional aspects of fortified gluten-free products, including their glycaemic index and antioxidant capacity [Capriles & Arêas, 2016; Taylor *et al.*, 2016; Tsatsaragkou & Mandala, 2016; Torres *et al.*, 2017]. The recent application and properties of additional ingredients in GFB will be discussed in the following sections of this review.

HOW TO IMPROVE GLUTEN-FREE BREAD?

In the last few decades, a great deal of attention has been paid to the development of novel and healthier GFBs able to fulfil all quality requirements for bakery products [Capriles *et al.,* 2016]. To this end, the special role of different protein sources has been strengthened and the use of alternative nutrient-dense raw materials and natural compounds has become increasingly popular.

Proteins of animal and plant origin

To build up a network similar to that formed by gluten in wheat bread production, the inclusion of other polymeric substances such as non-gluten proteins is a critical factor. Although proteins play a functional role in developing bread structure and texture, their inclusion in gluten-free formulae may also confer nutritional benefits to the final products [Ziobro *et al.,* 2013a]. Different types of proteins of both plant (such as cereals, pseudocereals, and legumes) and animal origin (such as dairy proteins and egg albumins) have been used to produce protein-enriched GFBs [Crockett *et al.*, 2011; Ziobro *et al.,* 2013a; Aguilar *et al.*, 2015; Collar *et al.,* 2015].

Dairy ingredients such as caseinates, whey proteins, and skim milk powder were widely used in gluten-free breadmaking due to their functional properties, which are similar to these of gluten, and to their high nutritional value, which entails an increase in calcium and protein content and supply of essential amino acids [Stathopoulos, 2008; Nunes *et al.,* 2009]. However, not all kinds of dairy proteins behave in the same way, as they possess different functionalities, which range from the ability of caseinates to stabilize the dough/batter, through the ability of isolated and concentrated whey proteins to form gels, to the high water-holding capacity of high-temperature skim milk powders [Nunes *et al.,* 2009]. Thus, the addition of dairy proteins in GF bread formulae could have several effects on the overall quality of the intermediate and end products: (a) at the dough level, they increase water binding capacity and enhance the handling properties of dough/batter; (b) at the bread level, they increase loaf volume, improve texture, enhance crust colour and aroma, and reduce the staling rate [Houben *et al.,* 2012]. Krupa-Kozak *et al.* [2013] evaluated the effects of the addition (12% and 24%) of four low lactose dairy proteins (calcium and sodium caseinate, dried whey protein isolate, and hydrolyzed whey proteins) on the behaviour of the dough and the quality properties of the resulting GFBs. They found that, although the GFBs exhibited higher protein content at all supplementation levels, breads prepared with the addition

of 12% milk powders where more than 5 times richer in protein than the control one; in addition, their crumb softness, specific volume, crust darkening, and crumb lightness were observed to significantly increase. However, since many newly diagnosed patients with coeliac disease commonly report a secondary lactose deficiency caused by an inadequate secretion of lactase (which is normally produced by the intestinal villi), the use of high-lactose dairy ingredients in the development of gluten-free bakery products must be carefully considered.

Other kinds of proteins which have shown a great potential in gluten-free breadmaking are egg proteins. The addition of egg proteins as gluten replacers is due to their foaming ability, stabilizing effect, and emulsifying properties. However, due to their allergenic character, their use as food products ingredients should be limited or carefully considered [Phongthai *et al.,* 2016].

Another way to include proteins in a GFB formulation is to use various types of legume grains. In fact, legumes offer a high nutritional value, suitable functional properties, and beneficial health effects. They are important sources of protein, whose content ranges from 18 to 25%. Soybeans, however, are unique in that they contain about 35–43% protein [Tharanathan & Mahadevamma, 2003]. Moreover, legume proteins, due to their high content of the essential amino acid – lysine which is often deficient in grain food, as well as a concomitant deficiency of methionine and cysteine, are nutritionally complementary to cereal proteins [Duranti, 2006]. Legumes are also good sources of minerals (such as calcium and iron), vitamins (especially B-group vitamins), and dietary fibre (both soluble and insoluble fractions). In addition, they are identified as low glycaemic index foods [Collar *et al.,* 2014]. When incorporated into food matrices, legume proteins show a range of functional properties, which include foaming, emulsifying, and gelation capabilities as well as texture, water/oil binding capacity, and viscosity [Maninder *et al.,* 2007]. In recent years, proteins from legume sources such as soybean, carob, pea, and lupine have been widely used in gluten-free breadmaking [Marco & Rosell, 2008a; Crockett *et al.,* 2011; Ziobro *et al.,* 2016; Horstmann *et al.,* 2017]. Soy proteins, which are usually added into the bread formulae either as soy protein isolates or high-protein soy flour, have for long been used to improve mechanical behaviour of doughs as well as textural properties, specific volume, and nutritional value of GFBs [Marco & Rosell, 2008b]. However, soy flour incorporation may be curbed by its "beany" flavour. Shin *et al.* [2013] found that the pre-treatment of soy flour can improve not only the flavour but also technological parameters of GFBs. Carob, which is the fruit of the carob tree (*Ceratonia siliqua* L.) (also called locust bean) is cultivated throughout the Mediterranean region for its edible pods. Carob flour is rich in proteins, dietary fibre, micronutrients, and polyphenols [Turfani *et al.,* 2017]. Moreover, it contains caroubin, a water-insoluble protein able to form wheat-like dough due to disulphide bonded high molecular weight proteins, which makes carob an interesting ingredient in gluten-free breadmaking [Smith *et al.,* 2012]. In fact, it was observed that carob germ flour (7%), when mixed with corn starch (93%), HPMC (2%) and water (80%), was able to form viscoelastic dough similar to that of wheat, resulting in breads with both a higher specific volume and crumb softness [Smith *et al.,* 2012]. Tsatsaragkou *et al.* [2014] used the Response Surface Methodology to optimise the levels of carob flour, resistant starch and water in rice-based GFBs. They reported that the use of 15% carob flour, 15% resistant starch, and 140% water resulted in GFBs rich in fibre (6.10 g/100g) and with softer crumb and improved porosity values. However, Miñarro *et al.* [2012] in their attempt to substitute soya protein with other legume proteins such as pea isolates, chickpea and carob germ flour, obtained opposite results. These authors found that, in spite of good rheological properties, the breads obtained with carob germ flour showed the lowest specific volume, the darkest crumb, and the highest values of hardness during 5 days of storage. On the contrary, the best overall behaviour (higher specific volume and softer crumb during storage) was observed in the breads produced with the addition of chickpea flour, probably due to the good emulsifying stability index of its proteins. Significant improvement in both the technological and the nutritional quality of GFB achieved *via* incorporation of chickpea flour has been recently reported also by other authors [Ouazib *et al.*, 2016; Santos *et al.*, 2018].

Pseudocereals

The rekindled interest in some under-utilized and naturally gluten-free plant species such as buckwheat, amaranth and quinoa derives from their excellent nutritional value. In fact, in addition to a superior protein profile, they have high contents of resistant starch, dietary fibre, and micronutrients, such as vitamins, minerals, and phenols. Although the number of gluten-free bakery products made of all three pseudocereals has increased significantly, buckwheat flour remains the most studied ingredient in the development of GFBs. On the contrary, only a few attempts have been made to include quinoa into GFB formulations [Capriles & Arêas, 2014].

Buckwheat belongs to the family *Poligonaceae* and genus *Fagopyrum,* which consists of about 15 species cultivated around the world. However, among them, only two species are utilized for food consumption: *Fagopyrum esculentum* and *Fagopyrum tataricum,* which are also known as common and tartary buckwheat, respectively. The nutritional value of buckwheat flour is highly connected with its protein composition. In fact, contrarily to the true cereals, where the main proteins are glutelin and prolamins, buckwheat proteins are composed mainly of the water-soluble globulins and albumins, which contain high levels of the essential amino acids lysine and arginine, resulting in a well-balanced amino acids composition. Due to that, despite a protein content close to that measured in common cereal grains (12%), buckwheat protein has a high biological value [Alvarez-Jubete *et al.,* 2010a,b; Giménez-Bastida *et al.,* 2017; Tömösközi & Langó, 2017]. Buckwheat is also a source of minor components such as minerals (potassium, calcium, copper, zinc, and manganese), vitamins (B-group and E), and phenolic compounds (hyperin, quercitrin, and quercetin). In particular, among pseudocereals, it is unique in containing the flavonol glycoside-rutin with recognized anti-inflammatory and antioxidant properties [Zhang *et al.,* 2012; Wronkowska *et al.,* 2016; Tömösközi & Langó, 2017]. Lukšič *et al.* [2016], evalu-

ating the impact of sourdough fermentation and thermal processes on the conversion of rutin to quercetin in bread made of common and tartary buckwheat, observed that the combined effects of sourdough fermentation and baking process only preserve quercetin, but not rutin, in tartary buckwheat breads. In turn, Torbica *et al.* [2010] found that the incorporation of different levels (up to 30%) of both husked and unhusked buckwheat flours in rice-based GFBs made the addition of hydrocolloids not necessary for a good development of the dough structure, without detrimental effects on the sensory bread acceptance (except for high levels of unhusked buckwheat flour). They also reported that the addition of increasing amounts of buckwheat flour reduced the starch retrogradation degree of the tested samples, leading to a final product with improved anti-staling properties. Other study assessed the effect of a dehulled and puffed buckwheat flour application at 40% substitution level on the nutritional value of two commercial GFB mixtures [Mariotti *et al.,* 2013]. Authors indicated that the inclusion of 40% dehulled buckwheat flour improved the leavening properties of the commercial GF mixtures. Moreover, the presence of a small amount of puffed buckwheat flour and HPMC limited the diffusion/loss of water from the bread crumb and the interactions between starch and protein macromolecules, resulting in a softer GFB crumb and reduced staling kinetics during storage. Krupa-Kozak *et al.* [2011b] reported that the addition of different levels of buckwheat flour (from 10% to 40%) improved the technological and nutritional quality of breads made with corn and potato starch. In particular, they observed that the increasing levels of buckwheat supplementation resulted in a proportional enrichment of the final products with both proteins and microelements (Cu and Mg). Turkut *et al.* [2016] demonstrated that, in gluten-free formulations based on rice flour, potato starch and buckwheat flour, the replacement of buckwheat with increasing levels of quinoa flour (up to 50%) can improve the bread sensory flavour and the overall liking, without detrimental effects on baking loss, specific volume and protein content, when levels of substitution were up to 25%.

In the last few years, apart from buckwheat flour, special emphasis has also been put on the inclusion of nutritious amaranth and quinoa flours into gluten-free formulations. Amaranth belongs to the family *Amaranthaceae* and genus *Amaranthus,* which consists of about 60 different species. Among them, *A. caudatus, A. cruentus,* and *A. hypochondriacus* are the three main species commonly used for food consumption. Quinoa belongs to the family *Chenopodiaceae* and genus C*henopodium*, in which, due to the presence of bitter tasting saponins whose contents can range from 0.03 to 2.05%, it is possible to identify sweet and bitter varieties [Schoenlechner *et al.,* 2008]. Amaranth and quinoa have higher protein contents (14–15% and 13–14%, respectively) compared to the true cereal grains. Furthermore, their amino acid compositions, with high levels of the essential amino acids lysine and methionine, are well-balanced and comparable (or even higher) to those of whole egg and casein, respectively [Schoenlechner *et al.,* 2008]. In addition to protein, amaranth and quinoa are rich in many nutrients, such as dietary fibre, folate, riboflavin, ascorbic acid, tocopherol, and phenolic compounds. Moreover, they have a total mineral content (calcium, magnesium, iron, potassium, and zinc) approximately 2 times higher than that of true cereals [Schoenlechner *et al.,* 2008]. The use of both amaranth and quinoa in GFB production has been recently investigated by Machado Alencar *et al.* [2015]. These authors reported that the supplementation of a starch-rich base formulation, containing cassava, potato, sour tapioca, and rice flour with amaranth, quinoa (20%) and different sweeteners (stevia, sucralose, and sucralose/acesulfame-K blend) led to breads with higher contents of protein, lipids and ash, without detrimental effects on the specific volume, firmness, and water activity. In particular, the best results were obtained in the experimental sample prepared with quinoa and stevia, which showed the highest specific volume and the lowest crumb firmness values.

Minor cereals: Sorghum and Teff

Sorghum (*Sorghum vulgare*) is a member of the grass family, which is more closely related to corn and wild rice than to wheat, rye and barley. For this reason, it is often used as a safe ingredient in gluten-free breadmaking [Schober *et al.*, 2007; Vallons *et al.*, 2010; Marston *et al.*, 2016]. In addition, the white and bland-taste flours obtained from the so-called food-grade sorghum lines, do not impart unusual colours or strong flavours to food products and, therefore, may be preferred over maize flour [Taylor *et al.,* 2006]. The major constituent of sorghum is starch, which constitutes about 72% of the whole grain. However, although sorghum has a similar chemical composition to that of maize, its starch tends to have a slow digesting profile and a higher gelatinization temperature, which could lead to an inadequate gelatinization during baking. Its protein content, ranging between 8.6% and 15.6%, is similar to that found in other cereal grains. Furthermore, sorghum proteins have poor nutritional quality (low lysine content) as well as poor functionality during processing. In fact, the prolamins of sorghum are located in protein bodies that have a relatively hydrophobic surface [Awika, 2017]. However, due to the large amount of bioactive compounds present in sorghum grain, it can be considered as a potential source of nutraceuticals [Taylor *et al.,* 2006]. In particular, sorghum contains phenolic acids and flavonoids, which are responsible for the different colour of sorghum grains. However, certain cultivars have higher contents of condensed tannins (proanthocyanidins) and, therefore, exhibit bitter taste [Awika, 2017]. Thus, the use of sorghum as the main ingredient in gluten-free breadmaking can have advantages but also limitations. Specifically, some physicochemical properties of sorghum flour negatively affect the breadmaking performance. In fact, during milling, the horny part of the endosperm forms coarse grits, which contribute to a coarse and sandy mouthfeel. The further milling of such grits into fine flour results in a high amount of damaged starch. Furthermore, when heated, sorghum proteins can form aggregates that, interfering with the starch gel, lead to breads with flat top and big holes in the crumb [Onyango *et al.,* 2011b]. As reported by Schober *et al.* [2005], the use of different varieties of sorghum can also affect the quality characteristics of the resulting breads. When comparing the breadmaking performance of 10 sorghum flours (70%) blended with 30%

corn starch, these authors observed significant differences in crumb hardness and crumb grain properties. They suggested that starch damage, which is influenced by kernel hardness, might have a key role in these differences. Moreover, despite no significant differences in the specific volume of the different breads, these authors concluded that larger bread volumes could be reached when corn starch is added to the sorghum flour. The importance of starch damage as well as sorghum flour composition and particles size on bread performance was also observed by Trappey *et al.* [2015]. In particular, they found that flour with a lower content of fibre and a smaller particle size enabled producing breads with more acceptable volume, crumb structure, colour, and texture. In their attempts to improve the quality of sorghum GFB, other authors adopted various promising approaches, including: (a) high-pressure processing [Vallons *et al.*, 2010] and heat treatment of sorghum flour [Marston *et al.*, 2016]; (b) the use of native and pregelatinised starch, and enzymes [Onyango *et al.,* 2010; 2011a, b]; and (c) the use of sourdough fermentation [Schober *et al.,* 2007].

Teff is an ancient cereal originating from Ethiopia where it is used to make traditional food preparations that range from typical baked goods, such as *injera* (leavened flatbread) and *kitta* (unleavened bread) through typical local spirits and opaque beer, such as *tella*, to porridge and soups [Bultosa & Taylor, 2002; Zhu, 2018]. Teff belongs to the family *Poaceae* and genus *Eragrostis*, which consists of over 350 species, of which *Eragrostis tef* is the only cultivated species. The renewed interest in the use of teff flour as a valuable ingredient in food processing is mainly due to its desirable nutritional properties and the absence of gluten. In terms of chemical composition, 73% of the total carbohydrates is composed of starch, which shows a low degree of retrogradation [Bultosa & Taylor, 2002]. The protein content of teff (8.7–11%) is close to that found in other cereals, such as wheat, maize, and barley but, unlike them, it has a well-balanced amino acid composition. Moreover, the essential amino acid profile of teff, except for its relatively lower lysine and isoleucine contents, is comparable to that of egg protein [Gebremariam *et al.,* 2014]. Since teff flour is usually obtained by grinding the whole tiny seeds, it has a high content of dietary fibre (9.8% dry basis) [Zhu, 2018]. Teff contains also considerable levels of vitamins and, compared to other cereals such as wheat, barley and sorghum, it is a good source of iron, calcium, and zinc. Furthermore, in addition to a lower lipid content (2–3%) [Schober *et al.,* 2007], teff flour is rich in unsaturated fatty acids (72.46%), including oleic acid (32.41%) and linoleic acid (23.83%) [Gebremariam *et al.,* 2014]. Although there has been a lot of research dealing with the physical, sensory, and nutritional performance of wheat breads supplemented with teff flours, only few studies have been published regarding its potential use as novel and nutritional ingredient in gluten-free breadmaking [Campo *et al.,* 2016; Marti *et al.,* 2017].

Dietary fibre

GFB is often characterised by a low content of dietary fibre, ranging from 1.2% to 7.2% in commercial GFBs [Thompson *et al.,* 2005; Segura & Rosell, 2011]. The refining process results in a decrease in fibre content as during this process the outer layer of grains containing most of the fibre is removed, and the starchy inner part is left. Dietary fibres consist of non-digestible carbohydrates and lignin that are intrinsic and intact in plants and should be resistant to the enzymes of the early-sections of the digestive tract and fermented in the colon by gut microbiota [Anderson *et al.,* 2009]. The daily intake of dietary fibre should range from 25 to 38 g [Drabińska *et al.,* 2018a]. The Joint WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases [Nishida *et al.,* 2004] stated that an adequate amount of fibre in the diet confers several health benefits including body mass management, as well as reduction of hypertension and risk of type 2 diabetes and cardiovascular diseases [Anderson *et al.,* 2009].

Inulin-type fructans (ITF) are commonly consumed soluble dietary fibres, which are more fermentable and viscous than the insoluble fibres [Slavin, 2013]. The structure of ITF, characterized by β-configuration, determines their ability to reach the colon in the intact form and their prebiotic properties [Drabińska *et al.,* 2018a]. ITF have not only nutritional quality, but can also affect the technological parameters of food products, which was widely studied in gluten-free breadmaking. Inulin can affect the quality of bread at every step of the production process. Hager *et al.* [2011] demonstrated that inulin influenced the moisture content of gluten-free batter. The inulin microcrystals led to the formation of a gel structure entrapping large volumes of water, which resulted in a less elastic and more viscous dough with a creamy texture. After baking, inulin-enriched GFB had a darker crust, but also harder crumb, and an increased rate of staling in comparison with the control bread without inulin [Slavin, 2013]. Similar findings were reported by Rodriguez *et al.* [2015], who analysed the addition of inulin to GFB fortified with bovine plasma proteins. Inulin-enriched GFB had smoother crust, increased loaf volume and reduced hardness, because of a decreased thickness of the walls surrounding the small air cells. The authors showed that inulin reduced the moisture loss and, simultaneously, delayed the staling of breads [Hager *et al.,* 2011], which in another study was found the reason behind the faster aging and staling of stored bread [Capriles & Arêas, 2013]. Korus *et al.* [2006] found that 5% enrichment of GFB with inulin resulted in an increased volume and decreased crumb hardness. However, the authors reported that too high addition of inulin may result in GFB with wrinkling crust and reduced cohesiveness and springiness of the crumb [Korus *et al.,* 2006].

ITF is a group of compounds varying in the length of the chemical chain, *i.e.* in the degree of their polymerization (DP), and consequently having different properties. Long-chain inulin significantly affects rheological and viscoelastic properties of gluten-free dough, causing a decrease in consistency and paste viscosity and an increase in the compliance values and gelatinization temperatures [Juszczak *et al.,* 2012]. On the other hand, short-chain inulin was found to increase bread volume, improve crumb texture (uniform and medium size porosity), and decrease the staling rate [Ziobro *et al.,* 2013b]. To obtain breads with various properties, a mixture of short- and long-chain ITF was also examined. Capriles & Arêas [2013] found that irrespectively of the amount of mixed

TABLE 2. By-products used in gluten-free breadmaking.

By products	Effect on gluten-free dough	Effect on gluten-free bread	References
Blackcurrant seeds	At the highest level (15%): Modified viscoelastic properties, Decreased values of consistency coefficients and flow indices	Decreases loaf volume, Slight increase in crumb hardness, gumminess and chewiness, Increased levels of proteins, dietary fibre, and polyphenols	[Korus *et al.,* 2012]
Strawberry seeds	At the highest level (15%): Modified viscoelastic properties, Decreased values of consistency coefficients and flow indices	Increased loaf volume Decreased crumb hardness, gumminess and chewiness Increased levels of proteins, dietary fibre, and polyphenols	[Korus *et al.,* 2012]
Orange pomace	Increased robustness of the batter, Decreased occurrence of starch gelatinization	Increased specific volume, Detrimental effect on crumb structure (dense and compact loaf), Decreased crumb hardness during storage (at lower level of addition), Increased levels of dietary fibre	[O'Shea *et al.,* 2013]

fructans added, the obtained breads were characterised by an enlarged volume and specific volume, probably due to the increased CO_2 retention capacity. Moreover, ITFs positively affected the crust colour of GFB, probably due to enhanced formation of brown nitrogenous polymers and melanoidins *via* Maillard reaction during baking. The authors also confirmed the improvement in the sensory quality of GFB fortified with ITFs. Irrespective of the amount of ITF added, the enriched breads received higher scores in terms of their appearance, colour, texture, and taste. Moreover, the fortified breads had a lower glycaemic index and glycaemic load and higher contents of dietary fibre [Capriles & Arêas, 2013]. The sensory acceptance was also found to be dependent on DP [Morais *et al.,* 2014]. GFB fortified with short-chain fructooligosaccharides received the highest scores for crust colour, porosity, and texture as well as for taste and flavour.

By-products

Recently, about one third of the fruit and vegetable, including peels, skins, outer leaves and seeds, is wasted during preparation and processing, which poses a huge problem for both the environment and the food industry. However, many of these by-products can be used as additives to gluten-free food products, thus finding a second life (Table 2). Fruit and vegetable by-products were found to be good sources of bioactive compounds, such as carotenoids, polyphenols, glucosinolates and vitamins, as well as dietary fibre [Domínguez-Perles *et al.,* 2010; O'Shea *et al.,* 2012; Radočaj *et al.*, 2014; Majzoobi *et al.*, 2016; Drabińska *et al.*, 2018b]. For that reason, nowadays, an increasing number of research focuses on the evaluation of the nutritional quality and further application of by- and waste products as low-cost sources of nutrients and functional ingredients.

Defatted strawberry and blackcurrant seeds have been used as additional sources of dietary fibre, protein, and polyphenols in GFB [Korus *et al.,* 2012]. The authors found that the addition of fruit seed modified the viscoelastic properties of gluten-free batter and decreased values of consistency coefficients and flow indices. Moreover, hardness of GFB was reduced by the addition of fruit by-products, especially strawberry seeds. The incorporation of defatted strawberry seed increased the loaf volume as compared to GFB enriched with blackcurrant seed and control bread, which was explained by a higher content of sugars which enhance the ability of yeast to produce CO_2 [Korus *et al.,* 2012].

Orange pomace, being a good source of dietary fibre (up to 40% DM) and bioactive compounds (minerals, vitamins), is another by-product that has been studied in GFB development [O'Shea *et al.,* 2013, 2015a,b; Talens *et al.,* 2017]. The incorporation of orange pomace into gluten-free batter improved the robustness and decreased starch gelatinisation [O'Shea *et al.,* 2013]. Furthermore, the obtained GFB enriched in orange pomace had a higher content of fibre (by 4%) [O'Shea *et al.,* 2015b]. In turn, Talens *et al.* [2017] showed that an attractive colour, flavour and texture of gluten-free muffins were obtained by the incorporation of a microwave-dried orange by-product to the batter.

The recent and on-going studies demonstrated that the use of by-products in the gluten-free food industry affords the possibility of improving the quality of many products. By-products are unused sources of dietary fibre, vitamins, minerals, and bioactive compounds, which can be used to increase the quality of gluten-free foodstuff which is usually poor. However, during the optimization of a novel gluten-free product, the pre-processing step should be considered as well.

CONCLUSIONS

The presence of gluten is considered fundamental for successful breadmaking. However, the elimination of gluten in cereal-based products has a detrimental effect on the breadmaking process and raises technological challenges in making good quality leavened bread. On the basis of the recent findings summarized in this review, it may be concluded that only the use of a combination of different ingredients and additives could result in GFBs with pleasant quality features that, in some cases, may be compared to those of the gluten-containing breads. In particular, the partial replacement of the conventional starchy ingredients with alternative nutrient-dense minor cereals, pseudocereals, and legumes allowed achieving promising results. The incorporation of low-cost sources of nutrients, such as different kinds of by- and waste

products, has also been suggested as an interesting approach to improve the quality of GFB.

However, despite the considerable advances made in this field, the nutritional quality of gluten-free products still remains a critical issue. Since low levels of protein, fibre, and essential nutrients, as well as high contents of fat and sugar are too often observed in GFB, further research aimed at exploring new strategies to overcome this major problem is needed.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

FUNDING STATEMENT

Supported by the grant of KNOW Consortium "Healthy Animal – Safe Food", MS&HE Decision No. 05–1/ KNOW2/2015.

REFERENCES

1. Aguilar, N., Albanell, E., Miñarro, B., Capellas, M. (2015). Chickpea and tiger nut flours as alternatives to emulsifier and shortening in gluten-free bread. *LWT – Food Science and Technology, 62*(1), 225–232.
2. Akhtar, S., Anjum, F.M., Anjum, M.A. (2011). Micronutrient fortification of wheat flour: Recent development and strategies. *Food Research International, 44*(3), 652–659.
3. Alvarez-Jubete, L., Arendt, E.K., Gallagher, E. (2010a). Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. *Trends in Food Science & Technology, 21*(2), 106–113.
4. Alvarez-Jubete, L., Auty, M., Arendt, E., Gallagher, E. (2010b). Baking properties and microstructure of pseudocereal flours in gluten-free bread formulations. *European Food Research and Technology, 230*(6), 437–445.
5. Anderson, J.W., Baird, P., Davis, R.H., Jr., Ferreri, S., Knudtson, M., Koraym, A., Waters, V., Williams, C.L. (2009). Health benefits of dietary fiber. *Nutrition Reviews, 67*(4), 188–205.
6. Awika, J.M. (2017). Sorghum: Its Unique Nutritional and Health-Promoting Attributes. In J.N.R. Taylor, J.M. Awika (Eds.), *Gluten-Free Ancient Grains: Cereals, Pseudocereals, and Legumes: Sustainable, Nutritious, and Health-Promoting Foods for the 21st Century.* Woodhead Publishing Limited, Cambridge, pp. 21–54.
7. Bathrellou, E., Kontogianni, M.D., Panagiotakos, D.B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review. *Maturitas, 112*, 29–33.
8. Bultosa, G., Taylor, J.R.N. (2002). Paste and gel properties and *in vitro* digestibility of teff [*Eragrostis tef* (Zucc.) Trotter] starch. *Starch/Stärke, 56,* 20–28.
9. Campo, E., del Arco, L., Urtasun, L., Oria, R., Ferrer-Mairal, A. (2016). Impact of sourdough on sensory properties and consumers' preference of gluten-free breads enriched with teff flour. *Journal of Cereal Science, 67*, 75–82.
10. Cappa, C., Lucisano, M., Mariotti, M. (2013). Influence of Psyllium, sugar beet fibre and water on gluten-free dough properties and bread quality. *Carbohydrate Polymers, 98*(2), 1657–1666.
11. Capriles, V.D., Arêas, J.A.G. (2013). Effects of prebiotic inulin-type fructans on structure, quality, sensory acceptance and glycemic response of gluten-free breads. *Food & Function, 4*(1), 104–110.
12. Capriles, V.D., Arêas, J.A.G. (2014). Novel approaches in gluten-free breadmaking: Interface between food science, nutrition, and health. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 871–890.
13. Capriles, V.D., Arêas, J.A.G. (2016). Approaches to reduce the glycemic response of gluten-free products: *In vivo* and *in vitro* studies. *Food & Function*, *7*(3), 1266–1272.
14. Capriles, V.D., dos Santos, F.G., Arêas, J.A.G. (2016). Gluten-free breadmaking: Improving nutritional and bioactive compounds. *Journal of Cereal Science, 67*, 83–91.
15. Collar, C., Conte, P., Fadda, C., Piga, A. (2015). Gluten-free dough-making of specialty breads: Significance of blended starches, flours and additives on dough behaviour. *Food Science & Technology International, 21*(7), 523–536.
16. Collar, C., Jiménez, T., Conte, P., Fadda, C. (2014). Impact of ancient cereals, pseudocereals and legumes on starch hydrolysis and antiradical activity of technologically viable blended breads. *Carbohydrate Polymers, 113*, 149–158.
17. Conte, P., Del Caro, A., Balestra, F., Piga, A., Fadda, C. (2018). Bee pollen as a functional ingredient in gluten-free bread: A physical-chemical, technological and sensory approach. *LWT – Food Science and Technology, 90*, 1–7.
18. Conte, P., Fadda, C., Piga, A., Collar, C. (2016). Techno-functional and nutritional performance of commercial breads available in Europe. *Food Science & Technology International, 22*(7), 621–633.
19. Cornejo, F., Rosell, C.M. (2015). Physicochemical properties of long rice grain varieties in relation to gluten free bread quality. *LWT – Food Science and Technology, 62*(2), 1203–1210.
20. Crockett, R., Ie, P., Vodovotz, I. (2011). Effects of soy protein isolate and egg white solids on the physicochemical properties of gluten-free breads. *Food Chemistry, 129*(1), 84–91.
21. de la Hera, E., Martinez, M., Oliete, B., Gómez, M. (2013). Influence of flour particle size on quality of gluten-free rice cakes. *Food and Bioprocess Technology, 6*(9), 2280–2288.
22. de la Hera, E., Rosell, C.M., Gomez, M. (2014). Effect of water content and flour particle size on gluten-free bread quality and digestibility. *Food Chemistry, 151*, 526–531.
23. Demirkesen, I., Kelkar, S., Campanella, O.H., Sumnu, G., Sahin, S., Okos, M. (2014). Characterization of structure of gluten-free breads by using X-ray microtomography. *Food Hydrocolloids, 36*, 37–44.
24. Department of Health. (1998). *Report on health and social subjects. No. 49. Nutrition and bone health with particular reference to calcium and vitamin D*. London: HMSO.
25. Domínguez-Perles, R., Martínez-Ballesta, M.C., Carvajal, M., García-Viguera, C., Moreno, D.A. (2010). Broccoli-derived by-products—A promising source of bioactive ingredients. *Journal of Food Science, 75*(4), C383–C392.
26. Drabińska, N., Ciska, E., Szmatowicz, B., Krupa-Kozak, U. (2018b). Broccoli by-products improve the nutraceutical potential of gluten-free mini sponge cakes. *Food Chemistry, 267*, 170–177.
27. Drabińska, N., Rosell, C.M., Krupa-Kozak, U. (2018a). Inulin-type fructans application in gluten-free products: Functionality

and health benefits. In J.-M. Mérillon & K. G. Ramawat (Eds.), *Bioactive Molecules in Food*. Springer International Publishing, Cham, pp. 1–40.
28. Drabińska, N., Zieliński, H., Krupa-Kozak, U. (2016). Technological benefits of inulin-type fructans application in gluten-free products – A review. *Trends in Food Science & Technology, 56*, 149–157.
29. Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*(2), 67–82.
30. Elgeti, D., Nordlohne, S. D., Föste, M., Besl, M., Linden, M. H., Heinz, V., Jeklea, M., Becker, T. (2014). Volume and texture improvement of gluten-free bread using quinoa white flour. *Journal of Cereal Science, 59*(1), 41–47.
31. Fadda, C., Sanguinetti, A.M., Del Caro, A., Collar, C., Piga, A. (2014). Bread staling: Updating the view. *Comprehensive Reviews in Food Science and Food Safety, 13*(4), 473–492.
32. Gallagher, E., Gormley, T.R., Arendt, E.K. (2003). Crust and crumb characteristics of gluten free breads. *Journal of Food Engineering, 56*(2–3), 153–161.
33. Gallagher, E., Gormley, T.R., Arendt, E.K. (2004). Recent advances in the formulation of gluten-free cereal-based products. *Trends in Food Science & Technology, 15*(3–4), 143–152.
34. Gebremariam, M.M., Zarnkow, M., Becker, T. (2014). Teff (*Eragrostis tef*) as a raw material for malting, brewing and manufacturing of gluten-free foods and beverages: A review. *Journal of Food Science and Technology, 51*(11), 2881–2895.
35. Giménez-Bastida, J.A., Zielinski, H., Piskula, M.K., Zielinska, D., Szawara-Nowak, D. (2017). Buckwheat bioactive compounds, their derived phenolic metabolites and their health benefits. *Molecular Nutrition & Food Research, 61*, art. no. 1600475.
36. Gómez, M., Sciarini, L.S. (2015). Gluten-free bakery products and pasta. In Arranz E., Fernandex Banares F., Rosell C.M. (Eds.) *Advances in the Understanding of Gluten Related Pathology and the Evolution of Gluten-Free Foods*. OmniaScience, Barcelona, pp. 565–604.
37. Gray, J.A., Bemiller, J.N. (2003). Bread staling: Molecular basis and control. *Comprehensive Reviews in Food Science and Food Safety, 2*(1), 1–21.
38. Gujral, H.S., Haros, M., Rosell, C.M. (2003). Starch hydrolyzing enzymes for retarding the staling of rice bread. *Cereal Chemistry, 80*(6), 750–754.
39. Hager, A.S., Ryan, L.A.M., Schwab, C., Gänzle, M.G., O'Doherty, J.V., Arendt, E.K. (2011). Influence of the soluble fibres inulin and oat β-glucan on quality of dough and bread. *European Food Research and Technology, 232*(3), 405–413.
40. Hager, A.S., Wolter, A., Czerny, M., Bez, J., Zannini, E., Arendt, E.K., Czerny, M. (2012). Investigation of product quality, sensory profile and ultrastructure of breads made from a range of commercial gluten-free flours compared to their wheat counterparts. *European Food Research and Technology, 235*(2), 333–344.
41. Haghighat-Kharazi, S., Milani, J.M., Kasaai, M.R., Khajeh, K. (2018). Microencapsulation of -amylase in beeswax and its application in gluten-free bread as an anti-staling agent. *LWT – Food Science and Technology, 92*, 73–79.
42. Haines, M.L., Anderson, R.P., Gibson, P.R. (2008). Systematic review: The evidence base for long-term management of coeliac disease. *Alimentary Pharmacology & Therapeutics, 28*(9), 1042–1066.
43. Hallert, C., Grant, C., Grehn, S., Grännö, C., Hultén, S., Midhagen, G., Ström, M., Svensson, H., Valdimarsson, T. (2002). Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Alimentary Pharmacology & Therapeutics, 16*(7), 1333–1339.
44. Hoefler, A.C. (2004). *Hydrocolloids, Eagan Press Handobook, St*. Minnesota, USA: Paul.
45. Horstmann, S.W., Axel, C., Arendt, E.K. (2018). Water absorption as a prediction tool for the application of hydrocolloids in potato starch-based bread. *Food Hydrocolloids, 81*, 129–138.
46. Horstmann, S.W., Foschia, M., Arendt, E. K. (2017). Correlation analysis of protein quality characteristics with gluten-free bread properties. *Food & Function, 8*(7), 2465–2474.
47. Houben, A., Höchstötter, A., Becker, T. (2012). Possibilities to increase the quality in gluten-free bread production: An overview. *European Food Research and Technology, 235*(2), 195–208. https://doi.org/10.1007/s00217–012–1720–0
48. Jnawali, P., Kumar, V., Tanwar, B. (2016). Celiac disease: Overview and considerations for development of gluten-free foods. *Food Science and Human Wellness, 5*(4), 169–176.
49. Juszczak, L., Witczak, T., Ziobro, R., Korus, J., Cieślik, E., Witczak, M. (2012). Effect of inulin on rheological and thermal properties of gluten-free dough. *Carbohydrate Polymers, 90*(1), 353–360.
50. Kadan, R.S., Robinson, M.G., Thibodeaux, D.P., Pepperman, A.B. (2001). Texture and other physicochemical properties of whole rice bread. *Journal of Food Science, 66*(7), 940–944.
51. Kiskini, A., Kapsokefalou, M., Yanniotis, S., Mandala, I. (2012). Effect of iron fortification on physical and sensory quality of gluten-free bread. *Food and Bioprocess Technology, 5*(1), 385–390.
52. Korus, J., Grzelak, K., Achremowicz, K., Sabat, R. (2006). Influence of prebiotic additions on the quality of gluten-free bread and on the content of inulin and fructooligosaccharides. *Food Science & Technology International, 12*(6), 489–495.
53. Korus, J., Juszczak, L., Ziobro, R., Witczak, M., Grzelak, K., Sójka, M. (2012). Defatted strawberry and blackcurrant seeds as functional ingredients of gluten-free bread. *Journal of Texture Studies, 43*(1), 29–39.
54. Korus, J., Witczak, M., Ziobro, R., Juszczak, L. (2009). The impact of resistant starch on characteristics of gluten-free dough and bread. *Food Hydrocolloids, 23*(3), 988–995.
55. Krupa-Kozak, U. (2014). Pathologic bone alterations in celiac disease: etiology, epidemiology, and treatment. *Nutrition, 30*(1), 16–24.
56. Krupa-Kozak, U., Altamirano-Fortoul, R., Wronkowska, M., Rosell, C.M. (2012). Breadmaking performance and technological characteristic of gluten-free bread with inulin supplemented with calcium salts. *European Food Research and Technology, 23*(3), 545–554.
57. Krupa-Kozak, U., Bączek, N., Rosell, C.M. (2013). Application of dairy proteins as technological and nutritional improvers of calcium-supplemented gluten-free bread. *Nutrients, 5*(11), 4503–4520.
58. Krupa-Kozak, U., Troszyńska, A., Bączek, N., Soral-Śmietana, M. (2011a). Effect of organic calcium supplements on the technological characteristic and sensory properties of gluten-free bread. *European Food Research and Technology, 232*(3), 497–508.
59. Krupa-Kozak, U., Wronkowska, M., Soral-Śmietana, M. (2011b). Effect of buckwheat flour on microelements and proteins contents in gluten-free bread. *Czech Journal of Food Sciences, 29*(2), 103–108.

60. Lazaridou, A., Duta, D., Papageorgiou, M., Belc, N., Biliaderis, C.G. (2007). Effects of hydrocolloids on dough rheology and bread quality parameters in gluten-free formulations. *Journal of Food Engineering, 79*(3), 1033–1047.
61. Lukšič, L., Bonafaccia, G., Timoracka, M., Vollmannova, A., Trček, J., Nyambe, T.K., Melini, V., Acquistucci, R., Germa, M., Kreft, I. (2016). Rutin and quercetin transformation during preparation of buckwheat sourdough bread. *Journal of Cereal Science, 69*, 71–76.
62. Machado Alencar, N.M., Steel, C.J., Alvim, I.D., de Morais, E.C., Andre Bolini, H.M. (2015). Addition of quinoa and amaranth flour in gluten-free breads: Temporal profile and instrumental analysis. *LWT – Food Science and Technology, 62*(2), 1011–1018.
63. Majzoobi, M., Poor, Z.V., Jamalian, J., Farahnaky, A. (2016). Improvement of the quality of gluten-free sponge cake using different levels and particle sizes of carrot pomace powder. *International Journal of Food Science & Technology, 51*(6), 1369–1377.
64. Mancebo, C.M., San Miguel, M.Á., Martínez, M.M., Gómez, M. (2015). Optimisation of rheological properties of gluten-free doughs with HPMC, psyllium and different levels of water. *Journal of Cereal Science, 61*, 8–15.
65. Maninder, K., Sandhu, K.S., Singh, N. (2007). Comparative study of the functional, thermal and pasting properties of flours from different field pea (*Pisum sativum* L.) and pigeon pea (*Cajanus cajan* L.) cultivars. *Food Chemistry, 104*(1), 259–267.
66. Marco, C., Rosell, C.M. (2008a). Breadmaking performance of protein enriched, gluten-free breads. *European Food Research and Technology, 227*(4), 1205–1213.
67. Marco, C., Rosell, C.M. (2008b). Functional and rheological properties of protein enriched gluten-free composite flours. *Journal of Food Engineering, 88*(1), 94–103.
68. Mariotti, M., Pagani, M.A., Lucisano, M. (2013). The role of buckwheat and HPMC on the breadmaking properties of some commercial gluten-free bread mixtures. *Food Hydrocolloids, 30*(1), 393–400.
69. Marston, K., Khouryieh, H., Aramouni, F. (2016). Effect of heat treatment of sorghum flour on the functional properties of gluten-free bread and cake. *LWT – Food Science and Technology, 65*, 637–644.
70. Marti, A., Marengo, M., Bonomi, F., Casiraghi, M.C., Franzetti, L., Pagani, M.A., Iametti, S. (2017). Molecular features of fermented teff flour relate to its suitability for the production of enriched gluten-free bread. *LWT – Food Science and Technology, 78*, 296–302. https://doi.org/10.1016/j.lwt.2016.12.042
71. Martin, J., Geisel, T., Maresch, C., Krieger, K., Stein, J. (2013). Inadequate nutrient intake in patients with celiac disease: results from a German dietary survey. *Digestion, 87*(4), 240–246. https://doi.org/10.1159/000348850
72. Martínez, M.M., Gómez, M. (2017). Rheological and microstructural evolution of the most common gluten-free flours and starches during bread fermentation and baking. *Journal of Food Engineering, 197*, 78–86.
73. Matos, M.E., & Rosell, C.M. (2015). Understanding gluten-free dough for reaching breads with physical quality and nutritional balance. *Journal of the Science of Food and Agriculture, 95*(4), 653–661.
74. Matos, M.E., Rosell, C.M. (2013). Quality indicators of rice-based gluten-free bread-like products: Relationships between dough rheology and quality characteristics. *Food and Bioprocess Technology, 6*(9), 2331–2341.
75. Meyer, D., Stavropolous, S., Diamond, B., Shane, E., Green, P.H.R. (2001). Osteoporosis in a north American adult population with celiac disease. *The American Journal of Gastroenterology, 96*(1), 112–119.
76. Miñarro, B., Albanell, E., Aguilar, N., Guamis, B., Capellas, M. (2012). Effect of legume flours on baking characteristics of gluten-free bread. *Journal of Cereal Science, 56*(2), 476–481.
77. Mir, S.A., Shah, M.A., Naik, H.R., Zargar, I.A. (2016). Influence of hydrocolloids on dough handling and technological properties of gluten-free breads. *Trends in Food Science & Technology, 51*, 49–57.
78. Mohammadi, M., Sadeghnia, N., Azizi, M.H., Neyestani, T.-R., Mortazavian, A.M. (2014). Development of gluten-free flat bread using hydrocolloids: Xanthan and CMC. *Journal of Industrial and Engineering Chemistry, 20*(4), 1812–1818.
79. Morais, E.C., Cruz, A.G., Faria, J.A.F., Bolini, H.M.A. (2014). Prebiotic gluten-free bread: Sensory profiling and drivers of liking. *LWT – Food Science and Technology, 55*(1), 248–254.
80. Morreale, F., Angelino, D., Pellegrini, N. (2018a). Designing a score-based method for the evaluation of the nutritional quality of the gluten-free bakery products and their gluten-containing counterparts. *Plant Foods for Human Nutrition (Dordrecht, Netherlands), 73*(2), 154–159.
81. Morreale, F., Garzón, R., Rosell, C.M. (2018b). Understanding the role of hydrocolloids viscosity and hydration in developing gluten-free bread. A study with hydroxypropylmethylcellulose. *Food Hydrocolloids, 77*, 629–635.
82. Naji-Tabasi, S., Mohebbi, M. (2015). Evaluation of cress seed gum and xanthan gum effect on macrostructure properties of gluten-free bread by image processing. *Journal of Food Measurement and Characterization, 9*(1), 110–119.
83. Nishida, C., Uauy, R., Kumanyika, S., Shetty, P. (2004). The joint WHO/FAO expert consultation on diet, nutrition and the prevention of chronic diseases: Process, product and policy implications. *Public Health Nutrition, 7*(1A, 1a), 245–250.
84. Nunes, M.H.B., Ryan, L.A.M., Arendt, E.K. (2009). Effect of low lactose dairy powder addition on the properties of gluten-free batters and bread quality. *European Food Research and Technology, 229*(1), 31–41.
85. O'Shea, N., Arendt, E.K., Gallagher, E. (2012). Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. *Innovative Food Science & Emerging Technologies, 16*, 1–10.
86. O'Shea, N., Doran, L., Auty, M., Arendt, E., Gallagher, E. (2013). The rheology, microstructure and sensory characteristics of a gluten-free bread formulation enhanced with orange pomace. *Food & Function, 4*(12), 1856–1863.
87. O'Shea, N., Ktenioudaki, A., Smyth, T.P., McLoughlin, P., Doran, L., Auty, M.A.E., Gallagher, E. (2015a). Physicochemical assessment of two fruit by-products as functional ingredients: Apple and orange pomace. *Journal of Food Engineering, 153*, 89–95.
88. O'Shea, N., Rößle, C., Arendt, E., Gallagher, E. (2015b). Modelling the effects of orange pomace using response surface design for gluten-free bread baking. *Food Chemistry, 166*, 223–230.
89. Onyango, C., Mutungi, C., Unbehend, G., Lindhauer, M.G. (2010). Rheological and baking characteristics of batter and bread prepared from pregelatinised cassava starch and sorghum and modified using microbial transglutaminase. *Journal of Food Engineering, 97*(4), 465–470.

90. Onyango, C., Mutungi, C., Unbehend, G., Lindhauer, M.G. (2011a). Modification of gluten-free sorghum batter and bread using maize, potato, cassava or rice starch. *LWT – Food Science and Technology, 44*(3), 681–686.
91. Onyango, C., Mutungi, C., Unbehend, G., Lindhauer, M.G. (2011b). Rheological and textural properties of sorghum-base formulations modified with variable amounts of native or pregelatinised cassava starch. *LWT – Food Science and Technology, 44*(3), 687–693.
92. Ouazib, M., Garzon, R., Zaidi, F., Rosell, C.M. (2016). Germinated, toasted and cooked chickpea as ingredients for breadmaking. *Journal of Food Science and Technology, 53(6),* 2664––2672.
93. Peña-Bautista, R.J., Hernandez-Espinosa, N., Jones, J.M., Guzmán, C., Braun, H.J. (2017). Wheat-based foods: Their global and regional importance in the food supply, nutrition and health. *Cereal Foods World, 62*(5), 231–249.
94. Penagini, F., Dilillo, D., Meneghin, F., Mameli, C., Fabiano, V., Zuccotti, G.V. (2013). Gluten-free diet in children: An approach to a nutritionally adequate and balanced diet. *Nutrients, 5*(11), 4553–4565.
95. Phongthai, S., D'Amico, S., Schoenlechner, R., Rawdkuen, S. (2016). Comparative study of rice bran protein concentrate and egg albumin on gluten-free bread properties. *Journal of Cereal Science, 72*, 38–45.
96. Pietzak, M. (2012). Celiac disease, wheat allergy, and gluten sensitivity: When gluten free is not a fad. *Journal of Parenteral and Enteral Nutrition, 36*(1, Suppl), 68S–75S.
97. Primo-Martín, C., van de Pijpekamp, A., van Vliet, T., de Jongh, H.H.J., Plijter, J.J., Hamerab, R.J. (2006). The role of the gluten network in the crispness of bread crust. *Journal of Cereal Science, 43*(3), 342–352.
98. Pruska-Kędzior, A., Kędzior, Z., Gorący, M., Pietrowska, K., Przybylska, A., Spychalska, K. (2008). Comparison of rheological, fermentative and baking properties of gluten-free dough formulations. *European Food Research and Technology, 227*(5), 1523–1536.
99. Radočaj, O., Dimić, E., Tsao, R. (2014). Effects of hemp (*Cannabis sativa* L.) seed oil press-cake and decaffeinated green tea leaves (*Camellia sinensis*) on functional characteristics of gluten-free crackers. *Journal of Food Science, 79*(3), C318–C325.
100. Rinaldi, M., Paciulli, M., Caligiani, A., Scazzina, F., Chiavaro, E. (2017). Sourdough fermentation and chestnut flour in gluten-free bread: A shelf-life evaluation. *Food Chemistry, 224*, 144–152.
101. Rodriguez Furlán, L.T., Pérez Padilla, A., Campderrós, M.E. (2015). Improvement of gluten-free bread properties by the incorporation of bovine plasma proteins and different saccharides into the matrix. *Food Chemistry, 170*, 257–264.
102. Ronda, F., Caballero, P.A., Quilez, J., Roos, Y.H. (2011). Staling of frozen partly and fully baked breads. Study of the combined effect of amylopectin recrystallization and water content on bread firmness. *Journal of Cereal Science, 53,* 97–103.
103. Ronda, F., Perez-Quirce, S., Lazaridou, A., Biliaderis, C.G. (2015). Effect of barley and oat β-glucan concentrates on gluten-free rice-based doughs and bread characteristics. *Food Hydrocolloids, 48*, 197–207. https://doi.org/10.1016/j.foodhyd.2015.02.031
104. Ronda, F., Perez-Quirce, S., Villanueva, M. (2017). Rheological Properties of Gluten-Free Bread Doughs: Relationship With Bread Quality. In J. Ahmed (Ed.), *Advances in Food Rheology and Its Applications*. Woodhead Publishing Series, pp. 297–334.
105. Rosell, C.M., Marco, C. (2008). Rice. In E.K. Arendt, F. Dal Bello (Eds.), *Gluten-Free Cereal Products and Beverages.* . Elsevier/Academic Press, London, pp. 81–111.
106. Różyło, R., Dziki, D., Gawlik-Dziki, U., Cacak-Pietrzak, G., Miś, A., Rudy, S. (2015). Physical properties of gluten-free bread caused by water addition. *International Agrophysics, 29*(3), 353–364.
107. Santos, F.G., Fratelli, C., Muniz, G., Capriles, V.D. (2018). Mixture design applied to the development of chickpea-based gluten-free bread with attractive technological, sensory, and nutritional quality. *Journal of Food Science, 83(1),* 188–197.
108. Saturni, L., Ferretti, G., Bacchetti, T. (2010). The gluten-free diet: safety and nutritional quality. *Nutrients, 2*(1), 16–34.
109. Scanlon, M.G., Zghal, M.C. (2001). Bread properties and crumb structure. *Food Research International, 34*(10), 841–864.
110. Scherf, K.A., Koehler P., Wieser, H. (2016). Gluten and wheat sensitivities – An overview. *Journal of Cereal Science* 67, 2–11.
111. Schober, T.J., Bean, S.R., Boyle, D.L. (2007). Gluten-free sorghum bread improved by sourdough fermentation: Biochemical, rheological, and microstructural background. *Journal of Agricultural and Food Chemistry, 55*(13), 5137–5146.
112. Schober, T.J., Bean, S.R., Boyle, D.L., Park, S.H. (2008). Improved viscoelastic zein-starch doughs for leavened gluten-free breads: Their rheology and microstructure. *Journal of Cereal Science, 48*(3), 755–767.
113. Schober, T.J., Messerschmidt, M., Bean, S.R., Park, S.-H., Arendt, E.K. (2005). Gluten-free bread from sorghum: quality differences among hybrids. *Cereal Chemistry, 82*(4), 394–404.
114. Schoenlechner, R., Siebenhandl, S., Berghofer, E. (2008). Pseudocereals. In E.K. Arendt, F. Dal Bello (Eds.), *Gluten-free cereal products and beverages.*. Elsevier/Academic Press, London, pp. 149–190.
115. Sciarini, L.S., Ribotta, P.D., León, A.E., Pérez, G.T. (2010). Influence of gluten-free flours and their mixtures on batter properties and bread quality. *Food and Bioprocess Technology, 3,* 577–585.
116. Sdepanian, V.L., de Miranda Carvalho, C.N., de Morais, M.B., Colugnati, F.A.B., Fagundes-Neto, U. (2003). Bone mineral density of the lumbar spine in children and adolescents with celiac disease on a gluten-free diet in São Paulo, Brazil. *Journal of Pediatric Gastroenterology and Nutrition, 37*(5), 571–576.
117. Segura, M.E., Rosell, C.M. (2011). Chemical composition and starch digestibility of different gluten-free breads. *Plant Foods for Human Nutrition (Dordrecht, Netherlands), 66*(3), 224–230.
118. Shin, D.-J., Kim, W., Kim, Y. (2013). Physicochemical and sensory properties of soy bread made with germinated, steamed, and roasted soy flour. *Food Chemistry, 141(1),* 517–523.
119. Simić, M., Žilić, S., Šimuruna, O., Filipčev, B., Škrobot, D., Vančetović J. (2018). Effects of anthocyanin-rich popping maize flour on the phenolic profile and the antioxidant capacity of mix-bread and its physical and sensory properties. *Polish Journal of Food and Nutrition Sciences, 68*(4), 299–308.
120. Singh, H., MacRitchie, F. (2001). Application of polymer science to properties of gluten. *Journal of Cereal Science, 33*(3), 231–243.
121. Slavin, J. (2013). Fiber and prebiotics: Mechanisms and health benefits. *Nutrients, 5*(4), 1417–1435.

122. Smith, B.M., Bean, S.R., Herald, T.J., Aramouni, F.M. (2012). Effect of HPMC on the quality of wheat-free bread made from carob germ flour-starch mixtures. *Journal of Food Science, 77*(6), C684–C689.
123. Stathopoulos, C.E. (2008). Dairy based ingredients. In E.K. Arendt, F. Dal Bello (Eds.), *Gluten-free cereal products and beverages,* Elsevier/Academic Press, London, pp. 217–236.
124. Talens, C., Álvarez-Sabatel, S., Rios, Y., Rodríguez, R. (2017). Effect of a new microwave-dried orange fibre ingredient vs. a commercial citrus fibre on texture and sensory properties of gluten-free muffins. *Innovative Food Science & Emerging Technologies, 44*, 83–88.
125. Taylor, J.N.R., Schober, T.J., Bean, S.R. (2006). Novel food and non-food uses for sorghum and millets. *Journal of Cereal Science, 44*(3), 252–271.
126. Taylor, J.R.N., Taylor, J., Campanella, O.H., Hamaker, B.R. (2016). Functionality of the storage proteins in gluten-free cereals and pseudocereals in dough systems. *Journal of Cereal Science*, *67*, 22–34.
127. Tharanathan, R.N., Mahadevamma, S. (2003). Grain legumes – a boon to human nutrition. *Trends in Food Science & Technology, 14*(12), 507–518.
128. Theethira, T.G., Dennis, M., Leffler, D.A. (2014). Nutritional consequences of celiac disease and the gluten-free diet. *Expert Review of Gastroenterology & Hepatology, 8*(2), 123–129.
129. Thompson, T. (1999). Thiamin, riboflavin, and niacin contents of the gluten-free diet: Is there cause for concern? *Journal of the American Dietetic Association, 99*(7), 858–862.
130. Thompson, T., Dennis, M., Higgins, L.A., Lee, A.R., Sharrett, M.K. (2005). Gluten-free diet survey: are Americans with coeliac disease consuming recommended amounts of fibre, iron, calcium and grain foods? *Journal of Human Nutrition and Dietetics, 18*(3), 163–169.
131. Tömösközi, S., Langó, B. (2017). Buckwheat: Its Unique Nutritional and Health-Promoting Attributes. In R.N. Taylor, J.M. Awika (Eds), *Gluten-free Ancient Grains: Cereals, Pseudocereals, and Legumes: Sustainable, Nutritious, and Health-Promoting Foods for the 21st Century*. Woodhead Publishing Limited, Cambridge, pp. 161–177.
132. Torbica, A., Hadnadev, M., Dapčević, T. (2010). Rheological, textural and sensory properties of gluten-free bread formulations based on rice and buckwheat flour. *Food Hydrocolloids, 24*(6–7), 626–632.
133. Torres, M., Arufe, S., Chenlo, F., Moreira, R. (2017). Coeliacs cannot live by gluten free bread alone – every once in a while they need antioxidants. *International Journal of Food Science and Technology 52,* 81–90.
134. Trappey, E.F., Khouryieh, H., Aramouni, F., Herald, T. (2015). Effect of sorghum flour composition and particle size on quality properties of gluten-free bread. *Food Science & Technology International, 21*(3), 188–202.
135. Tsatsaragkou K., Mandala, S.P. (2016). Structural role of fibre addition to increase knowledge of non-gluten bread. *Journal of Cereal Science, 67*, 58–67.
136. Tsatsaragkou, K., Gounaropoulos, G., Mandala, I. (2014). Development of gluten free bread containing carob flour and resistant starch. *LWT – Food Science and Technology, 58*(1), 124–129.
137. Turfani, V., Narducci, V., Durazzo, A., Galli, V., Carcea, M. (2017). Technological, nutritional and functional properties of wheat bread enriched with lentil or carob flours. *LWT – Food Science and Technology, 78*, 361–366.
138. Turkut, G.M., Cakmak, H., Kumcuoglu, S., Tavman, S. (2016). Effect of quinoa flour on gluten-free bread batter rheology and bread quality. *Journal of Cereal Science, 69*, 174–181.
139. Vallons, K.J.R., Ryan, L.A.M., Koehler, P., Arendt, E.K. (2010). High pressure-treated sorghum flour as a functional ingredient in the production of sorghum bread. *European Food Research and Technology, 231*(5), 711–717.
140. Wandersleben, T., Morales, E., Burgos-Diaz, C., Barahona, T., Labra, E., Rubilar, M., Salvo-Garrido, H. (2018). Enhancement of functional and nutritional properties of bread using a mix of natural ingredients from novel varieties of flaxseed and lupine. *LWT – Food Science and Technology, 91*, 48–54.
141. Wang, S., Karrech, A., Regenauer-Lieb, K., Chakrabati-Bell, S. (2013). Digital bread crumb: Creation and application. *Journal of Food Engineering, 116*(4), 852–861.
142. Wierdsma, N.J., van Bokhorst-de van der Schueren, M.A., Berkenpas, M., Mulder, C.J., van Bodegraven, A.A. (2013). Vitamin and mineral deficiencies are highly prevalent in newly diagnosed celiac disease patients. *Nutrients, 5*(10), 3975–3992.
143. Witczak, M., Juszczak, L., Ziobro, R., Korus, J. (2012). Influence of modified starches on properties of gluten-free dough and bread. Part I: Rheological and thermal properties of gluten-free dough. *Food Hydrocolloids, 28*(2), 353–360.
144. Witczak, M., Ziobro, R., Juszczak, L., Korus, J. (2016). Starch and starch derivatives in gluten-free systems – A review. *Journal of Cereal Science, 67*, 46–57.
145. Wronkowska, M., Piskuła, M.K., Zieliński, H. (2016). Effect of roasting time of buckwheat groats on the formation of Maillard reaction products and antioxidant capacity. *Food Chemistry, 196*, 355–358.
146. Wronkowska, M., Troszyńska, A., Soral-Smietana, M., Wołejszo, A. (2008). Effects of buckwheat flour [Fagopyrum esculentum Moench] on the quality of gluten-free bread. *Polish Journal of Food and Nutrition Sciences, 58*, 211–216.
147. Yazynina, E., Johansson, M., Jagerstad, M., Jastrebova, J. (2008). Low folate content in gluten-free cereal products and their main ingredients. *Food Chemistry, 111*(1), 236–242.
148. Yousif, E.I., Gadallah, M.G.E., Sorour, A.M. (2012). Physicochemical and rheological properties of modified corn starches and its effect on noodle quality. *Annals of Agricultural Sciences*, *57*, 19–27.
149. Zannini, E., Jones, J.M., Renzetti, S., Arendt, E.K. (2012). Functional replacements for gluten. *Annual Review of Food Science and Technology, 3*(1), 227–245.
150. Zhang, D., Mu, T., Sun, H. (2017). Comparative study of the effect of starches from five different sources on the rheological properties of gluten-free model doughs. *Carbohydrate Polymers, 176*, 345–355.
151. Zhang, Z-L., Zhou, M-L., Tang, Y., Li, F-L., Tang, Y-X., Shao, J. R., Xue, W-T., Wu, Y-M. (2012). Bioactive compounds in functional buckwheat food. *Food Research International, 49*, 389–395.
152. Zhu, F. (2018). Chemical composition and food uses of teff (Eragrostis tef). *Food Chemistry, 239,* 402–415.

153. Ziobro, R., Juszczak, L., Witczak, M., Korus, J. (2016). Non-gluten proteins as structure forming agents in gluten free bread. *Journal of Food Science and Technology, 53*(1), 571–580.
154. Ziobro, R., Korus, J., Juszczak, L., Witczak, T. (2013b). Influence of inulin on physical characteristics and staling rate of gluten-free bread. *Journal of Food Engineering, 116*(1), 21–27.
155. Ziobro, R., Witczak, T., Juszczak, L., Korus, J. (2013a). Supplementation of gluten-free bread with non-gluten proteins. Effect on dough rheological properties and bread characteristic. *Food Hydrocolloids, 32*(2), 213–220.

Submitted: 7 August 2018. Revised: 24 September 2018. Accepted: 17 October 2018. Published on-line: 3 December 2018.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 23–33
DOI: 10.31883/pjfns-2019-0003
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Phenolic Extracts from *Vaccinium corymbosum* L. Loaded in Microemulsions and Liposomes as Enhancers of Olive Oil Oxidative Stability

Nikolina Liović[1], Perica Bošković[2], Ivana Drvenica[3], Anet Režek Jambrak[4], Ana Marija Dropulić[5], Greta Krešić[1], Viktor Nedović[6], Branko Bugarski[7], Zoran Zorić[8], Sandra Pedisić[8], Tea Bilušić[5]*

[1]*Faculty of Tourism and Hospitality Management, University of Rijeka, Primorska 42 P.O. Box 97, 51410, Opatija, Croatia*
[2]*Faculty of Science, University of Split, Ruđera Boškovića 33, 21000 Split, Croatia*
[3]*Institute for Medical Research, University of Belgrade, Dr Subotića Starijeg 4, 11000 Belgrade, Serbia*
[4]*Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia*
[5]*Faculty of Chemistry and Technology, University of Split, Ruđera Boškovića 35, 21000 Split, Croatia*
[6]*Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia*
[7]*Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia*
[8]*Faculty of Food Technology and Biotechnology, Centre for Food Technology and Biotechnology, Petra Kasandrića 6, 23000 Zadar, Croatia*

Key words: phenolics, blueberry, microemulsions, liposomes, antioxidant, ultrasound

Natural phenolic compounds are recognized as bioactive ingredients in food but can also have a role as effective alternatives to synthetic antioxidants in stability improvement of foods prone to oxidation, such as edible oils. This study aimed at the preparation and HPLC-DAD characterization of phenolic extracts from *Vaccinium corymbosum* L. (raw, pasteurized, freeze-dried and treated with high-intensity ultrasound), and at testing their antioxidant potential in the prevention of olive oil oxidation in the native state and encapsulated into microemulsions and liposomes systems. Water-in-oil structured microemulsions used in this study were prepared using mechanical, ultrasonic, and high pressure homogenization. Liposomes with the average size of 589.1±2.9 nm were produced with the proliposome method using commercially available phosphatidylcholine – Phospolipon 90G. The obtained results showed significant prolongation of the oxidative stability of extra virgin olive oil enriched with encapsulated blueberry phenolic extracts than with native phenolic extracts, regardless of the method used for blueberry processing. Phenolic extracts encapsulated in microemulsions had a stronger effect on the prolongation of olive oil oxidative stability in comparison with the extracts encapsulated in liposomes. The average prolongation rate of oxidative stability was 45.65% by phenolic extracts encapsulated in microemulsions prepared by mechanical homogenization ($p=0.012$), and 58.72% by microemulsions prepared by ultrasound homogenization ($p=0.011$). Phenolic extracts encapsulated in microemulsions prepared by high pressure homogenization had no effect on oil oxidative stability prolongation.

INTRODUCTION

Phenolics are the most common phytochemicals in a human diet that comprise a variety of compounds with a great diversity of structures and biological functions (antioxidant, anti-inflammatory, antimicrobial, anticancer, antiproliferative, antiatherosclerotic properties) [Giampieri *et al*., 2017; Haminiuk *et al*., 2012]. In addition to being considered as potent bioactive food ingredients they are also used to enhance the color, the flavor and the shelf life/stability of different foods prone to oxidation, such as edible oils [Aladedunye *et al*., 2014; Asnaashari *et al*., 2015].

Among plant-derived sources of natural phenolics, blueberries (*Vaccinium corymbosum* L.) are one of the richest sources per serving size [Pérez-Jiménez *et al*., 2010]. Composition, stability and biological activity of phenolics originated from blueberry were already reported [Nile & Park, 2014; Rodríguez-Mateos *et al*., 2012]. Specifically, blueberries are characterized by a high content of anthocyanins that have been associated with a reduced risk of hypertension and myocardial infarction [Afrin *et al*., 2016], a preventive effect on metabolic syndrome [Basu & Lyons, 2012], beneficial effects on lipid profiles, and an inhibitory effect on atherosclerosis development [Huang *et al*., 2016]. The beneficial health effects of phenolic compounds depend not only on food intake but also on their stability which can vary due to the used post-harvest processing methods and the storage conditions of blueberry fruits [Michalska & Lysiak, 2015]. For instance, conventional fruit processing methods, such as pasteurization, which are still most commonly used, provide microbiological stability and longer shelf-life, but, on the other hand, their application can provoke the degradation of phenolic compounds. Therefore, researchers are nowadays more fo-

* Corresponding Author: Tel.: +385 51 294 707; Fax: +385 51 292 945; E-mail address: nikolina.liovic@fthm.hr (N. Lović)

cused on the usage of novel food processing technologies that are able to preserve valuable phenolic compounds, such as freeze-drying, pulsed electric fields, high-intensity ultrasound, and high pressure processing [Barba *et al*., 2017; Sablani *et al*., 2011].

Apart from the conventional and modern processing technologies used to preserve phenolics, the use of various encapsulation methods has become extremely important for further protection of these unstable compounds and prolongation of their biological activities. In that sense, the use of different encapsulation techniques (spray drying, coacervation, emulsions, liposomes, micellae, nanoparticles, freeze-drying, cocrystallization) in combination with phenolic extracts or pure phenolic compounds has been reported so far [Chatzidaki *et al*., 2015; Drvenica *et al*., 2017]. The use of emulsions is considered as one of the most promising and rapidly developing tools in the food industry due to their high-efficiency encapsulation rate, the maintenance of chemical stability of a targeted compound, and its controlled release [Lu *et al.*, 2016]. The mean droplet diameter of emulsions within food systems is typically somewhere between 100 nm and 100 μm. If dispersed phase droplets sizes are less than 100 nm, these (nano)emulsions are referred to as microemulsions [McClements, 2012]. Microemulsions, in particular, have beneficial properties that are suitable for designing functional foods due to their optical clarity, thermodynamical stability (high stability to gravitational separation, flocculation, and coalescence), and improved absorption, stability and bioavailability of bioactive components [Xu *et al*., 2017]. Liposomes are also interesting encapsulation systems, which have been widely used in the pharmaceutical and food industries. Due to the amphiphilic nature of the phospholipid bilayer, they have the ability to carry a variety of lipophilic and hydrophilic bioactive compounds. In recent years, liposomes have been used for the encapsulation of different phenolic compounds, such as hibiscus extract [Gibis *et al*., 2014], resveratrol [Isailović *et al*., 2013], green tea catechin and epigallocatechin gallate [Rashidinejad *et al*., 2014], as well as curcumin [Gómez-Mascaraque *et al*., 2017]. Gortzi *et al.* [2008] have proven that the antioxidant and antimicrobial activity of the *Myrtus communis* extract encapsulated in liposomes was higher compared to the extract in its pure form.

Despite the benefits arising from literature data on different processing methods used prior to the extraction of phenolics and their subsequent protection by encapsulation, there is a lack of such studies performed on blueberry fruits. Thus, the major objectives of this study were: (1) to determine and to compare the phenolic profile of blueberry extracts obtained from fruits in raw state and after pasteurization, freeze-drying, and high-intensity ultrasound by HPLC-DAD technique; (2) to prepare microemulsions using mechanical, ultrasound and high pressure homogenization, to characterize them and to encapsulate blueberry phenolic extracts into microemulsions; (3) to prepare and to characterize liposomes and to encapsulate blueberry phenolic extracts into liposome systems; and finally (4) to determine the effect of native and encapsulated blueberry phenolic extracts on olive oil oxidative stability by a Rancimat instrument.

TABLE 1. Experimental design of ultrasound treatment.

Sample	Amplitude (%)	Treatment time (min)	E (J)
A1	100	6	26621
A2	50	6	16154
A3	50	9	21385
A4	100	9	33623
A5	75	6	19904
A6	50	3	8625
A7	75	6	20391
A8	75	3	11101
A9	100	3	13902
A10	75	6	19899
A11	75	9	28762

MATERIAL AND METHODS

Sample preparation

Fresh blueberry samples were purchased in a local grocery store. One part of the samples was taken for dry matter determination and the other was blended into a blueberry puree. Part of the blueberry puree was separated and stored as a fresh control sample at –20°C under nitrogen atmosphere. The other part was subjected to subsequent processing. Total dry matter and moisture content were determined gravimetrically according to the AOAC 930.04 method by drying the samples in a thermal oven (Memmert, UF30, Schwabach, Germany) at 105°C until a constant weight has been achieved [Helrich, 1990].

Ultrasound treatment

High intensity ultrasound treatment was performed with an ultrasonic processor SONICATOR S-4000 (Misonix Sonicators, Newtown, CT, USA) with a probe diameter of 19.1 mm, at a frequency of 20 kHz. Blueberry puree (100 mL) was placed in a beaker (200 mL), which was used as a treatment vessel. Ultrasonication was carried out for 3, 6 and 9 min at an amplitude of 50, 75, and 100%. The experiment was designed in STATGRAPHICS Centurion (Stat Point Technologies, Inc., Warrenton, VA, USA) software using Multi-Factor Categorical Design. The experiment consisted of 11 experimental trials and one of them was replicated to get a good estimate of a potential experimental error (Table 1). Repetition experiments were carried out after other experiments followed by the order of runs designed by the program. After all treatments, the samples were stored at –20°C.

Freeze drying

Freeze drying was carried out in a freeze dryer (Labconco, Kansas City, USA) at a temperature of –55°C. Freeze-dried blueberries were finely ground into a powder and stored in a dry and dark place at the temperature below 20°C.

Pasteurization

Pasteurization was performed in a thermal oven (Memmert, UF30, Schwabach, Germany) at the temperature of 80°C for 5 min after which the sample was cooled down in cold water. The extraction of phenolics was done immediately after the pasteurization treatment.

Phenolics extraction and determination of total phenolic content

Extraction of phenolic compounds from blueberry samples was carried out according to the method described by Dragović-Uzelac *et al*. [2010]. Briefly, 5 g of blueberry puree and 0.5 g of freeze-dried blueberry powder were weighed and extracted using 20 mL of 80% (v/v) aqueous ethanol for 20 min under nitrogen atmosphere and filtered through filter paper Whatmann no. 40 (Whatmann International Ltd., Kent, UK) using a Büchner funnel. The extraction of the residue was repeated and the filtrates were combined and adjusted to 50 mL in a volumetric flask with 80% (v/v) aqueous ethanol.

The total phenolic content (TPC) in extracts was determined using the Folin-Ciocalteau reagent [Singleton & Rossi, 1965], and results were expressed as gallic acid equivalents (GAE) per 100 g of fresh weight (fw). All determinations were run in triplicate.

Analysis of phenolic compounds using HPLC-DAD

Chromatographic separation was performed using an HPLC instrument with Agilent 1260 Infinity quaternary LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array detector (PDA), an automatic injector, and ChemStation software. Extracts were previously filtered through a 0.45-μm pore size membrane filter (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The separation of compounds was performed on a Nucleosil 100–5C18, 5 μm (250 mm × 4.6 mm i.d.) column (Macherey--Nagel GmbH & Co. KG, Düren, Germany). Solvent composition and gradient conditions were as previously described by Zorić *et al*. [2014].

For gradient elution, mobile phase A contained 3% of formic acid in water (v/v), while solution B contained 3% of formic acid in 80% acetonitrile (v/v). The elution program was as follows: 0–28 min 0% B, 28–35 min 25% B, 35–40 min 50% B, 40–45 min 80% B, and for the last 10 min again 0% B at a flow rate of 0.8 mL/min. The injection volume was 5 μL. Calculation of the concentrations was based on the external standard method and the compounds were identified by comparing their retention times and absorption spectra with those of the authentic standards (anthocyanins were identified at 520 nm, phenolic acid at 280 nm and flavonol glycoside at 360 nm).

All anthocyanin standards, delphinidin-3-glucoside (D--3-G), cyanidin 3-glucoside (C-3-G), petunidin 3-glucoside (Pt-3-G), peonidin 3-glucoside (Pn-3-G), and malvidin 3-glucoside (M-3-G) were prepared as stock solutions in acidified methanol (1% of formic acid in methanol, v/v) at a concentration of 100 mg/L. Working standard solutions were prepared by diluting the stock solution to yield five concentrations in a range from 20 to 100 mg/L.

Standards of quercetin-3-glucoside (Q-3-G) and chlorogenic acid (CA) were prepared as stock solutions in ethanol/water (8:2, v/v) at the following concentrations: Q-3-G 400 mg/L and CA 52 mg/L. Working standard solutions were prepared by diluting the stock solution to yield five concentrations in the range from 80 to 400 mg/L for Q-3-G and from 10.4 to 52 mg/L for CA. The results were expressed in mg per 100 g of fresh weight, as mean value ± standard deviation (N=2 replicates).

Preparation of microemulsions

Two microemulsion samples (ME-1 and ME-2) were prepared using the best combination of extra virgin olive oil as an oil phase at the percentage obtained from preliminary studies by diluting the combination of oil and a surfactant/cosurfactant mixture with water using a magnetic stirrer and an Ultra-Turrax T25 Homogenizer (IKA Labortechnik, Staufen, Germany) for 5 min. The surfactant to cosurfactant ratio (*i.e.* Tween 80 to ethanol) was 9:1 (w/w). The mixture turned from turbid through opaque to a translucent yellow mixture, forming a microemulsion. The obtained microemulsions contained: 70% (w/w) of the surfactant/cosurfactant mixture, 20% (w/w) of extra virgin olive oil, and 10% of aqueous phase in the case of ME-1 sample; and 70% (w/w) of the surfactant/cosurfactant mixture, 10% (w/w) of extra virgin olive oil, and 20% of aqueous phase in the case of ME-2 sample. Apart from the preparation of microemulsions by mechanical homogenization, a previously mentioned mixture containing water, olive oil, Tween 80 and ethanol was treated in a Ultrasonic Lab Homogenizer UP200S (Hielscher Ultrasonics GmbH, Teltow, Germany) for 5 min. The frequency was 20 kHz with an amplitude of 70%. The third way to prepare emulsion systems was to use a High-Pressure Homogenizer (EmulsiFlex-C3, Avestin Europe GmbH, Mannheim, Germany). Three cycles under 1000 bar were applied with the constant flow at 50 mL/min.

Preparation of liposomes

Liposomes with phenolic extracts were prepared using the proliposome method as described by Pravilovic *et al*. [2015]. This method, easy scalable for industrial application, implies the initial formation of a proliposome mixture containing lipid, ethanol, and water, which is eventually converted into liposomes by a simple dilution step [Perrett *et al*., 1991]. Commercially available soy lecithin Phospholipon 90G (Phospholipid GmbH, Köln, Germany), which consists of phosphatidylcholine (min. 94%), was used for the preparation of liposomes. Namely, the mixture of Phospholipon 90G with an adequate amount of ethanol and water (1:0.8:2, w/w/w) was heated up to 60°C in a sealed beaker with continuous stirring at 800 rpm until a homogenous dispersion has been obtained. After cooling the mixture down to 25°C, a small portion of phenolic extract was added to the dispersion while stirring at 800 rpm during the next 30 min to obtain the final liposomal formulation. The ratio between phenolic extracts and Phospholipon 90G used in liposomes was 1:10 (w/w).

Characterization of microemulsions and liposomes

Droplet size, polydispersity index, and zeta potential determination

The droplet size, polydispersity index (PDI), and zeta potential of microemulsions and liposome samples were

measured with photon correlation spectroscopy using Zetasizer Nano ZS (Malvern Panalytical Ltd, Malvern, UK). Each sample was measured three times at the room temperature and the averages were taken as a result. Furthermore, the liposome samples were diluted by 1000 with ultra-pure water before the measurements to avoid multiple scattering effects. Physical stability of the prepared liposomes stored at +4°C was monitored through repeated photon correlation spectroscopy measurements after 7, 14, and 21 days.

Liposome encapsulation efficiency

The encapsulation efficiency in liposomes was calculated based on the determination of TPC through centrifugal separation of un-encapsulated phenolic extract from loaded liposomes, as described by Isailović *et al*. [2013].

Viscosity, conductivity, and surface tension measurements

The viscosity of microemulsion samples was measured at 25°C with a Brookfield viscometer (LVDV-E, Brookfield Engineering Laboratories, Middleboro, MA, USA) using spindle no. 61. with a shear rate of 30 rpm. The electrical conductivity and surface tension of microemulsion samples were also measured at 25°C with a conductivity meter (inoLab 7310, WTW GmbH, Weilheim, Germany) using conductivity cells with a cell constant of 1.0 and a tensiometer (SIGMA 703D, Biolin Scientific Oy, Espoo, Finland) using the ring method, respectively. The conductivity of liposome samples was determined using a Zetasizer Nano ZS apparatus (Malvern Panalytical Ltd, Malvern, UK). All measurements were performed in triplicate.

Physical stability study

The physical stability of microemulsions (ME-1 and ME-2) was studied with regard to the thermal stability and centrifugation effects. Microemulsions were kept at different temperatures (4°C, 25°C, and 35°C) and observed for phase separation, precipitation or flocculation. Also, microemulsions were centrifuged (Sorvall RC 5B Superspeed Centrifuge, Dupont, Newton, USA) at 8000 rpm for 20 min at the temperature of 25°C and inspected for any change in their homogeneity.

Evaluation of the antioxidant potential of phenolic extracts with the Rancimat method

The oxidative stability of extra virgin olive oil enriched with phenolic extracts from blueberries (in the native form and encapsulated in microemulsions and liposomes) was evaluated using a Rancimat 743 instrument (Metrohm, Herisau, Switzerland) to monitor the progress of accelerated oxidation at the temperature of 120°C. The olive oil samples (3 g) were enriched with 0.2 g of phenolic extracts and with 0.4 g of microemulsions and liposomes containing phenolic extracts (2:1 ratio).

The samples were tested at a temperature of 120°C (ΔT= 1.4°C) with a constant air flow of 20 L/h. The conductivity was measured as a function of time and the results are expressed as a prolongation of the induction time (in hours). All determinations were performed in triplicate, and the results are presented as mean value ± standard deviation.

Statistical analysis

Data analyses were performed using MedCalc Statistical Software version 14.8.1 (MedCalc Software, Ostend, Belgium; http://www.medcalc.org; 2014). Since the data could not meet the criteria of variance homogeneity (Levene's test) and normal distribution (determined with Shapiro–Wilks test), the analysis was performed with Kruskal-Wallis test. In all analyses, p-values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Influence of different processing treatments on blueberry phenolic profile and TPC

To the best of our knowledge, this study is the first report that compares the phenolic profile and TPC of blueberry extracts obtained from raw plant material, and from plant material treated by conventional (pasteurization) and modern processing technologies (freeze-drying, high-intensity ultrasound).

Table 2 shows contents of individual anthocyanins, flavonol glycosides, and chlorogenic acid in different blueberries extracts. As expected, anthocyanins were the most abundant phenolic compounds and five anthocyanidin glycosides were identified. The predominant anthocyanidin glycosides in fresh (A0), freeze-dried (AL), and in all ultrasound-treated samples (A1-A11) were malvidin and delphinidin glycosides that together accounted for almost 80% of all detected anthocyanins. The obtained results are in accordance with previously reported data [Neveu *et al*., 2010]. In the pasteurized sample (AP), due to thermal degradation, only malvidin and peonidin glycosides were detected and total anthocyanin content was reduced by almost 80%. Total anthocyanin content calculated as the sum of all individual anthocyanidin glycosides was lower in comparison to other studies [Može *et al*., 2011; Rodríguez-Mateos *et al*., 2012]. The content of chlorogenic acid in all samples was remarkably lower and that of flavonol glycosides was higher than previously reported data [Može *et al*., 2011; Neveu *et al*., 2010; Rodríguez-Mateos *et al*., 2012]. These differences may be attributed to the inherent variability in the plant material due to growing location and conditions but also because of different methodologies used to quantify phenolic compounds.

Concerning the TPC among different extracts (Table 3), the obtained results indicate that the freeze-dried extract had a 33% higher phenolic content as compared to the raw plant material (fresh, untreated puree). This is in accordance with the findings of Sablani *et al*. [2011] who reported improved retention of phytochemicals after freeze-drying treatment. Freeze-drying may lead to a higher extraction efficiency due to the formation of ice crystals which cause the rupturing of cell structure and thus better solvent access during the extraction [Keinänen & Julkunen-Tiitto, 1996]. Brownmiller *et al*. [2008] investigated the effect of thermal treatment on blueberry products and found a significant loss of anthocyanins (28 to 95%), which is in accordance with results of this study. Pasteurized blueberry puree had a significantly lower amount of total phenolics as compared to the raw sample due

TABLE 2. Concentration of phenolic compounds in blueberry extracts.

Compound	Samples (mg/100 g fw)													
	A0	AP	AL	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Anthocyanins														
Del-3-gal	14.71±0.03	–	11.29±0.01	6.55±0.02	5.91±0.01	7.19±0.01	7.02±0.00	5.32±0.01	7.10±0.00	6.56±0.01	7.24±0.00	6.74±0.05	6.27±0.00	6.45±0.06
Del-3-glu	–	–	–	2.01±0.01	1.42±0.02	2.02±0.00	1.74±0.05	1.46±0.04	1.73±0.04	1.78±0.01	1.85±0.00	1.60±0.03	1.43±0.00	1.31±0.15
Cy-3-gal	2.03±0.03	–	1.89±0.01	1.06±0.02	1.01±0.01	1.16±0.00	1.03±0.01	0.97±0.06	1.20±0.00	1.14±0.03	1.26±0.01	1.08±0.01	1.08±0.03	0.93±0.06
Del-3-ara	9.11±0.01	–	6.35±0.04	5.36±0.00	4.91±0.01	6.14±0.09	5.31±0.01	4.66±0.05	5.60±0.06	5.18±0.11	5.82±0.06	5.05±0.05	5.08±0.01	4.89±0.01
Pet-3-gal	9.10±0.00	–	7.32±0.04	3.84±0.05	3.69±0.02	4.23±0.01	3.98±0.03	3.41±0.01	3.99±0.01	3.64±0.07	4.07±0.04	3.74±0.00	3.51±0.01	3.52±0.03
Pet-3-glu	0.89±0.01	–	0.86±0.02	2.30±0.01	2.10±0.08	2.59±0.02	2.22±0.03	2.08±0.01	2.23±0.01	2.18±0.01	2.35±0.02	2.05±0.06	2.00±0.00	1.91±0.00
Peo-3-gal	0.78±0.02	1.35±0.07	0.45±0.04	0.59±0.01	0.66±0.01	0.56±0.00	0.62±0.02	0.51±0.03	0.50±0.01	0.40±0.00	0.61±0.02	0.51±0.01	0.53±0.01	0.40±0.02
Peo-3-glu	3.91±0.02	–	3.57±0.0	3.49±0.04	2.99±0.02	3.30±0.01	2.88±0.04	2.60±0.08	2.80±0.00	2.78±0.01	3.29±0.01	2.89±0.02	2.35±0.00	2.52±0.02
Mal-3-gal	31.54±0.13	14.86±0.06	25.46±0.06	17.69±0.04	17.62±0.05	18.96±0.02	17.59±0.03	16.35±0.07	17.75±0.06	16.59±0.11	18.20±0.01	17.18±0.03	16.90±0.03	15.95±0.00
Mal-3-glu	0.84±0.03	4.47±0.00	1.11±0.03	8.45±0.01	8.26±0.04	9.13±0.01	7.85±0.04	7.35±0.02	7.94±0.08	7.78±0.01	7.82±0.03	7.45±0.00	7.11±0.01	7.12±0.7
Mal-3-ara	16.63±0.09	–	16.52±0.01	12.33±0.04	12.00±0.01	13.05±0.05	11.54±0.02	10.94±0.04	11.73±0.04	11.28±0.03	11.96±0.00	11.25±0.03	10.73±0.00	10.34±0.04
Total	**89.54**	**20.63**	**74.82**	**63.67**	**60.57**	**68.15**	**61.78**	**55.65**	**62.57**	**59.31**	**64.47**	**59.54**	**56.9**	**55.34**
Other phenolics														
Q (der.1)	4.54±0.08	–	10.40±0.04	7.31±0.01	5.00±0.04	5.51±0.01	5.03±0.02	5.99±0.02	6.47±0.04	5.99±0.02	7.79±0.02	7.76±0.06	6.98±0.05	6.45±0.01
Q (der.3)	2.79±0.02	0.17±0.00	–	–	–	1.34±0.01	–	–	–	–	–	–	–	–
Q-3-glu	4.55±0.01	2.01±0.01	–	–	–	–	0.97±0.6	–	0.93±0.04	0.90±0.00	1.00±0.02	0.89±0.01	0.98±0.02	0.83±0.00
Q (der.5)	1.72±0.04	0.73±0.04	2.39±0.01	1.77±0.01	1.70±0.01	2.03±0.00	1.67±0.00	1.61±0.01	1.68±0.03	2.75±0.00	2.04±0.01	2.05±0.05	1.95±0.06	2.95±0.07
CA	7.74±0.06	5.64±0.06	9.68±0.02	3.04±0.05	2.01±0.01	3.23±0.01	3.04±0.02	7.58±0.00	2.07±0.00	3.36±0.01	2.78±0.01	2.95±0.07	2.32±0.01	2.90±0.02
Total	**21.34**	**8.55**	**22.47**	**12.12**	**8.71**	**12.11**	**10.71**	**15.18**	**11.15**	**13.00**	**13.61**	**13.65**	**12.23**	**13.13**

Results are expressed as mean value ± SD. Dry matter content in freeze-dried sample was 98% and in other samples 15.88%.
Untreated (fresh) sample was denoted as A0, pasteurized sample as AP, freeze-dried (lyophilized) sample as AL and ultrasound treated samples as A1-A11 (see Table 1).
Q = Quercetin; Q-3-glu = Quercetin-3-glucoside; CA = Chlorogenic acid; Del-3-gal = Delphinidin-3-galactoside; Del-3-glu = Delphinidin-3-glucoside; Cy-3-gal = Cyanidin-3-galactoside; Del-3-ara = Delphinidin-3-arabinoside; Pet-3-gal = Petunidin-3- galactoside; Pet-3-glu = Petunidin-3-glucoside; Peo-3-gal = Peonidin-3-galactoside; Peo-3-glu = Peonidin-3-glucoside; Mal-3-gal = Malvidin-3-galactoside; Mal-3-glu = Malvidin-3-glucoside; Mal-3-ara = Malvidin-3-arabinoside.

TABLE 3. Total phenolic content (TPC) of blueberry extracts.

Sample	TPC (mg GAE/100 g fw)		
	Median	Min	Max
A0	312.58[b]	302.02	338.98
AP	276.67[a,c,d,f,i,l,m,n]	197.47	288.28
AL	417.54[b,d,e,g,h,j,k,m]	391.87	419.25
A1	327.36[b,c]	280.90	333.70
A2	284.06[c,f,i,l]	274.56	287.23
A3	321.02[b,e,h]	315.74	341.09
A4	290.40[c]	285.12	308.35
A5	289.34[c,f,i]	268.22	292.51
A6	332.64[b,e,h,j]	325.25	332.64
A7	284.06[c,i]	254.50	326.30
A8	307.30[c]	283.01	319.97
A9	355.87[b,e]	279.84	365.38
A10	321.02[b,c]	283.01	350.59
A11	325.25[b]	295.68	326.30
p-value	*0.041*		

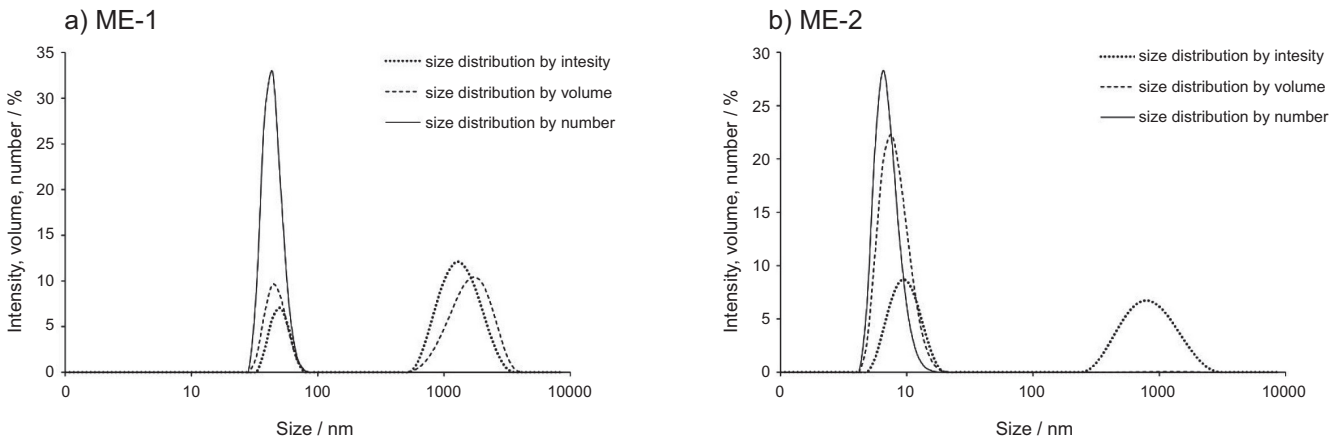
Medians compared with Kruskal Wallis test. Statistically significant differences ($p<0.05$) from: [a]A0, [b]AP, [c]AL, [d]A1, [e]A2, [f]A3, [g]A4, [h]A5, [i]A6, [j]A7, [k]A8, [l]A9, [m]A10, [n]A11.
A0 = Untreated (fresh) sample; AP = pasteurized sample; AL = freeze-dried (lyophilized) sample; A1-A11 = ultrasound treated samples (see Table 1).

to thermal sensitivity of phenolics. However, another study has shown that pasteurization significantly decreased only anthocyanin content but not TPC which could be explained by condensation reactions between anthocyanins and other polymeric compounds resulting in the formation of polymerized phenolic compounds that can be detected with the Folin-Ciocalteau reagent [Casati *et al*.2017]. In addition, an interesting study conducted by Howard *et al*. [2016] showed that stability of anthocyanins during pasteurization can be achieved with previous acidification of juice samples to pH 2.1. No significant changes in TPC were observed after ultrasound treatments, however, the recovery of phenolics mostly increased after the shortest treatment (3 min) at amplitudes of 50 and 100%. A slight decrease in total phenolic content was found at higher temperatures at the end of the process and this effect was more pronounced with longer treatments time (6 and 9 min). Overall, the obtained results showed that none of the ultrasound treatments had negative effects on the TPC of the blueberry fruits. As previous studies showed, ultrasonic treatments can ensure the microbial safety of foods, while their influence on phenolic compounds is minimal [Golmohamadi *et al*., 2013].

Characterization of microemulsions and liposomes

For the characterization of microemulsions and liposome systems, particle size as well as particle size distribution represent indispensable factors due to their effect on the emulsification properties, viscosity, and stability.

Emulsion systems used in this study were prepared by mechanical, ultrasound and high pressure homogenization to obtain microemulsions with enhanced stability. Regardless of the preparation technique, the microemulsion samples had a droplet size distribution with a PDI value of 0.683 ± 0.015 and droplet size of 50.43 ± 8.17 nm for microemulsion 1 (ME-1) and a PDI value of 0.722 ± 0.023 and droplet size of 9.87 ± 1.63 nm for microemulsion 2 (ME-2), which indicates that a stable microemulsion was formed. Focusing on size distribution by intensity in Figure 1, large peaks around 1000 nm may be caused by few large particles (commonly called "dust") which are also the main reason of higher PDI. A confirmation of these previous statements is the absence of these peaks in size distribution by number.

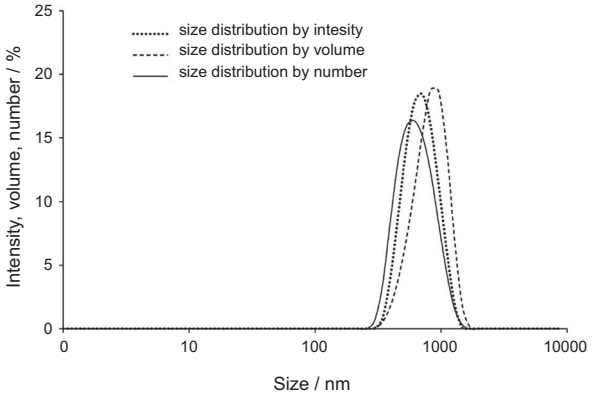


Formulation	Refractive index	Zeta-potential (mV)	Conductivity (ms/cm)	Surface tension (nM/m)	Viscosity (cP)
ME-1	1.4616	-7.16	0.0352	32.20	907
ME-2	1.4510	-2.11	0.0280	31.87	1105

FIGURE 1. The droplet size distribution by intensity, volume, and number for: a) ME-1 and b) ME-2 microemulsion, as well as a refractive index, zeta-potential, conductivity, surface tension, and viscosity. Composition of ME-1 and ME-2 is provided in Materials and Methods section.

The obtained results are in accordance with the study by Jafari *et al.* [2006], who concluded that emulsification required optimal conditions for both methods beyond which the emulsion sizes would either increase or have little change with further processing. It is well known that there is a strong correlation between the specific structure of the microemulsion systems and their electrical conductive behavior, where phase systems ("oil in water" or "water in oil") of the microemulsions were determined by measuring specific conductivity [Eicke *et al*., 1989]. In this study, microemulsion formulations had a specific conductivity of 0.035 mS/cm for ME-1 and 0.028 mS/cm for ME-2, which indicates the "water in oil" structure of microemulsions. Also, "water in oil" structure of microemulsions is confirmed with color tests. The mean viscosity of formulations was 907 cps for ME-1 and 1105 cps for ME-2. The highest viscosity was found for ME-2, which indicates a possible change from "water in oil" to a bicontinuous structure. The surface tension of the formulations was 32.20 mN/m for ME-1 and 31.67 mN/m for ME-2. It can be concluded that both samples had a "water-in-oil" structure because the surface tension and refractive index values were very close to the values of an oil continuous phase. The visual examination experiment was carried out over a period of 6 months in weekly intervals for the first 3 months and monthly intervals for the subsequent months. The visual observation showed no evidence of phase separation or any precipitation or flocculation. These samples also revealed no sign of phase separation under centrifugation stress at 8000 rpm for 20 min. The centrifugation tests showed that microemulsions remained homogenous without any phase separation, which points to their good physical stability.

The two produced microemulsions, ME-1 and ME-2, were used as a "premix" for further investigation on the blueberry phenolic extract incorporation by an appropriate method in a portion of 30% (v/v). Interestingly, the microemulsion containing phenolic extracts from blueberries had nearly the same size distribution as the one without extracts (data not shown). Incorporation of various phenolic compounds in microemulsions results in small changes in the apparent hydrodynamic diameter of the aqueous droplets [Chatzidaki *et al*., 2015]. This result was also confirmed by Jafari *et al*. [2006] that a critical point above which the emulsions become "over-processed" exists and that only slight effect on emulsion droplet size can be achieved from "premix" (ME-1 and ME-2) to final emulsions. This outcome was established even when different methods were combined (mechanical homogenization for premix production and high-pressure homogenization for final emulsion formation), as shown by the study of Juttulapa *et al.* [2017].



Formulation	Zeta-potential (mV)	Conductivity (ms/cm)
LIPOSOMES	-39.31±0.9	0.00879±0.01

FIGURE 2. The size distribution by intensity, volume, and number of liposomes used in the study, as well as the zeta-potential and conductivity.

The average size of liposomes (z-average) used in this study was 589.1±2.9 nm. The size distribution of liposomes shown in Figure 2 by intensity, volume, and number %, revealed very monodispersed distribution with a PDI of 0.172±0.019. This result was in agreement with a study of Isailović *et al*. [2013], where in a comparison of a few different methods for the preparation of liposome, the proliposome method gave the lowest PDI value (below 0.2.), confirming once more its efficacy and relevance for industrial application. Liposome vesicles had a negative surface charge with mean zeta potential of -39.1±0.9, suggesting good electrostatic stabilization of dispersion against aggregation [Isailović *et al*., 2013; Rashidinejad *et al*., 2014]. This was also confirmed by the stability test results, where the mean diameter of liposomes did not change by more than 9% during three weeks of storage at +4°C (data not shown). Liposomes containing phenolic extracts from blueberries had nearly the same size as liposomes without extracts. This outcome is consistent with the results reported by Gibis *et al*. [2014] who encapsulated hibiscus extract in soy lecithin liposomes. Furthermore, loaded liposomes showed no significant change of mean zeta potential, indicating that phenolics are located inside the vesicles, most likely *via* hydrogen bonding between polar head groups of liposomes and phenolics, and hydrophobic interactions of hydrophobic moieties of the phenolics and the fatty acid tails of phospholipids in liposomes [Gibis *et al*., 2012]. The encapsulation efficiency of the produced liposomal formulation was 70.5±0.8%, which is very similar to other reports of lecithin liposomes loading green tea and hibiscus extracts [Gibis *et al*., 2014; Rashidinejad *et al*., 2014].

The effect of native and encapsulated blueberry phenolic extracts on the oxidative stability of edible oil

The use of antioxidants in the food industry has an important role since oxidation of edible oils is one of the major challenges. There are few studies that evaluated the effect of fruit phenolic compounds on the prolongation of the oxidative stability of edible oils [Aladedunye *et al*., 2014; Asnaashari *et al*., 2015]. However, there is a lack of studies focused on the use of microemulsions and/or liposomes to enhance the effect of phenolic compounds in the prolongation of the edible oil oxidative stability. In that sense, this is the first comparative study about the effect of native and encapsulated blueberry phenolic extracts on the oxidative stability of extra virgin olive oil measured with the Rancimat apparatus. Extra virgin olive oil was used as a matrix because it represents one of the healthiest

TABLE 4. The prolongation of the lag phase of the oxidative stability of olive oil (in hours) with native phenolic extracts from *Vaccinium corymbosum* L. and loaded in microemulsions (ME-1 and ME-2) and liposomes.

Sample	Phenolic extracts	ME-1		ME-2		Liposomes
		Mechanical homogenization	Ultrasound homogenization	Mechanical homogenization	Ultrasound homogenization	
A0	0.00	3.90±0.31*	3.45±0.20*	1.20±0.06	5.78±0.28*	3.05±0.11*
AP	0.62±0.13*	0.78±0.31	2.77±0.13*	4.62±0.33*	5.76±0.17*	8.87±0.20*
AL	0.00	2.88±0.06*	7.42±0.07*	1.99±0.20*	5.55±0.06*	3.63±0.14*
A1	0.46±0.34*	3.46±0.07*	9.89±0.21*	1.39±0.07*	6.86±0.23*	2.27±0.21
A2	0.94±0.25*	4.72±0.31*	10.29±0.16*	4.35±0.33*	3.71±0.17	3.24±0.40*
A3	0.75±0.17*	9.91±0.27*	3.73±0.11*	7.42±0.31*	9.68±0.18*	3.40±0.20*
A4	0.95±0.13*	4.72±0.17*	15.15±0.18*	4.71±0.07*	11.02±0.13*	4.34±0.13*
A5	0.00	10.22±0.20*	11.16±0.11*	4.89±0.11*	5.14±0.34*	3.19±0.31*
A6	0.00	1.86±0.31*	12.71±0.17*	6.07±0.10*	12.27±0.34*	2.54±0.20
A7	0.02±0.14	3.95±0.38*	5.21±0.06*	3.01±0.33*	6.73±0.24*	2.67±0.28*
A8	0.03±0.17	3.49±0.35*	2.80±0.14*	1.43±0.33*	4.05±0.31*	2.81±0.24*
A9	0.00	4.06±0.13*	8.05±0.20*	2.93±0.30*	8.26±0.35*	2.81±0.16*
A10	0.00	6.91±0.17*	14.18±0.13*	8.50±0.14*	8.25±0.23*	3.14±0.27*
A11	0.00	2.69±0.11*	5.19±0.21*	4.30±0.20*	13.26±0.13*	2.58±0.14
p-value	*0.029*	*0.012*	*0.011*	*0.012*	*0.011*	*0.021*

Results are expressed as mean value ± SD. Values marked with * within column are significantly different ($p<0.05$) from the control sample (induction time of pure extra virgin olive oil = 10.43h; induction time of extra virgin olive oil with mechanically homogenized microemulsion = 9.41h; induction time of extra virgin olive oil with ultrasonically homogenized microemulsion = 13.28h; induction time of extra virgin olive oil with liposomes = 6.98h) compared by Kruskal-Wallis test.
A0 = Untreated (fresh) sample; AP = pasteurized sample; AL = freeze-dried (lyophilized) sample; A1-A11 = ultrasound treated samples (see Table 1).

cooking oils which is characteristic for the Mediterranean diet. The effect of different blueberry phenolic extracts on the oxidative stability of extra virgin olive oil is shown in Table 4. The highest prolongation of the oxidative stability of olive oil was achieved with extracts A2 and A4, obtained after treatment with high intensity ultrasound ($p=0.029$). Interestingly, many extracts did not show any effect on the prolongation of the oxidative stability of the olive oil. Despite significant reduction of phenolic content in the pasteurized sample, its detected antioxidant capacity expressed through the effect on the prolongation of the oil oxidative stability may be due to the formation of anthocyanin polymers or formation of Maillard reaction products in response to thermal treatment [Brownmiller *et al*., 2008; Casati *et al*., 2017]. There are some speculations about the correlation between the TPC and the prolongation of the oxidative stability [Tiveron *et al*., 2012].

Blueberry extracts encapsulated in microemulsions prepared by mechanical homogenization ensured a significant prolongation of the oxidative stability of extra virgin olive oil (Table 4). In comparison to the control sample (oil enriched only with mechanically homogenized microemulsions), it was observed that olive oil needed 9.41 hours to oxidize, while the olive oil containing phenolic compounds encapsulated in ME-1 and ME-2 needed on average 13.94 hours and 13.46 hours, which meant prolongation by 48.22% and 43.09% ($p=0.012$), respectively. The microemulsions prepared with an ultrasonic homogenizer generally showed a higher effect on the prolongation of the lag phase of the oxidative stability in comparison with microemulsions prepared with a mechanical homogenizer (Table 4). The prolongation of the lag phase was minimally 20.86% and maximally 114.08% for ME-1 ($p=0.011$) enriched with phenolic extracts. For ME-2 enriched with phenolic extracts, the prolongation rate varied from 27.94 to 99.85% ($p=0.011$). The effect of liposome-phenolic extract systems on the prolongation of the oil oxidative stability was also very high and the average prolongation rate was 43.68% ($p=0.021$) (Table 4). Microemulsions prepared with high pressure homogenizer and enriched with phenolic extracts had no effect on the prolongation of the oxidative stability of extra virgin olive oil (data not shown). Although Fernandez-Avila & Trujillo [2017] stated that the use of high pressure homogenization produced the emulsion with better physical stability during storage, maintaining optimal oxidative stability until 3 months, this study did not prove the effect of microemulsions produced by high pressure homogenization on the prolongation of the oxidative stability of the extra virgin olive oil.

To the best of our knowledge, there are no previous data on the use of blueberry phenolic extracts encapsulated in microemulsions and liposomes on the prolongation of the oxidative stability of edible oils. Mohideen *et al.* [2015] studied the effects of blueberry juices on lipid oxidation during spray drying of menhaden oil and they found that 10% of blueberry juices

reduced lipid oxidation. Li *et al.* [2015] reported that blueberry extract can be successfully used to delay the lipid oxidation during spray drying and the storage of Pollock liver oil. Although it was expected, a correlation between a lower droplet size of ME-2 and a better antioxidant effect of incorporated blueberry extracts than in the case of ME-1 was not found. This indicates that olive oil portion as the main difference in composition between ME-1 and ME-2 could contribute to the final physical properties of the produced emulsion due to its own phenolic content [Giacintucci *et al.*, 2016]. Since the stronger effect on the prolongation of the lag phase of the oxidative stability of microemulsions produced by ultrasound homogenizer in comparison with microemulsions prepared with a mechanical homogenizer cannot be explained by differences in their droplet characteristics, this phenomenon merits further elucidation through investigation on added phenolic extracts partitioning and interface organization induced by these methods. Comparing two delivery systems in our study it was concluded that emulsions gave better results than liposomes. This could be explained by the fact that encapsulated phenolics also protect liposomal phospholipids from peroxidation, through acting as high-level scavengers of radicals [Balanč *et al.*, 2015], as confirmed in the case of resveratrol, rutin, and quercetin encapsulation in liposomes *via* inhibition of malondialdehyde formation [Balanč *et al*., 2015; Cesquini *et al*., 2003]. In that sense, the antioxidant capacity of phenolics is probably partially involved in the anti-peroxidation of phospholipids, not only its action on olive oil, while in the case of microemulsions their antioxidant potential could be fully engaged in prolonging the oxidative stability of the olive oil.

CONCLUSIONS

The present study indicates that the use of freeze drying enhances the phenolic yield in extracts from blueberry fruits in comparison with raw plant material. Influence of high-intensity ultrasound on phenolic compounds was minimal while pasteurization caused significant losses. The most abundant phenolic constituents detected *via* HPLC analysis in all samples were malvidin glycosides.

The obtained results showed that the use of microemulsions and liposome systems can greatly enhance the effect of blueberry phenolic extracts on the prolongation of the oxidation process of extra virgin olive oil. The effect of native blueberry phenolic extracts on the prolongation of the oxidative stability of olive oil was very weak in comparison with the effect of extracts encapsulated in microemulsions and liposomes. Furthermore, the microemulsions prepared by an ultrasound homogenizer showed a higher effect on the prolongation of the oxidative stability than microemulsions prepared with mechanical and high pressure homogenizer. Accordingly, encapsulated natural antioxidants from blueberry may be considered as potential candidates for preserving the oxidative stability of lipid-containing food.

RESEARCH FUNDING

The authors are grateful to the Ministry of Science and Technology of the Republic of Croatia and the Ministry of Education, Science and Technological Development of the Republic of Serbia for having financed bilateral project entitled “Enhancement of the stability and the bioavailability of selected phytochemicals using different delivery systems and mathematical modelling of an *in vitro* digestion model”.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Robert Belužić from the Department of Molecular Medicine from the Rudjer Boskovic Institute in Zagreb (Croatia) for the technical help using the high pressure homogenization.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES

1. Afrin, S., Gasparrini, M., Forbes-Hernandez, T.Y., Reboredo-Rodriguez, P., Mezzetti, B., Varela-Lopez, A., Giampieri, F., Battino, M. (2016). Promising health benefits of the strawberry: A focus on clinical studies. *Journal of Agricultural and Food Chemistry*, *64*(22), 4435–4449.
2. Aladedunye, F., Przybylski, R., Niehaus, K., Bednarz, H., Matthäus, B. (2014). Phenolic extracts from *Crataegus×mordenensis* and *Prunus virginiana*: Composition, antioxidant activity and performance in sunflower oil. *LWT – Food Science and Technology*, *59*(1), 308–319.
3. Asnaashari, M., Tajik, R., Khodaparast, M.H.H. (2015). Antioxidant activity of raspberry (*Rubus fruticosus*) leaves extract and its effect on oxidative stability of sunflower oil. *Journal of Food Science and Technology – Mysore*, *52*(8), 5180–5187.
4. Balanč, B.D., Ota, A., Djordjević, V.B., Šentjurc, M., Nedović, V.A., Bugarski, B.M., Ulrih, N.P. (2015). Resveratrol-loaded liposomes: interaction of resveratrol with phospholipids. *European Journal of Lipid Science and Technology*, *117*(10), 1615–1626.
5. Barba, F.J., Mariutti, L.R.B., Bragagnolo, N., Mercadante, A.Z., Barbosa-Cánovas, G.V., Orlien, V. (2017). Bioaccessibility of bioactive compounds from fruits and vegetables after thermal and nonthermal processing. *Trends in Food Science & Technology*, *67*(September), 195–206.
6. Basu, A., Lyons, T.J. (2012). Strawberries, blueberries, and cranberries in the metabolic syndrome: Clinical perspectives. *Journal of Agricultural and Food Chemistry*, *60*(23), 5687–5692.
7. Brownmiller, C., Howard, L.R., Prior, R.L. (2008). Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. *Journal of Food Science*, *73*(5), H72–H79.
8. Casati, C.B., Baeza, R., Sanchez, V. (2017). Comparison of the kinetics of monomeric anthocyanins loss and colour changes in thermally treated Blackcurrant, Maqui Berry and Blueberry pulps from Argentina. *Journal of Berry Research*, *7*(2), 85–96.
9. Cesquini, M., Torsoni, M.A., Stoppa, G.R., Ogo, S.H. (2003). t-BOOH-induced oxidative damage in sickle red blood cells and the role of flavonoids. *Biomedicine & Pharmacotherapy*, *57*(3–4), 124–129.
10. Chatzidaki, M.D., Mitsou, E., Yaghmur, A., Xenakis, A., Papadimitriou, V. (2015). Formulation and characterization of food-

grade microemulsions as carriers of natural phenolic antioxidants. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *483*(October), 130–136.
11. Dragović-Uzelac, V., Savić, Z., Brala, A., Levaj, B., Kovačević, D.B., Biško, A. (2010). Evaluation of phenolic content and antioxidant capacity of blueberry cultivars (*Vaccinium corymbosum* L.) grown in the northwest Croatia. *Food Technology and Biotechnology*, *48*(2), 214–221.
12. Drvenica, I., Đorđević, V., Trifković, K., Bojana, B., Steva, L., Bugarski, B., Nedović, V. (2017). Industry-Relevant Encapsulation Technologies for Food and Functional Food Production. In M. Krokida (Ed.), *Thermal and Nonthermal Encapsulation Methods* (1st edition, pp. 221–263). Boca Raton, Florida: CRC Press.
13. Eicke, H.F., Borkovec, M., Das-Gupta, B. (1989). Conductivity of water-in-oil microemulsions: a quantitative charge fluctuation model. *The Journal of Physical Chemistry*, *93*(1), 314–317.
14. Fernandez-Avila, C., Trujillo, A.J. (2017). Enhanced stability of emulsions treated by Ultra-High Pressure Homogenization for delivering conjugated linoleic acid in Caco-2 cells. *Food Hydrocolloids*, *71*(October), 271–281.
15. Giacintucci, V., Di Mattia, C., Sacchetti, G., Neri, L., Pittia, P. (2016). Role of olive oil phenolics in physical properties and stability of mayonnaise-like emulsions. *Food Chemistry*, *213*(December), 369–377.
16. Giampieri, F., Forbes-Hernandez, T.Y., Gasparrini, M., Afrin, S., Cianciosi, D., Reboredo-Rodriguez, P., Varela Lopez, A., Quiles, J.L., Mezzetti, B., Battino, M. (2017). The healthy effects of strawberry bioactive compounds on molecular pathways related to chronic diseases. *Annals of the New York Academy of Sciences*, *1398*(1), 62–71.
17. Gibis, M., Vogt, E., Weiss, J. (2012). Encapsulation of polyphenolic grape seed extract in polymer-coated liposomes. *Food & Function*, *3*(3), 246–254.
18. Gibis, M., Zeeb, B., Weiss, J. (2014). Formation, characterization, and stability of encapsulated hibiscus extract in multilayered liposomes. *Food Hydrocolloids*, *38*(July), 28–39.
19. Golmohamadi, A., Möller, G., Powers, J., Nindo, C. (2013). Effect of ultrasound frequency on antioxidant activity, total phenolic and anthocyanin content of red raspberry puree. *Ultrasonics Sonochemistry*, *20*(5), 1316–1323.
20. Gómez-Mascaraque, L.G., Casagrande Sipoli, C., de La Torre, L.G., López-Rubio, A. (2017). Microencapsulation structures based on protein-coated liposomes obtained through electrospraying for the stabilization and improved bioaccessibility of curcumin. *Food Chemistry*, *233*(October), 343–350.
21. Gortzi, O., Lalas, S., Chinou, I., Tsaknis, J. (2008). Reevaluation of bioactivity and antioxidant activity of *Myrtus communis* extract before and after encapsulation in liposomes. *European Food Research and Technology*, *226*(3), 583–590.
22. Haminiuk, C.W.I., Maciel, G.M., Plata-Oviedo, M.S.V., Peralta, R.M. (2012). Phenolic compounds in fruits – an overview. *International Journal of Food Science and Technology*, *47*(10), 2023–2044.
23. Helrich, K. (1990). *Official Methods of Analysis: 930.04 Moisture Content in Plants* (15th editi, Vol. 1). Arlington, Virginia, USA: AOAC.
24. Howard, L.R., Brownmiller, C., Mauromoustakos, A., Prior, R.L. (2016). Improved stability of blueberry juice anthocyanins by acidification and refrigeration. *Journal of Berry Research*, *6*(2), 189–201.
25. Huang, H., Chen, G., Liao, D., Zhu, Y., Xue, X. (2016). Effects of berries consumption on cardiovascular risk factors: a meta-analysis with trial sequential analysis of randomized controlled trials. *Scientific Reports*, *6*(March), 23625.
26. Isailović, B.D., Kostić, I.T., Zvonar, A., Đordević, V.B., Gašperlin, M., Nedović, V.A., Bugarski, B.M. (2013). Resveratrol loaded liposomes produced by different techniques. *Innovative Food Science and Emerging Technologies*, *19*(July), 181–189.
27. Jafari, S.M., He, Y., Bhandari, B. (2006). Nano-emulsion production by sonication and microfluidization – A comparison. *International Journal of Food Properties*, *9*(3), 475–485.
28. Juttulapa, M., Piriyaprasarth, S., Takeuchi, H., Sriamornsak, P. (2017). Effect of high-pressure homogenization on stability of emulsions containing zein and pectin. *Asian Journal of Pharmaceutical Sciences*, *12*(1), 21–27.
29. Keinänen, M., Julkunen-Tiitto, R. (1996). Effect of sample preparation method on birch (*Betula pendula* Roth) leaf phenolics. *Journal of Agricultural and Food Chemistry*, *44*(9), 2724–2727.
30. Li, J., Solval, K.M., Alfaro, L., Zhang, J., Chotiko, A., Delgado, J.L.B., Chouljenko, A., Bankston, D., Bechtel, P.J Sathivel, S. (2015). Effect of blueberry extract from blueberry pomace on the microencapsulated fish oil. *Journal of Food Processing and Preservation*, *39*(2), 199–206.
31. Lu, W., Kelly, A.L., Miao, S. (2016). Emulsion-based encapsulation and delivery systems for polyphenols. *Trends in Food Science & Technology*, *47*(January), 1–9.
32. McClements, D.J. (2012). Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, *8*(6), 1719–1729.
33. Michalska, A., Lysiak, G. (2015). Bioactive compounds of blueberries: Post-harvest factors influencing the nutritional value of products. *International Journal of Molecular Sciences*, *16*(8), 18642–18663.
34. Mohideen, F.W., Stine, J., Bechtel, P.J., Solval, K.M., Bankston, J.D., Sathivel, S. (2015). Effects of blueberry (*Vaccinium corymbosum*) juice on lipid oxidation during spray drying of microencapsulated menhaden Oil. *International Journal of Food Properties*, *18*(5), 1139–1153.
35. Može, Š., Polak, T., Gašperlin, L., Koron, D., Vanzo, A., Ulrih, N.P., Abram, V. (2011). Phenolics in Slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.). *Journal of Agricultural and Food Chemistry*, *59*(13), 6998–7004.
36. Neveu, V., Perez-Jiménez, J., Vos, F., Crespy, V., du Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D., Scalbert, A. (2010). Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database*, art. no. bap024.
37. Nile, S.H., Park, S.W. (2014). Edible berries: Bioactive components and their effect on human health. *Nutrition*, *30*(2), 134–144.
38. Pérez-Jiménez, J., Neveu, V., Vos, F., Scalbert, A. (2010). Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. *European Journal of Clinical Nutrition*, *64*(Suppl 3), S112–S120.
39. Perrett, S., Golding, M., Williams, W.P. (1991). A simple method for the preparation of liposomes for pharmaceutical applications: Characterization of the liposomes. *Journal of Pharmacy and Pharmacology*, *43*(3), 154–161.

40. Pravilovic, R., Radunovic, V., Boskovic-Vragolovic, N., Bugarski, B., Pjanovic, R. (2015). The influence of membrane composition on the release of polyphenols from liposomes. *Hemijska Industrija*, *69*(4), 347–353.
41. Rashidinejad, A., Birch, E.J., Sun-Waterhouse, D., Everett, D.W. (2014). Delivery of green tea catechin and epigallocatechin gallate in liposomes incorporated into low-fat hard cheese. *Food Chemistry*, *156*(August), 176–183.
42. Rodríguez-Mateos, A., Cifuentes-Gomez, T., Tabatabaee S., Lecras, C., Spencer, J.P. E. (2012). Procyanidin, anthocyanin and chlorogenic acid contents of highbush and lowbush blueberries. *Journal of Agricultural and Food Chemistry*, *60*(23), 5772–5778.
43. Sablani, S.S., Andrews, P.K., Davies, N.M., Walters, T., Saez, H., Bastarrachea, L. (2011). Effects of air and freeze drying on phytochemical content of conventional and organic berries. *Drying Technology*, *29*(11), 205–216.
44. Singleton, V.L., Rossi, J.A.J. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(3), 144–158.
45. Tiveron, A.P., Melo, P.S., Bergamaschi, K.B., Vieira, T.M.F.S., Regitano-d'Arce, M.A.B., Alencar, S.M. (2012). Antioxidant activity of brazilian vegetables and its relation with phenolic composition. *International Journal of Molecular Sciences*, *13*(7), 8943–8957.
46. Xu, S., Ni, Z., Ma, L., Zheng, X. (2017). Control of alternaria rot of cherry tomatoes by food-grade *Laurus nobilis* essential oil microemulsion. *Journal of Food Safety*, *37*(1), e12286.
47. Zorić, Z., Dragović-Uzelac, V., Pedisić, S., Kurtanjek, Ž., Garofulić, I.E. (2014). Kinetics of the degradation of anthocyanins, phenolic acids and flavonols during heat treatments of freeze-dried sour cherry Marasca paste. *Food Technology and Biotechnology*, *52*(1), SI, 101–108.

Submitted: 6 May 2018. Revised: 13 August 2018. Accepted: 17 October 2018. Published on-line: 3 December 2018.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 35–44
DOI: 10.31883/pjfns-2019-0002
http://journal.pan.olsztyn.pl

Original research article
Section: Nutrition Section

Modulation of Caecal Microbiome in Obese Mice Associated with Administration of Amaranth or Soybean Protein Isolates

Diana Olguín-Calderón[1,+], Jorge L. González-Escobar[1,+], Rosalva Ríos-Villa[1], Elena Dibildox-Alvarado[2], Antonio De León-Rodríguez[1], Ana P. Barba de la Rosa[1,]*

[1]IPICYT, Instituto Potosino de Investigación Científica y Tecnológica A.C. Camino a la Presa San José 2055, Lomas 4a sección, San Luis Potosí, S.L.P., 78216 Mexico
[2]Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí. Av. Dr. Salvador Nava 6, Zona Universitaria, San Luis Potosí, S.L.P., 78210 Mexico

Key words: amaranth, caecal microbiome, obesity, protein intake, short-chain fatty acids

Obesity is defined as abnormal or excessive body fat accumulation that may have negative effects on health. Healthy diet induces a balance of gut microbiota, helping in turn to combat this metabolic disorder. Amaranth is well known because of its beneficial properties on health, but its effects on microbiota profile are still unknown. The aim of this study was to analyse the changes of gut microbiota in diet-induced obese mice due to amaranth protein consumption and to compare them with the changes due to soybean protein intake. Male C57BL/6 mice were fed for 8 weeks with regular (RD) or high-fat (HF) diet, without or with complementation with amaranth or soybean protein isolates. Morphological changes in caecum ultra-thin sections were measured after hematoxylin/eosin staining. Microbiota was isolated from the caecum and *16S rRNA* gene was sequenced. Caecal Short Chain Fatty Acids (SCFAs) were quantified by gas chromatography. The consumption of soybean protein induced the ectopic deposition of fat in the whole intestine while amaranth proteins increased caecal crypt depth and calceiform cells number sustaining its beneficial effect on health. The count of Ruminococcacea family bacteria was increased in mice fed with HF diet, but amaranth proteins intake reduced its abundance. In turn, Lachnospiraceae bacteria abundance decreased in mice fed the Control-HF and amaranth HF diets, but increased in mice fed the soybean diets. In mice fed the RD diets, amaranth induced the abundance of Prevotellaceae, an acetate-producing bacteria. Study results indicate that the modulation of caecal microbiota could be one of the mechanisms by which amaranth exerts its beneficial effects on health.

INTRODUCTION

Obesity is a positive imbalance of energy intake and expenditure with excessive weight gain and is related to comorbidities as diabetes mellitus, cardiovascular, and metabolic diseases. Obesity has increased the international morbidity rate and has become one of the most disquieting health problems of the 21st century with over 1.9 billion overweight adults from which approximately 600 million are obese [WHO, 2016]. The increase in obesity condition has been observed in both developed and developing countries and does not exclusively afflict adults; more than 41 million children under five are overweight or obese. Reports indicate that Mexico is the second country with the highest obesity rates, after the United States, and first one regarding child overweight and obesity ratios [OECD, 2015].

There are several treatments for the obese condition that range from lifestyle modification, pharmacological or psychological therapy, surgery, and even the implementation of new procedures as a faecal transplant [Marotz & Zarrinpar, 2016]. Drugs and surgery are not appropiate for each patient and represent the last options due to their side effects, hence diet modification will have the greatest impacts in preventing the development of comorbidities and improving the quality of life of patients who present an excessive weight gain. In the pursuit of health-beneficial foods, the incorporation of nutraceuticals in diets is gaining increased attention [Houston, 2013].

Amaranth is considered a nutraceutical and evidences of its health benefits have been reported [Gómez-Cardona *et al*., 2017]. Amaranth seeds have high dietary fiber contents, are rich in unsaturated fatty acids, and their proteins are rich in essential amino acids (lysine, tryptophan, and sulfur amino acids) [Bressani *et al*., 1987]. It is known that the amaranth lipid fraction has hypocholesterolaemic properties due to its high quantities of squalene [Martirosyan *et al*., 2007], while amaranth protein consumption induces the accumulation of antioxidant proteins such as paraoxonase/arylestereas 1 (PON1) [Velarde-Salcedo *et al*., 2017]. It has also been observed that the consumption of amaranth proteins increases leptin levels, a molecule that is involved in appetite control. Moreover, these proteins reduce concentrations of ghrelin, an orexigenic hormone [Gómez-Cardona *et al*., 2017].

* Corresponding Author: Tel.:+52 444 8342082; Fax: +52 444 8342000
E-mail: apbarba@ipicyt.edu.mx (AP Barba de la Rosa).
+These authors contributed equally.

Nutraceutical food and microbiota are extremely associated with the nutritional and health status of the host. Dietary components are susceptible to be metabolized by the bacteria during the gastrointestinal passage for their subsequent absorption [Laparra & Sanz, 2010]. Bacteroidetes and Firmicutes are the principal phyla that suffer alterations in abundance and diversity when feeding habits and compounds of diet are modified in obese individuals, but results are still unclear. Reports using mice models have indicated a significant increase in the Firmicutes/Bacteroidetes ratio [Turnbaugh *et al*., 2008], while Jiao *et al*. [2018] reported no differences when comparing obese with lean rodents. Others works have indicated that Bacteroidetes increase in human fecal microbiota in overweight subjects compared with lean ones [Schwiertz *et al*., 2010]. Nonetheless, in the distal colon these phyla are important for dietary fibers fermentation in order to produce end products such as Short-Chain Fatty Acids (SCFAs), that not only improve the microbial environment but also regulate several biochemical processes in the host [Kasubuchi *et al*., 2015].

To date, there are no reports regarding how amaranth proteins, which have a good balance of essential amino acids, can modify the profile of the caecal microbiota and influence the generation of SCFAs, and whether this potential change could have positive effects on human health. Therefore, the aim of this study was to assess the effect of the gut microbiota profile on diet-induced obese mice due to a daily amaranth protein intake and to compare it with the effects due to soybean protein consumption.

MATERIALS AND METHODS

Animals and diets

Male 6-week old C57BL/6 mice were obtained from "Unidad de Producción y Experimentación de Animales de Laboratorio UPEAL" (UAM, Xochimilco, Mexico). Mice were housed in controlled conditions at 21 ± 2°C and dry air humidity at 50% ± 15 g H_2O/g with a 12 h light/dark cycle in groups of four to five mice in a standard stainless steel cage. Animals were randomly assigned to six groups containing eight mice per group. In the two first groups, the control groups, animals were fed with regular diet (Ctrl-RD) and high-fat diets (Ctrl-HF). In the third and fourth groups, mice were fed with regular diet or high-fat diets supplemented with amaranth protein isolate (AMA-RD and AMA-HF, respectively). In the fifth and sixth groups, the mice diets were supplemented with soybean protein isolate (SOY-RD and SOY-HF, respectively).

The regular diet groups received the commercial Teklad (Envigo, Huntingdon, UK) diet 2018S containing (in 100 g diet): 18.6 g protein (from wheat, corn, soybean), 44.3 g carbohydrates, and 6.2 g fat. High-fat diet was supplied with Teklad TD 06414 containing (in 100 g diet): 23.5 g protein (casein), 27.3 g carbohydrates, and 34.3 g fat. The food consumption and mice weight were recorded every week. Protein isolates from amaranth and soybean, with a purity of 76.5% and 85.5%, respectively, were obtained as described elsewhere [Escobedo-Moratilla *et al*., 2017]. Amaranth or soybean protein was administered once daily using a stainless steel oral gavage at a dose of 10 mg protein/kg BW (body weight). Food and water were provided *ad libitum* during the study.

Procedures for animal housing and care were assessed according to the Mexican regulatory standard (NOM-062-ZOO-1999) and animal experiments were approved by the Institutional Research Bioethics Committee at IPICYT Code: LPBM-AMA-C57/002 and ratified by the Ethics Research Committee Code: DIX.UC-EB-17–001 (Registration code: CONBIOETICA24CEI00320130722). At the end of the experiment, animals were fasted for 6 h on paper bedding before euthanized; all segments after stomach were collected, placed in liquid nitrogen, and stored at -80°C until microbial analysis.

Histomorphometry appearance of the caecum

Frozen caecum sample was dissected into 7 mm sections cutting 0.6 mm approximately from the ileum for each randomly selected gut. Samples were immersed in 10 mL PBS/100 mL formaldehyde (pH=6.4), de-hydrated, clarificated, and embedded in paraffin. Three serial sections of 7 μm from each animal were collected in silanized slides at one location of caecum and stained with hematoxylin-eosin. The analysis was carried out blind to the treatment to evaluate the histological changes and the Lieberkühn cryps depth. The morphometrical analysis was achieved with the light microscope Zeiss Axio Imager M2 (Carl Zeiss Co. Oberkochen, German). Crypt depth measurements were done by triplicate in 10 well-oriented Lieberkühn crypts by field from the base to the highest point still visible with 40-fold magnification using ImageJ software v1.46r.

Sequencing and analysis of bacterial *16S rRNA* gene

Caecal contents were obtained by extrusion from each thawed intestinal tissue. DNA extraction was achieved using the DNeasy UltraClean microbial kit (Qiagen, Hilden, Germany) from 200 mg of intestinal digesta following the manufacturer's instructions. The obtained DNA was quantified by spectrophotometry with NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and stored at -80°C until use.

Paired-end sequencing with a read length of 2×250 bp was performed using the Illumina Miseq platform by the molecular sequencing laboratory Research and Testing Laboratory (Lubbock, Texas, USA). The V3-V4 regions of *16S rRNA* gene were amplified by PCR using the universal primers containing Illumina adapter sequences (357wF/785R). The obtained sequences were merged and filtered by quality using Pear from the free online software Galaxy v 0.9.6.0 (http://www.usegalaxy.org). A specify P value of 0.01, 35 as minimum overlap size, 200 bp as the minimum possible length of the assembled sequences, and a Phred score of 25 were applied in the analysis [Kautz *et al*., 2013]. The filtered sequences were dereplicated and cleaned of chimeric sequences with UCHIME with the reference database GoldFasta using USEARCH-Tool-Suite. Resulting data were grouped into operational taxonomic units (OTU's) with 97% of identity using the database from Ribosomal Data Project (RDP) available online in GALAXY VGL 4.0.1 (http://galaxy-qld.genome.edu.au, accessed APRIL/2017). Explicet v2.10.5 software was used to make the OTU Stacket Bar plot to represent bacterial relative abundances between groups. OTU table was analyzed to survey the relationship of metadata characteristics

for multiple diversity measurements. Shannon, Good's coverage, and Rarefaction were calculed using iNEXT [Hsieh *et al*., 2016] and Spade (http://chao.stat.nthu.edu.tw/wordpress/software).

Measurement of caecal Short-Chain Fatty Acids (SCFAs)

Caecal digesta were used for SCFAs quantification. Briefly, samples (100 mg) were homogenized in one mL of ultrapure MilliQ® water (Merck, Darmstadt, DE), and mixed on a vortex mixer for 2 min. The homogenized sample was incubated for 20 min in an ice-water bath and centrifuged at 4ºC and 4800×*g* for 20 min. Supernatant was recovered and this procedure was repeated two times for clarifying. The sample was filtered through 0.22 μm Millipore filter (Merck, Darmstadt, Germany) before injection into the chromatographic system.

Analysis of SCFAs was performed on an Agilent 6890 N GC system using a 30 m x 0.25 mm I.D. HP-INNOwax GC capillary column with a film thickness of 0.5 μm (Agilent Technologies Inc, CA, USA). Helium was used as carrier gas at a flow rate of 1.5 mL/min with a split ratio of 1^{e+001}:1 and the set temperatures for the injector and flame ionization detector (Agilent Technologies Inc.) were 220 and 250ºC, respectively. The flow rates of hydrogen and air were 30 and 300 mL/min, respectively. The volume of the injected sample was 1 μL, and the running time was 20.5 min. The determinations were performed on three individual samples for each group. Calibration curves were performed from 1.5 to 100 mg/L for acetic acid, 3.1 to 50 mg/L for propionic acid and 1.87 to 30 mg/L for butyric acid (5 concentration levels, 3 replicates for each level).

Statistic analysis

Quantitive data was evaluated using SigmaPlot 12.3 software (Systat Inc., Illinois, USA), through a Kolmogórov-Smirnov normality test followed by a one-way analysis of variance (ANOVA) with a post hoc Tukey test ($p<0.05$) with a desired statistical power of 0.8 and a Kruskal-Wallis with a post hoc Dunn test for non-parametrical data.

RESULTS AND DISCUSSION

The incidence of obesity-related diseases has increased and currently the intake of a high protein diet is attracting particular attention due to its impact on gut microbiota. Amaranth proteins, which present a good balance of essential amino acids as well as an excellent digestibility [Bressani *et al*., 1987], are the ideal protein source for food supplementation. Therefore, we explore the effects of amaranth protein on modulation of intestinal microbiota in obese mice. However, several studies suggest that body weight, food intake, and gut microbiota composition may vary depending on interactions with sex [Yang *et al.*, 2014] and then only male C57BL/6 mice were used in the present study.

Physiognomic parameters measurements

The physiognomic parameters showed that at the end of the experiment, the mice fed the high-fat (HF) diet had the highest weight gain. No changes in body weight were observed in mice treated with the regular diet supplemented with amaranth (AMA-RD), but mice fed with soybean (SOY-RD) showed a tendency to increase weight (Table 1).

At the beginning of the experiment, the highest feed intake was observed in the mice fed the SOY-RD diet, but in all HF diets, the mice feed intake was similar in all groups ($p<0.001$). At the end of the experiment, it was observed a similar amount of feed ingested by the mice treated with AMA diets, but not in the mice fed the SOY-HF diets, which showed a significant decrease ($p<0.05$) (Table 1). The weight gain of the whole intestine was correlated with the feed intake, where SOY-HF diet-fed mice showed the lowest intestinal weight. With regard to epididymal fat tissue, it was similar among groups fed either with RD or HF diet (Table 1). Overall, the physiognomic parameters showed slight changes as it has been observed in previous studies with HF diet [Tomas *et al*., 2016]. However, an anatomically important observation of intestines in mice displayed that samples from the SOY-HF diet-fed mice were coated with fat (Figure 1). These results potentially indicate that fat accumulation is linked with the consumption of soy protein because mice fed amaranth protein presented morphology comparable to control groups (Figure 1).

Studies have suggested that the high consumption of soy protein decreases food intake and body weight compared to other protein sources, like whey [Li *et al*., 2016], which is also in agreement with the present results. However, an excessive ectopic deposition of fat in intestine was observed in the SOY-HF diet-fed mice, in spite of a lower feed intake. Interestingly, other studies have already detected the deposi-

TABLE 1. Physiognomic parameters at different nutritional conditions in all groups at the beginning and after eight weeks of protein consumption.

Parameters	Ctrl-RD	Ctrl-HF	AMA-RD	AMA-HF	SOY-RD	SOY-HF
Body weight (g)	14.6 ± 6.01^{b}	41.2 ± 20.4^{a}	19.3 ± 7.76^{b}	36.2 ± 13.6^{a}	$25.1\pm8.28^{a,b}$	35.7 ± 10.7^{a}
Feed intake at T_o (g)	$3.21\pm0.30^{c,d}$	2.30 ± 1.50^{d}	$4.10\pm0.28^{b,c}$	2.30 ± 0.29^{d}	5.46 ± 0.14^{a}	$3.15\pm0.23^{c,d}$
Feed intake at T_8 (g)	2.93 ± 0.49^{b}	$2.30\pm0.79^{b,c}$	$3.35\pm0.51^{a,b}$	$2.22\pm0.38^{b,c}$	$3.51\pm0.38^{a,b}$	1.36 ± 0.49^{c}
Intestinal weight (g)	$1.88\pm0.22^{a,b}$	$1.70\pm0.30^{b,c}$	2.02 ± 0.22^{a}	$1.66\pm0.29^{b,c}$	$1.88\pm0.15^{b,c}$	1.47 ± 0.27^{d}
EFT weight (g)	$0.38\pm0.17^{b,c}$	1.30 ± 0.56^{a}	$0.38\pm0.12^{b,c}$	1.38 ± 0.49^{a}	0.61 ± 0.18^{b}	1.20 ± 0.27^{a}

T_0=values at the beginning of the experiment. T_8=values after 8 weeks of protein intake. EFT= Epididymal fat tissue. Values represent the mean of total population in each group (n=8) ±standard deviation (SD). Grams (g). Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Different superscript letters along rows indicate statistical differences at $p<0.05$ in a Kruskal-Wallis with a post hoc Dunn test.

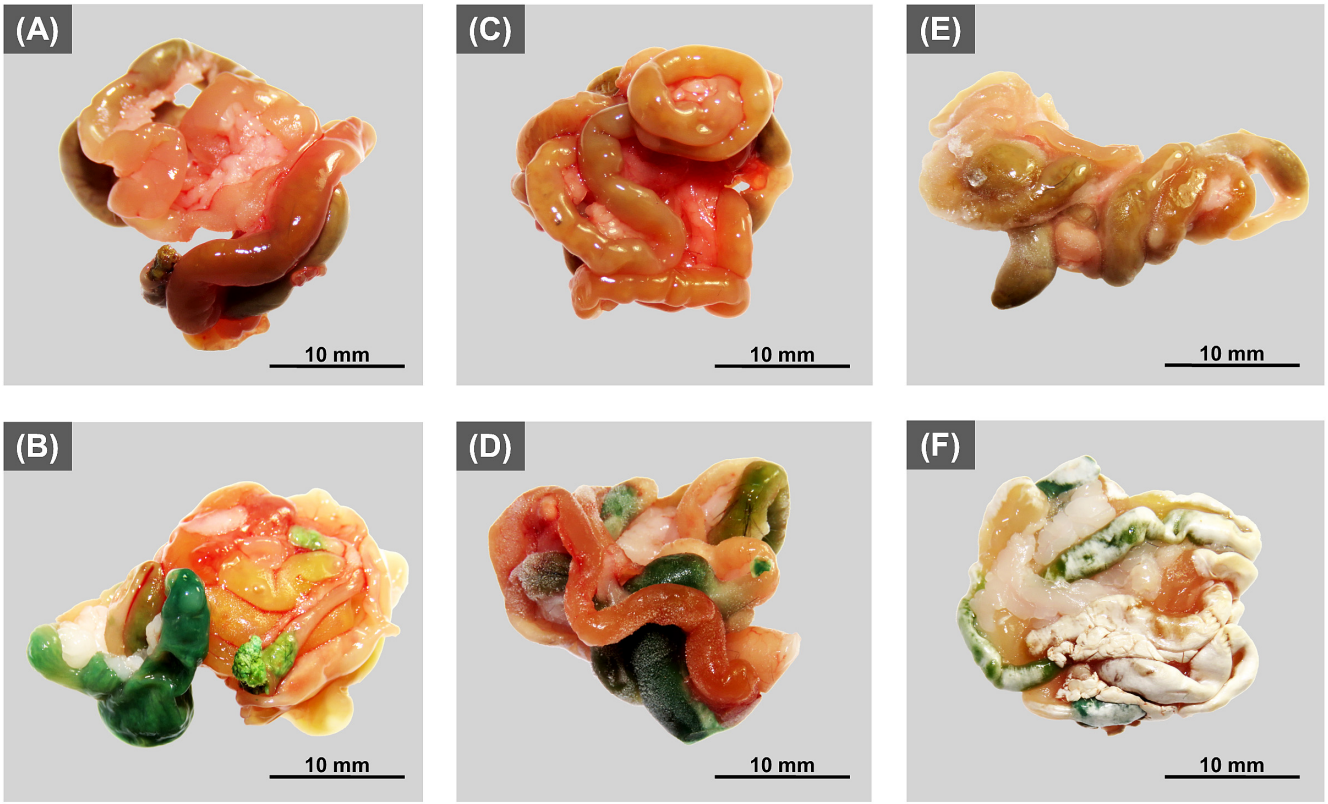


FIGURE 1. Macroscopical appearance of the intestinal tissue of mice fed with different diets. Digital images show fat coating the gut structures: (A) Ctrl-RD; (B) Ctrl-HF; (C) AMA-RD; (D) AMA-HF; (E) SOY-RD; and (F) SOY-HF. Ctrl=control, RD=regular diet, HF=high fat diet, AMA=amaranth proteins, SOY=soybean proteins.

tion of fat in the intestinal mucosa by triacylglycerol accumulation in mice fed a high fat diet compared to mice fed a low fat diet [Douglass *et al*., 2012]. This abnormal fat accumulation could be the result of metabolic and endocrine changes that may trigger both oxidative stress and inflammation at tissue and systemic levels with the development of obesity, lipotoxicity, and insulin resistance [Barazzoni *et al*., 2018]. This is in agreement with previous results that have shown that amaranth protein supplementation was able to reduce the insulin levels in mice plasma but mice fed soy proteins tended to generate insulin resistance [Escobedo-Moratilla *et al*., 2017].

Caecum histomorphometric appearance

The morphological changes of the caecum due to the diet are shown in Figure 2A, and the crypt depth dimensions are shown in Figure 2B. A significant decrease in crypt depth was observed in mice fed the Ctrl-HF diet when compared with samples from mice fed the Ctrl-RD diet (Figure 2Aa and 2b). Amaranth diets caused also a significant decrease in crypt depth, when compared with Ctrl-RD diets (Figure 2B). However, the crypt of mice fed the AMA-HF showed more epithelial cells, as well as the number and size of calceiform cells by crypt (Figure 2Ac and 2d), which resemble those observed in the Ctrl-RD fed-mice group. Alike, SOY-RD diet (Figure 2Ae) elicited a similar effect as Ctrl-RD, but SOY-HF diet reduced significantly crypt depths (Figure 2Af) even more than the Ctrl-HF diet did (Figure 2Ab). Furthermore, it was observed the loss of the intestinal epithelial structure in the mice fed the SOY-HF diet, which may be related to the inflammatory state (Figure 2Af).

The gut epithelium is broadly folded into crypts and villi, which increase the contact surface for secretory, absorptive, and digestive activities. These functions contribute to homeostatic regulation that impacts mucosal defences, but also the dynamics of intestinal microbiota [Jakobsson *et al*., 2015]. Therefore, structural changes or alterations in gut epithelium are extremely linked with dysbiosis and permeation of luminal noxious molecules, triggering the dysregulation of inflammation that promotes the pathogenesis of intestinal and systemic diseases [Battson *et al*., 2018].

Our results revealed that the caecum appearance was altered not only by the HF diet, but also by the type of proteins in diet. The SOY-HF diet had a greater impact on the mice epithelial tissue integrity with a considerable increase of epithelial cells density. This effect of soy protein consumption in conjunction with the previously reported insulin resistance [Escobedo-Moratilla *et al*., 2017], could be a consequence of a grade of tissues inflammation. In addition, the accumulation of fat observed in mice intestines (Figure 1) could be accompanied by secretory products and inflammatory cytokines that promote expansion of tissues as reported elsewhere [Magnuson *et al*., 2015]. Therefore, these modifications of the epithelium potentially suggest that the HF treatments caused a degree of dysbiosis, and that the soy protein does not help to mitigate the effects caused by the HF diet as the amaranth proteins do.

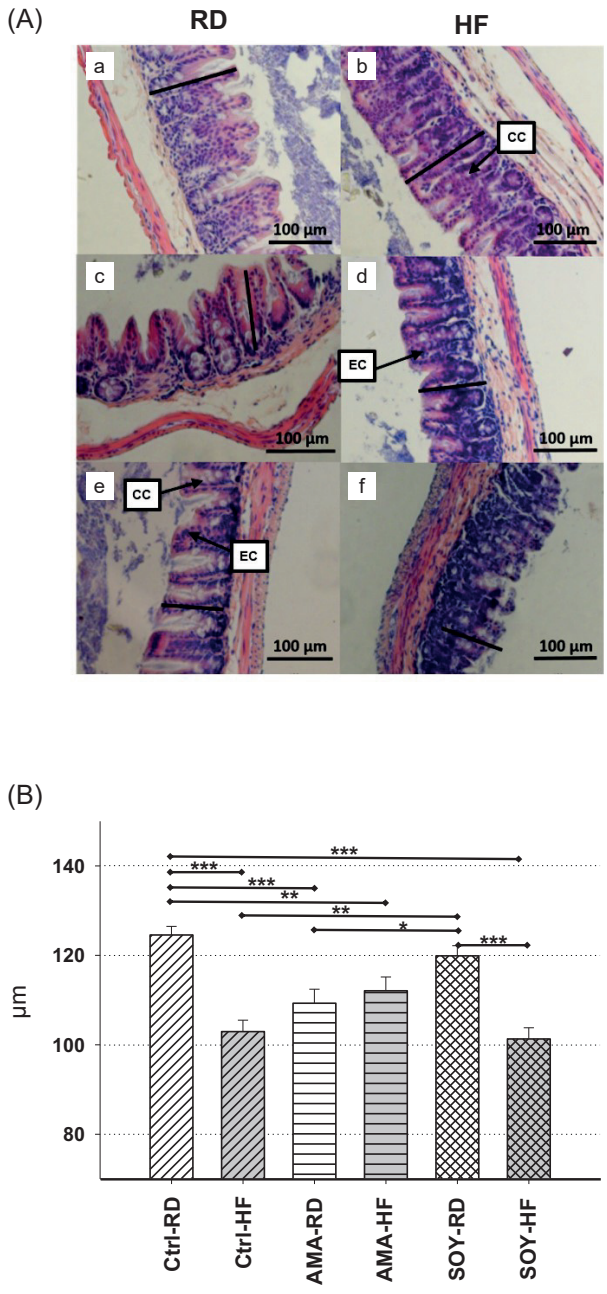


FIGURE 2. Morphometric analysis of the caecum of mice fed with different diets. (A) Representative micrographics of hematoxylin/eosin stained cuts of caecum samples from: a and b=control animals; c and d=from animals feed with amaranth proteins; e and f=from animals feed with soybean proteins. RD=regular diet or HF=high-fat diet. Stained cuts were observed in a Zeiss Axio Imager microscope. (B) Crypt depth modification of intestinal cecum. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Bars indicate mean and SEM values of each ultrathin slide. Black lines in A) represent a crypt depth measure in each treatment. CC=calceiform cells; EC=epithelial cells. Asterisks denote significant differences (*$p<0.05$, **$p<0.01$ and ***$p<0.001$ in the Kruskal-Wallis with a post hoc Dunn test).

Sequence analysis of the caecum bacteria population

Obesity-related changes in the gut microbiota have been linked to the decrease in species diversity. However, recent works have reported an increase of gut bacteria diversity in mice fed high fat diets [Zeng *et al*., 2016]. Our results showed that AMA and SOY protein diets also increased species diversity in the mice caecal microbiota. Ctrl-HF diet intake was characterized by a trend to increase numbers of OTUs compared with Ctrl-RD (Table 2). Mice fed the AMA and SOY diets did not differ significantly concerning numbers of OTUs, but a significant increase was observed in OTUs count compared with the mice group fed the Ctrl-RD diet (Table 2). These changes in bacterial diversity are associated with the relative abundance of sequences, but not with the OTUs distribution as observed in Good's coverage and Shannon index (Table 2).

It is known that gut microbiota in the vertebrates is dominated by Firmicutes and Bacteroidetes, which constitute 80–90% of the total resident bacteria [Cani & Knauf, 2016]. Some authors have related the presence of Firmicutes to an obese or overweight status [Turnbaugh *et al*., 2008], while others have had associated them to the lean population [Schwiertz *et al*., 2010; Ravussin *et al*., 2012]. Jiao *et al*. [2018] reported that there were no significant differences in Bacterioidetes to Firmicutes (B/F) ratio between obese and lean rodents. Although this relationship is still not well understood, this could be attributed to different model species, kind of diet, laboratory conditions, and also study design [Aguirre & Venema, 2015]. However, a notable change in gut microbial ecology has been observed in periods of nutrient deprivation or fasting [Beli *et al*., 2018]. We observed high levels of Firmicutes (over 90%) and low levels of Bacteroidetes (about 8%), which could be associated with the fasting period (6 h) before mice euthanasia (Figure 3A and 3B). A similar microbial profile was reported not only in mice fed with regular diet (control) but also in mice fed with high-fat diet fasted for 16 h [Zarrinpar *et al*., 2018]. This tendency is related to the ability to harvest energy from endogenous substrates in the absence of food during extended fasting; in this situation the gut microbiome could affect the generation of metabolites such as bile acids and SCFAs [Beli *et al*., 2018].

The phylum Firmicutes was mainly composed by Ruminococcaceae and Lachnospiraceae families (Figure 3A), while in the non-firmicutes the main family was Porphyromonadaceae with a notable reduction of Helicobacteraceae when protein isolates were included in diets (Figure 3B). Mitigation of Helicobacteraceae family abundance is related to the improvement of inflammation in colitis and bowel diseases [Rooks *et al*., 2014]. Regarding to Ruminococcaceae family, an increase in abundance was observed in caecal contents in mice fed with Ctrl-HF and SOY-diets compared with AMA-diets fed mice (Figure 4A). High abundance of Ruminococcaceae has been observed in other studies following HF diets [Zhang *et al*., 2010]. It has stated that Ruminoccocaceae induced expression of genes involved in inflammation process, such as *Angpl*4, in diet-induced obese mice [Ravussin *et al*., 2012]. These bacteria have been also observed in leptin-resistant obese and diabetic mice; this correlates with a previous work, which showed that mice fed with SOY protein tended to have insulin resistance [Escobedo-Moratilla *et al*., 2017]. The Lachnospiraceae family showed the same abundance levels in mice fed with Ctrl- and AMA diets but in caecal digesta of mice fed with SOY-diets an increase in abundance was observed (Figure 4B).The Lachnospiraceae have been detected in infants of overweight mothers [Tun *et al*.,

TABLE 2. Relative abundance, number of OTUs, and alpha diversity measures of mice caecal microbiota.

	Ctrl		AMA		SOY	
	RD	HF	RD	HF	RD	HF
Total sequence	11,733±2252^{c}	20,167±1020^{a}	15,237±21.0^{b}	15,176±1251^{ab}	15,824±1624^{ab}	21,050±7295^{a}
Total OTUs[1]	6152±503^{c}	7324±700^{b}	8468±413^{a}	7976±1449^{ab}	9757±1588^{a}	9710±1655^{a}
Rarefaction*	9150±3039^{a}	7047±4373^{a}	9650±4551^{a}	8701±2459^{a}	9775±3653^{a}	7660±1450^{a}
Shannon index	8.80±2.81^{a}	9.17±3.55^{a}	8.07±3.13^{a}	8.75±2.73^{a}	8.37±2.41^{a}	9.05±3.41^{a}
Good's coverage	0.67±0.05^{ab}	0.64±0.01^{b}	0.75±0.14^{ab}	0.65±0.07^{ab}	0.72±0.02^{a}	0.63±0.09^{b}

[1]Data was grouped into operational taxonomic units (OTUs) with 97% identity. Values represent the mean of total population ±standard deviation (SD). Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Different superscript letters along rows indicate statistical differences at $p<0.05$ in a Kruskal-Wallis with a post hoc Dunn test. *Sample-size-based rarefaction was computed with an extrapolation extends up to the maximum size of each sample.

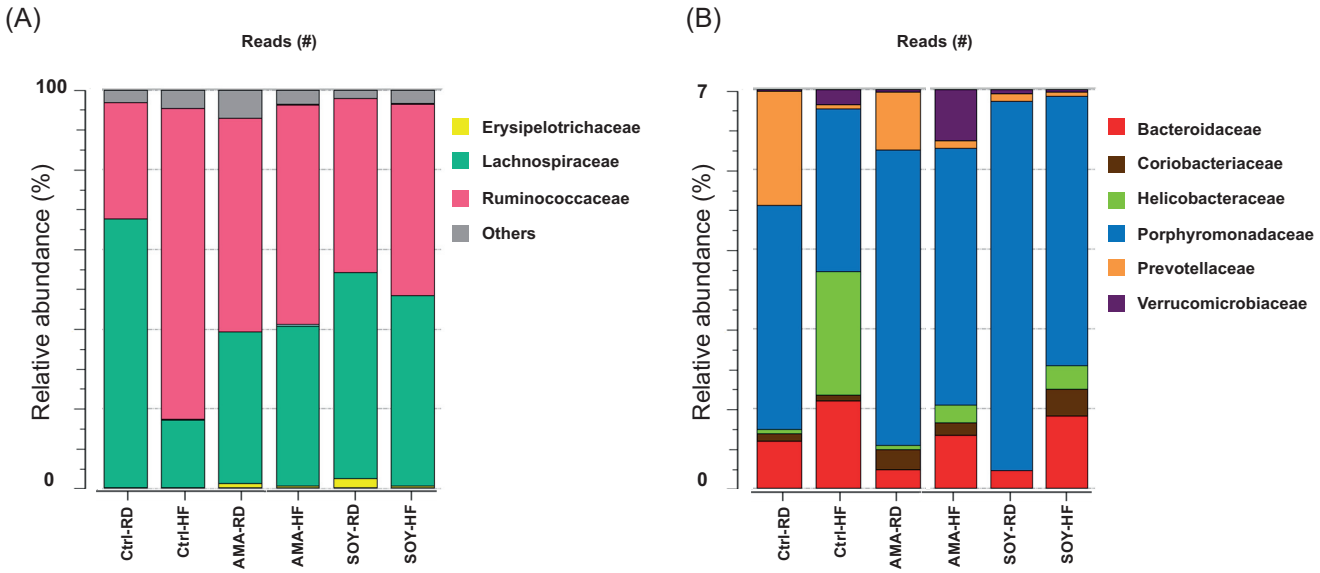


FIGURE 3. Modulation of the composition of mice caecal microbiota by different diets. The average relative abundance was expressed as a percentage of the total population at the family level in each group. (A) Families belonging to the Firmicutes phylum, and (B) families belonging to the phyla of Bacteroidetes, Verrucomicrobia, and Proteobacteria.

2018], the tendency to an increased abundance of Lachnospiraceae in SOY diets is potentially linked with the obesity status observed in those SOY- groups.

Within the Bacteroidetes families, Prevotellaceae showed higher relative abundance in caecal contents of mice fed with AMA-RD (Figure 4C), while Bacteroidaceae were abundant in mice fed with Ctrl-HF diet (Figure 4D). *Prevotella* belonging to the Prevotellaceae family possess the enzymatic machinery involved in sensing and hydrolysing complex carbohydrates and proteinaceous compounds with acetate as a product of fermentation [Hahnke *et al*., 2015]. Among the Bacteroidaceae family, *Bacteroides thetaiotaomicron* has been associated with the damage of epithelial barrier. According to Wrzosek *et al*. [2013], this bacterium together with bacteria that consume acetate and produce butyrate can interfere goblet cell differentiation, glycosylation, and mucin production in the colonic epithelium. Moreover, the increase of *Bacteroidales* has been detected in overweight women [Tagliabue & Elli, 2013]. These data could explain the occurrence of Bacteroidaceae family in mice fed with Ctrl-HF diet as a potential indicator of the epithelial damage caused by dysbiosis.

Protein administration generates similar values of caecal Short Chain Fatty Acids (SCFAs)

Gut microbiota uses products of fermentation of dietary fiber and in lesser extent dietary, endogenous proteins, to produce Short Chain Fatty Acids (SCFAs), which have emerged as important signalling molecules with a diverse physiological effects including stimulation of ileal motility and mucus production [Battson *et al*., 2018].

Acetic acid, propionic acid, and butyric acid are the most abundant SCFAs and constitute 95% of all those found in the body. In lean individuals, intestinal SCFAs concentration is mainly constituted by acetic acid, followed by propionic acid and butyric acid in a molar ratio of 60:20:20, respectively. Our results showed that mice fed the Ctrl-RD presented a SCFAs ratio of 75:2:23 (acetic, propionic, and butyric acids, respectively), ratio that was significantly decreased in caeca

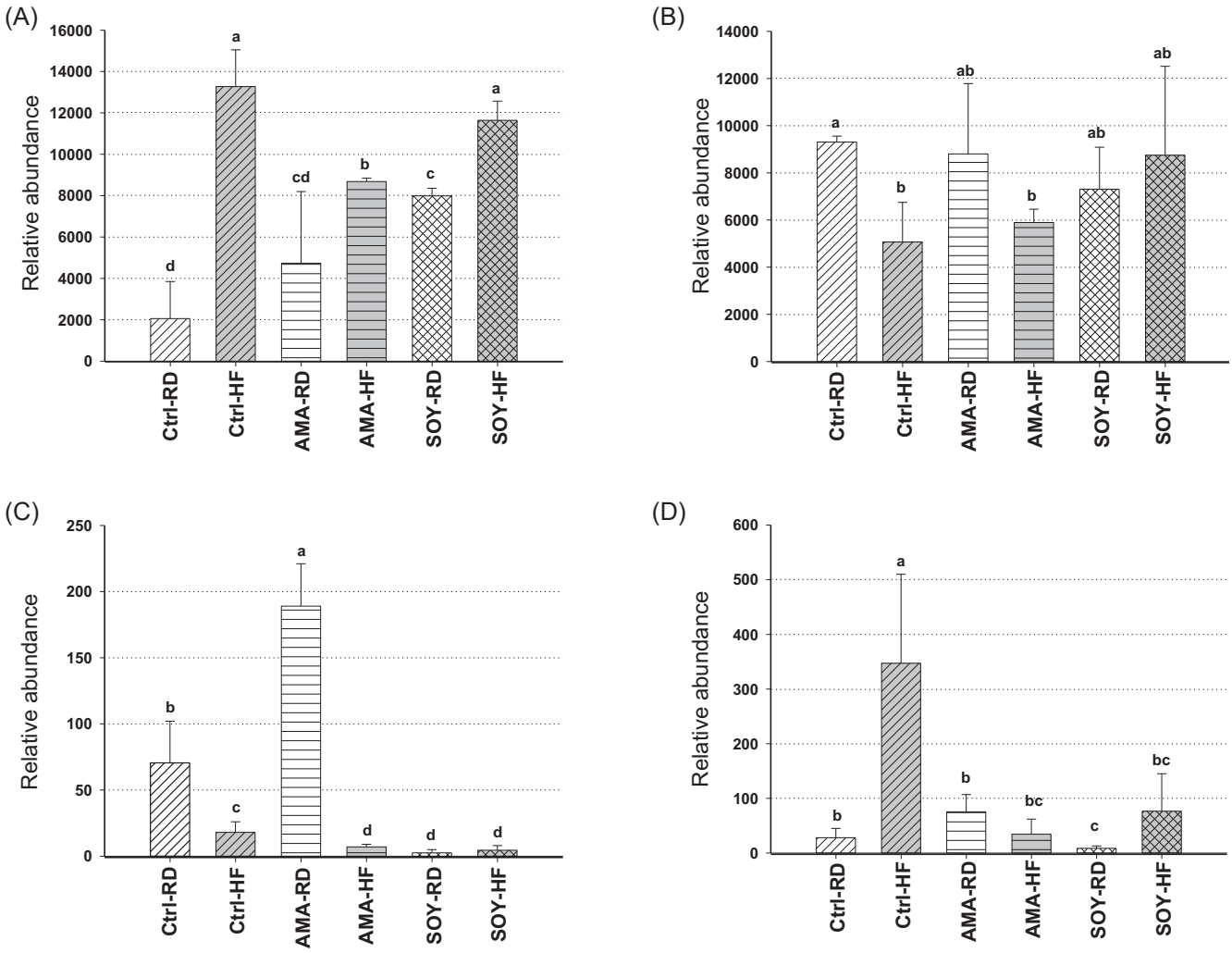


FIGURE 4. Family-level distributions of the microbial communities. Average of the relative abundance of microorganism family in each mice group. (A) Ruminococcaceae, (B) Lachnospiraceae, (C) Prevotellaceae, and (D) Bacteroidaceae. Different superscript letters indicate statically significant differences at $p<0.05$ in the Kruskal-Wallis with a post hoc Dunn test.

of mice fed the following diets: Ctrl-HD (80:5:15), AMA-RD (82:4:14), AMA-HD (84:5:11), SOY-RD (80:3:17), and SOY--HD (86:4:10) (Figure 5). This marked difference was observed due to a statistical increase in acetic acid and a decrease in propionic acid contents (Figure 5B and C) that potentially are associated with the consumption of proteins. Hedemann *et al*. [2009] reported a decrease in propionic acid in the caecum and colon of rats fed with cellulose, in a molar ratio of 83:6:11 and 80:4:6, respectively.

Gullan *et al*. [2016] reported that faecal cultures from healthy individuals fed with amaranth or quinoa diet showed an increase of SCFAs and Prevotellaceae family, as observed in this work. Prevotellaceae and Lachnospiraceae families are acetate-producing bacteria [Ferrario *et al*., 2017], their presence could be associated with acetic acid content increase (Figure 4B and 4C). Bacteroidetes richness has been correlated with faecal propionic acid levels in humans [Salonen *et al*., 2014]; in this context the decrease of propionic acid could be linked to the lack of those bacterial groups in the microbial composition (Figure 3B). However, Vital *el al*. [2015] indicate that there is no clear association between the butyric acid production with the abundance of Ruminococcaceae and Lachnospiracea that exhibit alternative, protein-fed, butyrate-synthesis pathways.

CONCLUSION

Not only the amount of protein is important, but also the source of protein has a significant impact on health. Although soybean meal has been reported to provide benefits to health, basically for the contents of isoflavones, proteins alone could have undesirable results. Our results have shown that soybean proteins consumption tends to increase fat accumulation, in contrast to amaranth proteins. The macroscopic analysis of the caecum also showed that amaranth consumption, under HF diets, had a tendency to increase the number and size of calceiform cells by crypt, similarly to the control diets.

The consumption of both amaranth and soybean proteins caused a reduction of Helicobacteracea, which is related with a decrease in pathogen families. But the intake of amaranth proteins, which have an excellent essential amino acid balance and good digestibility values, led to an improved microbial profile of the Prevotellaceae and Ruminococcaceae families,

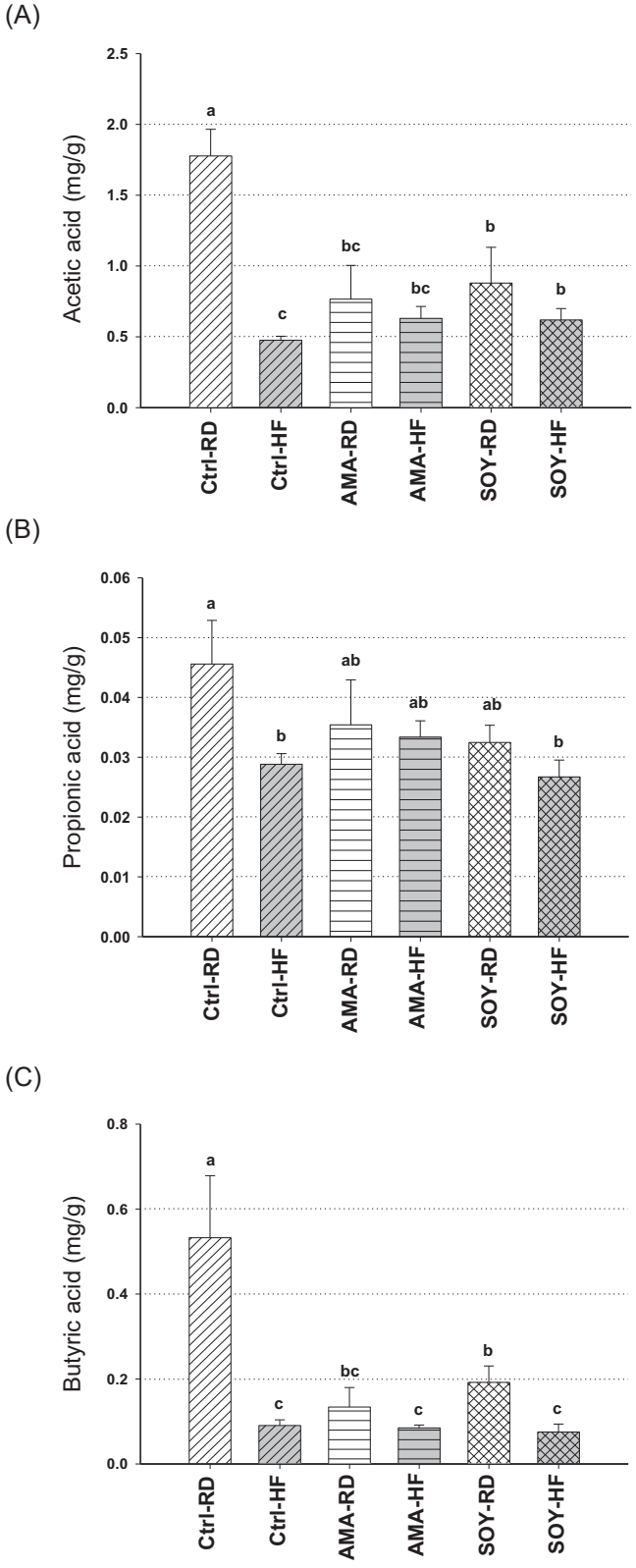


FIGURE 5. Short-chain fatty acid accumulation in caecal digesta of mice fed with different diets. (A) acetic acid; (B) propionic acid; and (C) butyric acid. Means values with SEM for each group. Ctrl=Control, AMA=amaranth, SOY=soybean, RD=regular diet, HF=high fat diet. Different superscript letters indicate statically differences at $p<0.05$ in the Kruskal-Wallis with a post hoc Dunn test.

which are microorganisms related with the intestinal barrier recovery. Results of our study suggest that amaranth could exert a modulation of caecal microbiota, which could be a new mechanism of action by which it exerts its health benefits. This finding opens new avenues to future research on amaranth mechanisms of action.

ACKNOWLEDGEMENTS

DOC and JLGE thank CONACYT fellowships nos. 590392 and 386100, respectively and thank A. Escobedo-Moratilla and A.J. Velarde-Salcedo for their help with mice work. Thanks to Araceli Patron, Alberto Barrera, and Victor Balderas for their technical support. We also thank to Problemas Nacionales "Amaranto en la Soberanía Alimentaria" Project No. 248415.

CONFLICT OF INTEREST

Authors declare no conflict of interests.

REFERENCES

1. Aguirre, M., Venema, K. (2015). Does the gut microbiota contribute to obesity? Going beyond the gut feeling. *Microorganisms*, *3*(2), 213–235.
2. Barazzoni, R., Cappellari, G.G., Ragni, M., Nisoli, E. (2018). Insulin resistance in obesity: an overview of fundamental alterations. *Eating Weigh Disorders*, *23*(2), 149–157.
3. Battson, M.L., Lee, D.M., Weir, T.L., Gentile Ch.L. (2018). The gut microbiota as a novel regulator of cardiovascular function and disease. *The Journal of Nutritional Biochemistry*, *56*, 1–15.
4. Beli, E., Yan, Y., Moldovan, L., Vieira, C.P., Gao, R., Duan, Y., Prasad, R., Bhatwadekar, A., White, F.A., Townsend, S., Chan, L., Ryan, C.N., Morton, D., Moldovan, E.G., Chu, F.I., Oudit, G.Y., Gavin Y., Derendorf, H., Adorini, L., Wang, X.X.X., Evans-Molina, C., Mirmira, R.G., Boulton, M.E., Yoder, M.C., Li, Q.H., Levi, M., Busik, J.V., Grant, M.B. (2018). Restructuring of the gut microbiome by intermittent fasting prevents retinopathy and prolongs survival in db/db mice. *Diabetes*, *67*(9), 1867–1979. Doi. org/10.2337/db18–0158.
5. Bressani, R., Elias, L.G., González, J.M., Gómez-Brenes, R. (1987). The chemical composition and protein quality of amaranth grain germplasm in Guatemala. *Archivos Latinoamericanos de Nutrición*, *37*(2), 364–377.
6. Cani, P.D., Knauf, C. (2016). How gut microbes talk to organs: The role of endocrine and nervous routes. *Molecular Metabolism*, *5*(9), 743–752.
7. Douglass, J.D., Malik, N., Chon, S.H., Wells, K., Zhou, Y.X., Choi, A.S., Joseph, L.B., Storch, J. (2012). Intestinal mucosal triacylglycerol accumulation secondary to decreased lipid secretion in obese and high fat fed mice. *Frontiers in Physiology*, *3*, art. no. 25. doi: 10.3389/fphys.2012.00025.
8. Escobedo-Moratilla, A., Velarde-Salcedo, A.J., Magaña-Hernández, C.V., Barrera-Pacheco, A., Espitia-Rangel, E., Herrera-Estrella, A., Barba de la Rosa, A.P. (2017). Amaranth protein improves lipid profile and insulin resistance in a diet-induced obese mice model. *Journal of Food and Nutrition Research*, *5*(12), 914–924.

9. Gullan, B., Gullón, P., Tavaria, F., Yáñez, R. (2016). Assessment of the prebiotic effect of quinoa and amaranth in the human intestinal ecosystem. *Food and Function*, *7*(9), 3782–3788.
10. Ferrario, C., Statello, R., Carnevali, L., Mancabelli, L., Milani, C., Mangifesta, M., Duranti, S., Lugli, G.A., Jimenez, B., Lodge, S., Viappiani, A., Alessandri, G., Dall'Asta, M., Del Rio, D., Sgoifo, A., van Sinderen, D., Ventura, M., Turroni, F. (2017). How to feed the mammalian gut microbiota: bacterial and metabolic modulation by dietary fibers. *Frontiers in Microbiology, 8*, art. no. 1749, doi:10.3389/fmicb.2017.01749.
11. Gómez-Cardona, E.E., Hernández-Domínguez, E.E., Huerta-Ocampo, J.Á., Jiménez-Islas, H., Díaz-Gois, A., Velarde-Salcedo, J., Barrera-Pacheco, A, Goñi-Ochoa, A., Barba de la Rosa, A.P. (2017). Effect of amaranth consumption on diabetes-related biomarkers in patients with diabetes. *Diabetes, Obesity & Metabolic Disorders 3*, 5–10. [http://www.kenkyugroup.org/article/16/71/Effect-of-amaranth-consumption-on-diabetes-related-biomarkers-in-patients-with-diabetes].
12. Hahnke, S., Maus, I., Wibberg, D., Tomazetto, G., Pühler, A., Klocke, M., Schlüter, A. (2015). Complete genome sequence of the novel *Porphyromonadaceae bacterium* strain ING2-E5B isolated from a mesophilic lab-scale biogas reactor. *Journal of Biotechnology*, *193*, 34–36.
13. Hedemann, M.S., Theil, P.K., Bach Knudsen, K.E. (2009). The thickness of the intestinal mucous layer in the colon of rats fed various sources of non-digestible carbohydrates is positively correlated with the pool of SCFA but negatively correlated with the proportion of butyric acid in digesta. *British Journal of Nutrition*, *102*(1), 117–125.
14. Houston, M. (2014). The role of nutrition and nutraceutical supplements in the prevention and treatment of hypertension. *World Journal of Cardiology*, *6*(2), 38–66..
15. Hsieh, T.C., Ma, K.H., Chao, A. (2016). iNEXT: An R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods in Ecology and Evolution*, *7*(12), 1451–1456.
16. Jakobsson, H.E., Rodríguez-Piñeiro, A.M., Schütte, A., Ermund, A., Boysen, P., Bemark, M., Sommer, F., Bäckhed, F., Hansson, G.C., Johansson, M.E. (2015). The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Reports*, *16*(2), 164–177.
17. Jiao, N., Baker, S.S., Nugent, C.A., Tsompana, M., Cai, L.T., Wang, Y., Buck, M.J., Genco, R.J., Baker, R.D., Zhu, R.Y., Zhu, L. (2018). Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: a meta-analysis. *Physiological Genomics*, *50*(4), 244–254.
18. Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A., Kimura, I. (2015). Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients*, *7*(4), 2839–2849.
19. Kautz, S., Rubin, B.E.R., Russell, J.A., Moreaua, C.S. (2013). Surveying the microbiome of ants: comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. *Applied and Environmental Microbiology*, *79*(2), 525–534.
20. Laparra, J.M., Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research*, *61*(3), 219–225.
21. Li, J., Armstrong, C.L.H., Campbell, W.W. (2016). Effects of dietary protein source and quantity during weight loss on appetite, energy expenditure, and cardio-metabolic responses. *Nutrients*, *8*(2), art. no. 63. doi: 10.3390/nu8020063.
22. Magnuson, A., Fouts, J., Booth, A., Foster, M. (2015). Obesity-induced chronic low-grade inflammation : Gastrointestinal and adipose tissue crosstalk. *Integrative Obesity and Diabetes*, *1*(5), 103–108.
23. Marotz, C.A., Zarrinpar, A. (2016). Treating obesity and metabolic syndrome with fecal microbiota transplantation. *Yale Journal of Biology Medicine*, *89*(3), 383–388.
24. Martirosyan, D.M., Miroshnichenko, L.A., Kulakova, S.N., Pogojeva, A.V, Zoloedov, V.I. (2007). Amaranth oil application for coronary heart disease and hypertension. *Lipids in Health and Disease*,.6, art. no. 1. doi: 10.1186/1476–511X-6–1.
25. OECD. (2015). Organization for Economic Co-operation and Development. Key Facts – Mexico, Update 2014. Obesity and the Economics Prevention: Fit not Fat., 1–5. [https://www.oecd.org/mexico/Obesity-Update-2014-MEXICO_EN.pdf].
26. Ravussin, Y., Koren, O., Spor, A., LeDuc, C., Gutman, R., Stombaugh, J., Knight, R., Ley, R.E., Leibel, R.L. (2012). Responses of gut microbiota to diet composition and weight loss in lean and obese mice. *Obesity (Silver Spring)*, *20*(4). doi: 10.1038/oby.2011.111.
27. Rooks, M.G., Veiga, P., Wardwell-Scott, L.H., Tickle, T., Segata, N., Michaud, M., Gallini, C.A., Beal, C., van Hylckama-Vlieg, J.E.T., Ballal, S.A., Morgan, X.C., Glickman, J.N., Gevers, D., Huttenhower, C., Garrett, W.S. (2014). Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *The ISME Journal*, *8*(7), 1403–1417.
28. Salonen, A., Lahti, L., Salojärvi, J., Holtrop, G., Korpela, K., Duncan, S.H., Date, P., Farquharson, F., Johnstone, A.M., Lobley, G.E., Louis, P., Flint, H.J., de Vos, W.M. (2014). Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *The ISME Journal, 8*, 2218–2230.
29. Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N.A., Donus, C., Hardt P.D. (2010). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity*, *18*(1), 190–195.
30. Tagliabue, A., Elli, M. (2013). The role of gut microbiota in human obesity: Recent findings and future perspectives. *Nutrition, Metabolism and Cardiovascular Diseases*, *23*(3), 160–168.
31. Tomas, J., Mulet, C., Saffarian, A., Cavin, J.B., Ducroc, R., Regnault, B., Tan, C.K., Duszka, K., Burcelin, R., Wahli, W., Sansonetti, P.J., Pédron, T., (2016). High-fat diet modifies the PPAR-pathway leading to disruption of microbial and physiological ecosystem in murine small intestine. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(40), E5934-E5943.
32. Turnbaugh, P.J., Bäckhed, F., Fulton, L., Gordon, J.I. (2008). Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host & Microbe*, *3*(4), 213–223.
33. Tun, H.M., Bridgman, S.L., Chari, R., Field, C.J., Guttman, D.S., Becker, A.B., Mandhane, P.J, Turvey, S.E., Subbarao, P., Sears, M.R., Scott, J.A., Kozyrskyi, A.L. (2018). Roles of birth mode and infant gut microbiota in intergenerational transmission of overweight and obesity from mother to offspring. *JAMA Pediatrics*, *172*(4), 368–377.
34. Velarde-Salcedo, A.J., Regalado-Rentería, E., Velarde-Salcedo, R., Juárez-Flores, B.I., Barrera-Pacheco, A., de Mejía, E.G., Barba de la Rosa, A.P. (2017). Consumption of amaranth induces

the accumulation of the antioxidant protein paraoxonase/arylesterase 1 and modulates dipeptidyl peptidase iv activity in plasma of streptozotocin-induced hyperglycemic rats. *Journal of Nutrigenetics and Nutrigenomics*, *10*, 181–193.

35. Vital, M., Gao, J., Rizzo, M., Harrison, T., Tiedje, J.M. (2015). Diet is a major factor governing the fecal butyrate-producing community structure across Mammalia, Aves and Reptilia. *The ISME Journal*, *9*(4), 834–843.
36. WHO, World Health Organization. Obesity and overweight, fact sheet No311, 2016 [http://www.who.int/mediacentre/factsheets/fs311/en/] (accessed Jul 20, 2017).
37. Wrzosek, L., Miquel, S., Noordine, M.L., Bouet, S., Chevalier-Curt, M.J., Robert, V., Philippe, C., Bridonneau, C., Cherbuy, C., Robbe-Masselot, C., Langella, P., Thomas, M. (2013). *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. *BMC Biology*, *11*, art. no. 61. doi: 10.1186/1741–7007–11–61.
38. Zarrinpar, A., Chaix, A., Yooseph, S., Panda, S. (2014). Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. *Cell Metabolism*, *20*(6), 1006–1017.
39. Zeng, H., Ishaq, S.L., Zhao, F.Q., Wright, A.D.G. (2016). Colonic inflammation accompanies an increase of β-catenin signaling and Lachnospiraceae/Streptococcaceae bacteria in the hind gut of high-fat diet-fed mice. *The Journal of Nutritional Biochemistry*, *35*, 30–36.
40. Zhang, C., Zhang, M.H., Wang, S.Y., Han, R.J., Cao, Y.F., Hua, W.Y., Mao, Y.J., Zhang, X.J., Pang, X.Y., Wei, C.C., Zhao, G.P., Chen, Y., Zhao, L.P. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *The ISME Journal*, *4*, 232–241.

Submitted: 27 March 2018. Revised: 19 July and 20 August 2018. Accepted: 23 October 2018. Published on-line: 17 December 2018.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 45–52
DOI: 10.31883/pjfns-2019-0004
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Behavior of *Listeria innocua* Strains Under Pressure Treatment – Inactivation and Sublethal Injury

Justyna Nasiłowska[1], Barbara Sokołowska[1,2], Monika Fonberg-Broczek[2]*

[1]Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology, Department of Fruit and Vegetable Product Technology, 36 Rakowiecka str., 02–532 Warsaw, Poland
[2]Institute of High Pressure Physics, Polish Academy of Sciences, Laboratory of Biological Materials, 29/37 Sokołowska str., 01–142 Warsaw, Poland

Key words: *Listeria innocua*, beetroot juice, high hydrostatic pressure, sublethal injury, transmission electron microscopy

The inactivation and sublethal injury of two strains of *Listeria innocua* (one collection strain and one wild strain isolated from beetroot juice) suspended in beetroot juice and in model solutions, after high hydrostatic pressure (HHP) were investigated. Changes within the population assessed by plating count methods of both *L. innocua* strains suspended in a buffer pH 4.0 were more noticeable than in the natural beetroot juice environment. In beetroot juice the lethal effect was reported after 1 min of pressure treatment at 400 MPa for the collection strain. In the case of the wild type strain, exposure to the maximal parameters of the compression process (400 MPa, 10 min) decreased the population number below 1 log (CFU/mL) but did not cause complete injury. The collection strain of *L. innocua* was easier to inactivate in beetroot juice than the strain isolated from this environment. The maximum level of sublethal injury was observed when the cells were suspended in a buffer pH 7.0. Structural damage in cell membranes after HHP processing was observed using a transmission electron microscope (TEM).

INTRODUCTION

Beetroot is a traditional vegetable distributed in many parts of the world and has been used commercially to produce juice and natural pigments. One of the leading red beet producers is Poland, where fresh beetroot juice has nowadays become increasingly popular because of its multiple health benefits, such as anticancer activity and protection against degenerative diseases [Clifford *et al.*, 2015]. Beetroot contains dietary fiber and carbohydrates of a moderate caloric value. It is a rich source of minerals and important vitamins, and therefore it can play an essential role in the composition of a well-balanced diet [USDA Food Composition Database, 2018; Zielińska-Przyjemska *et al*., 2009]. Due to the fact that edible parts of root vegetables have a direct contact with soil, beetroot juice is one of the most contaminated among the commercially available fresh juices and can be a source of undesirable microbiota including pathogenic microorganisms [Sapers, 2003; Sokołowska *et al.*, 2011]. One of the most virulent foodborne pathogens, widely distributed in the natural environment, is *Listeria monocytogenes*. It has been detected in fruit and vegetables that are contaminated by the soil or by manure used as a fertilizer. Among the investigated samples of unpasteurized commercial root vegetable and fruit juices, 29% (n=17) have been reported to contain *Listeria monocytogenes* [Sokołowska *et al.*, 2011]. This pathogen can survive short pasteurization or freezing, and can be resistant to treatment with food preservatives. Moreover, it can grow in acidic foods traditionally considered as of low risk [Jordan *et al.*, 2001]. The infective dose of *L. monocytogenes* depends on the resistance of the individual host. Fresh fruit and vegetables which are consumed without any further thermal treatment, and are contaminated with *L. monocytogens* at more than 100 CFU/g, are considered to pose a direct risk to human health [Commission Regulation 2073/2005]. Exceeding this number is dangerous, especially for people with compromised immunity, children, the elderly and pregnant women, as it may induce listeriosis and even sepsis [Goulet *et al.*, 2008].

The High Hydrostatic Pressure (HHP) is a technology used worldwide for the preservation of various commercial products, including vegetable juices. However, this technology has not yet been implemented on the industrial scale in Poland. HHP allowed reducing counts of microbes responsible for spoilage and for shortening the shelf-life of beetroot juice [Sokołowska *et al.*, 2013, 2014, 2017], while not markedly changing the sensory and nutritional attributes of the product. Mild, non-thermal technologies used in food preservation, apart from the inactivation, trigger the sublethal injury of bacterial cells. Injury caused by high hydrostatic pressure has been observed in many bacterial cells [Patterson *et al.*, 1995; Yang *et al.*, 2012; Sokołowska *et al.*, 2014; Wang *et al.,* 2016]. The mechanism of microbial inactivation by HHP is re-

* Corresponding Author: Tel.: +48 22 606 3605;
E-mail: nasilowska@ibprs.pl (J. Nasiłowska)

lated to the morphological changes in the cell, modification of the cytoplasmic membrane, damage to the genetic mechanism, and adverse biochemical reaction [Hoover *et al.*, 1989]. Changes in the bacterial cell can be reversible or irreversible depending on the level of environmental stress and physiological condition of the cell. Even though the membrane damage plays a major role in HHP inactivation, the partial loss of its functionality does not always lead to cell death. In a consequence, some of the cells in the population will be sublethally injured [Wesche *et al.*, 2009]. However, the sublethally injured cells may reveal increased sensitivity to inhibitors, which are ingredients of selective agar media [Espina *et al.*, 2016]. The injured survivors are able to recover and resume growth if suitable environmental conditions emerge, and therefore may become dangerous to customers [Mackey *et al.*, 2000]. This is the reason why appropriate identification and quantification of the sublethally injured population play a key role in food safety. An indirect method for evaluating the number of sublethally injured cells is the plating technique which utilizes a selective medium with the addition of NaCl [Yuste *et al.*, 2004; Sokołowska *et al.* 2014], because immediately after HHP processing the damaged cells have no, or a lower, ability to grow on this medium.

This work describes results of the investigation of the survival, sublethal injury, and diversity of the resistance of *Listeria innocua* strains in pasteurized beetroot juice and in model solutions: buffers pH 4.0 and 7.0, after high hydrostatic pressure treatment.

MATERIALS AND METHODS

Microorganisms and growth conditions

Listeria innocua was used in this study. This bacteria is physiologically very close to the previously mentioned *L. monocytogenes*, and is frequently found in the same food products, therefore it is often used for experiments [Escolar *et al.,* 2017].

Two strains of *Listeria innocua* were used in this study: CIP80.11T obtained from the Culture Collection of the Institut Pasteur (Paris, France) and 23/2013 (wild type strain) isolated from unpasteurized Polish beetroot juice obtained from the own collection of the Department of Fruit and Vegetable Product Technology at IAFB (Warsaw, Poland). The strains were stored in Cryobank at a temperature below -27°C±3°C. Broth subcultures were prepared by inoculating a tube containing 10 mL of sterile Brain Heart Infusion (BHI) broth (BioMerieux, I'Etoile, France) with a pure culture immobilized on sterile beads. After inoculation, the tubes were incubated at 37°C for 24 h and then each overnight culture was moved with a 0.1 mL loop on a Petri dish with Tryptic Soy Yeast Extract (TSYE) agar (Biocar Diagnostics, Beauvais, France). Next, the culture from the plate with a 0.1 mL loop was added to 250 mL Erlenmeyer flasks containing 200 mL of Tryptic Soy Broth with Yeast Extract (TSBYE) (Biocar Diagnostics, Beauvais, France) to prepare the second subculture, which was incubated at 37°C for 18 h to obtain the stationary phase culture. Then, 10 mL of the second subculture were added to fresh sterile broth (TSB or TSYEB) and incubated at 37°C for 18 h. The cultures were then harvested by centrifugation (4000×*g*, 10 min, 4°C). The sedimented cells were aseptically re-suspended in phosphate-buffered saline (PBS, pH 7.2) and again centrifuged. The washing procedure was repeated twice. After that, model suspensions of *L. innocua* were prepared in PBS (1:9, v/v). Just before HHP treatment, McIlvain buffers (0.1 M citric acid, 0.2 M disodium phosphate) in pH 4.0 and pH 7.0, and beetroot juice were inoculated with *L. innocua* cells in a concentration of about 10^7 CFU/mL and transferred into sterile polyethylene tubes (Sarstedt, Newton, USA) in 13 mL portions in duplicate.

Model suspensions and beetroot juice

McIlvaine buffers pH 4.0 and pH 7.0, and pasteurized beetroot juice, acidified with citric acid to pH from 3.98 to 4.17 (produced by Victoria Cymes, Poland) were used.

HHP treatment

High pressure treatment was performed using a U 4000/65 device (Unipress, Warsaw, Poland). The apparatus was capable of operating up to 600 MPa, at temperatures ranging from −10°C to +80°C. The maximum volume of the treatment chamber was 0.95 L. The pressure--transmitting fluid was distilled water and polypropylene glycol (1:1, v/v). Each two independent samples were treated in two independent cycles. The treatment was performed at pressures of 200 MPa, 300 MPa, and 400 MPa, at 20°C for 1, 5, and 10 min. Pressure of up to 400 MPa was generated in 70–80 s and the release time was 2–4 s. The total process time did not include the come-up and come-down time of pressurization. After the treatment, the samples were removed from the chamber and placed immediately on ice. The control samples were unpressurized.

Plate count analytical methods

The HHP-treated samples were analyzed immediately after processing. Ten-fold serial dilutions in Tryptone Salt broth (Biokar Dignostics, Beauvais, France) of each sample were prepared. Appropriate dilutions of samples were spread on agars. Counts of total viable cells were determined by spread plate on TSYE agar, while TSYE agar supplemented with 5% NaCl (POCh, Gliwice, Poland) was used to determine uninjured cells in the population [Yuste *et al*., 2004]. This concentration of NaCl was estimated in the laboratory as the maximum concentration that did not change the morphology and number of unstressed *L. innocua* cells. The number of sublethally injured survivors was estimated by the difference between the counts of total viable and uninjured cells in the population [Yuste *et al.*, 2004; Espina *et al*., 2016]. Plates with TSYE agar were incubated for 24 h/37°C, and these with TSYE agar+5% NaCl for 48 h/37°C [Espina *et al*., 2016]. The plates containing less than 300 CFU/mL were selected for counting [Yuste *et al*., 2004].

Cell morphology assessment by transmission electron microscopy (TEM)

After exposure to 400 MPa for 5 min, the bacteria cells in the PBS buffer (pH 7.2) were fixed with 2.5% glutaraldehyde cacodylic buffer and incubated for one hour, then washed with 0.1 M cacodylic buffer. Next, they were postfixed in 1% OsO_4 in ddH_2O for 1 h and washed three times in ddH_2O. Af-

ter postfixation, the samples were dehydrated through a graded series of EtOH (30% – 10 min, 50% – 10 min, 70% – 24 h, 80% – 10 min, 90% – 10 min, 96% – 10 min, anhydrous EtOH – 10 min, acetone – 10 min) and infiltrated with epon resin in acetone (1:3 – 30 min, 1:1 – 30 min, 3:1 – 2h), infused twice for 24 h in pure epon resin and polymerized at 60°C for 24 h. Next, 60 nm sections were prepared using RMC ultramicrotome MT-X (RMC Boeckeler Instruments, Tucson, USA), contrasted with uranyl acetate and lead citrate according to Reynolds [1983], and examined on LIBRA 120 electron microscope produced by Zeiss (Oberkochen, Germany). Images were captured with the Slow-Scan CCD camera (Proscan) using EsiVision Pro 3.2 software (Soft Imaging Systems GmbH). Measurements were performed using the analySIS® 3.0 image-analytical software (Soft Imaging Systems GmbH, Münster, Germany).

Statistical analysis

The results of survival and sublethal injuries of bacteria were analyzed by two-way ANOVA statistical model with Scheffe's test using Statistica version 13 (TIBCO Software Inc., Palo Alto, CA, USA). Statistical comparison was made for results obtained at different times of the process. The differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Effect of HHP on bacterial cells

The results of the experiment showed that the inactivation and injury of *L. innocua* cells subjected to HHP depended on the origin of the strain, as well as the medium and parameters of the process. Survival rates of the population under the studied conditions and in all media tested are presented in Figures 1–3.

For both strains suspended in beetroot juice, increasing the pressurization time from 1 to 10 min under the pressure of 200 MPa had no significant effect on their survival ($p\geq0.05$) (Figure 1). The maximum reduction was less than 1.1 log (CFU/mL). A higher reduction was achieved when the pressure was increased up to 300 MPa. After 5 min of treatment, the population of the collection strain suspended in beetroot

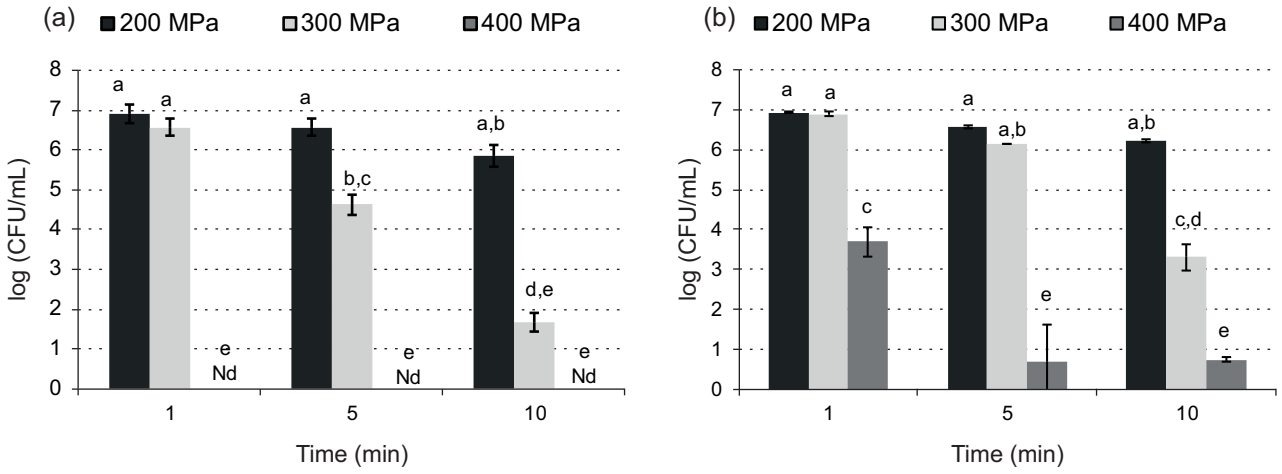


FIGURE 1. Effect of high hydrostatic pressure on the survival of *L. innocua* CIP80.11T (a) and wild type strain 23/2013 (b) in beetroot juice. The bars with different letters are significantly different at $p<0.05$; Nd – not detected.

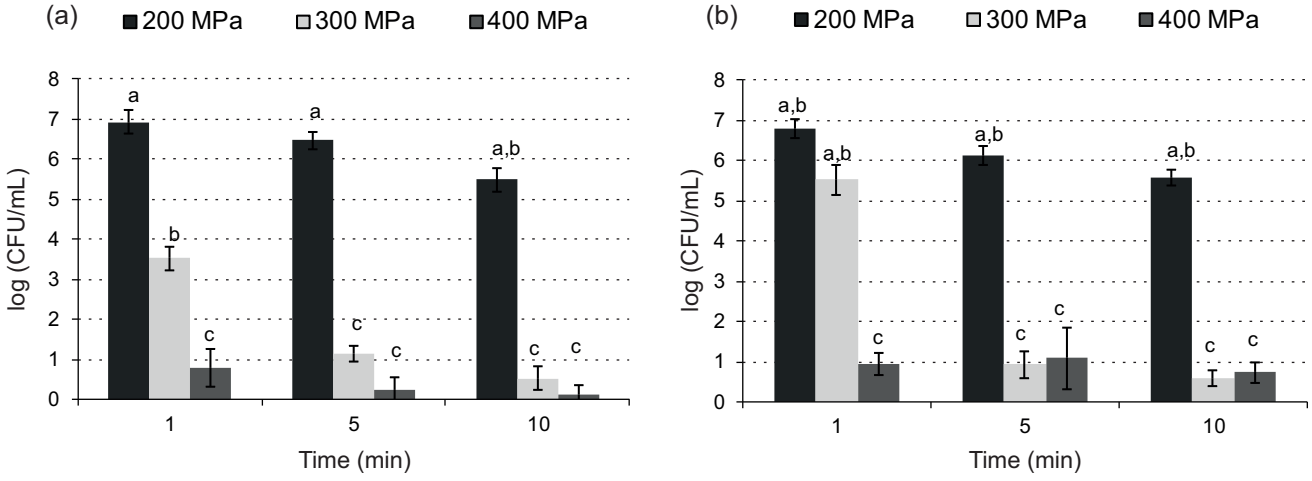


FIGURE 2. Effect of high hydrostatic pressure on the survival of *L. innocua* CIP80.11T (a) and wild type strain 23/2013 (b) in buffer pH 4. The bars with different letters are significantly different at $p<0.05$.

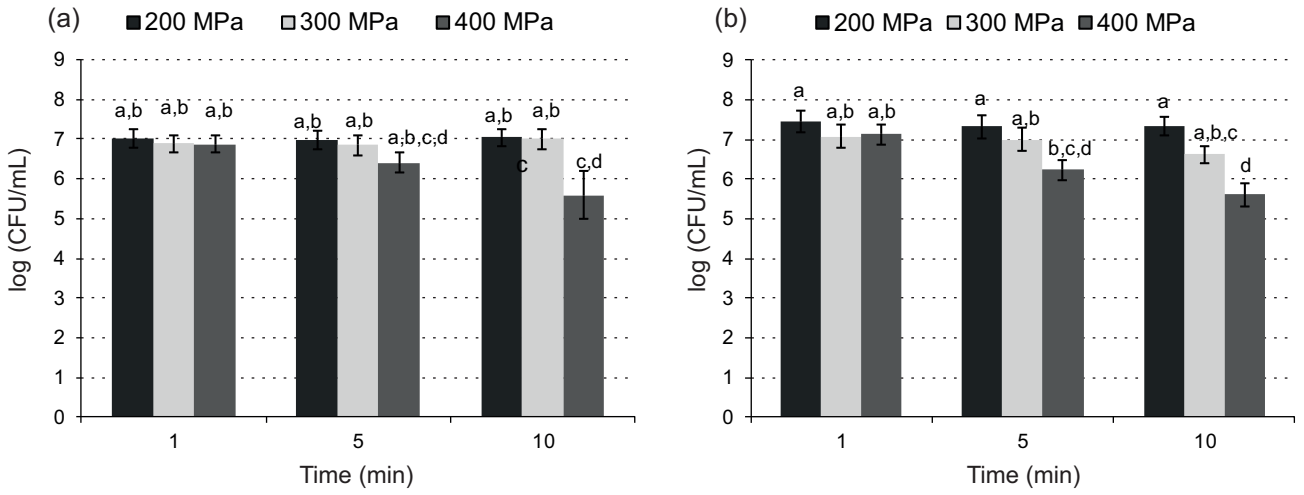


FIGURE 3. Effect of high hydrostatic pressure on the survival of *L. innocua* CIP80.11T (a) and wild type strain 23/2013 (b) in buffer pH 7. The bars with different letters are significantly different at $p<0.05$.

juice decreased by 2.3 log (CFU/mL), while under the same conditions the reduction for the wild type strain was 0.9 log (CFU/mL). When the treatment time was extended up to 10 min, the inactivation of both strains increased by 2.9 log (CFU/mL). In the beetroot juice samples, after the application of 400 MPa for 1 min the collection strain of *L. innocua* was not detected, while the inactivation of the wild type strain was 3.3 log (CFU/mL). Increasing the time of exposure up to 10 min resulted in a significant decrease ($p<0.05$) in the population number of the wild type strain but did not provide its complete inactivation. Our previous studies have shown that the HPP treatment at 400 MPa and 20°C for 10 min of the same beetroot juice (pH 4.18, °Bx 12.35), resulted in 6.2 log (CFU/mL) reduction of *E. coli* ATCC 7839, whereas HPP treatment at 300 MPa and 20°C for 10 min caused about 3.5 log (CFU/mL) reduction of *Saccharomyces cerevisiae* NCFB 3191 [Sokołowska *et al.*, 2013, 2014].

Changes within the population of *L. innocua* suspended in buffer pH 4.0 were more noticeable (Figure 2) than in a natural beetroot juice environment, however there were no significant differences between both strains ($p\geq0.05$). This was most probably due to the presence of molecules, such as lipids and carbohydrates, in product. This modified the effect of HHP on microorganisms, which was confirmed in our previous study [Sokołowska *et al.*, 2013]. After 10 min of the treatment under 200 MPa, the population numbers of the collection and wild type strains decreased by 1.4 and 1.6 log (CFU/mL), respectively. When the samples of the collection strain in an acid model solution were treated under 300 MPa for 1 min, their inactivation reached 3.3 log (CFU/mL). Under the same conditions, the level of reduction of the wild type strain was only 1.6 log (CFU/mL). A decline in the population numbers of both strains at the level of about 1 log (CFU/mL) was observed after 5 min of the treatment. Further enhancement of the process parameters had no significant effect on the studied bacterial populations ($p\geq0.05$). Jofré *et al*. [2010] studied the inactivation of five strains of *L. monocytogenes* of different origins suspended in a complex medium (pH: 5.1; 6.3, 7.4). The results have demonstrated that treatment at 400 MPa for 10 min greatly affected the viability of each strain. We have shown similar findings in an acid model solution. However, the results of bacterial inactivation, in a near-neutral pH environment, were totally different. In our study, the survival rates of *L. innocua* suspended in buffer 7.0 under pressure reaching up to 400 MPa for 5 min have shown no significant differences ($p\geq0.05$) (Figure 3). Maximum inactivation of both strains was observed after the treatment at 400 MPa for 10 min and was below 1.5 log (CFU/mL). On the contrary to our results. Patterson *et al.* [1995] showed 5 log (CFU/mL) reduction of *L. monocytogenes* in a phosphate buffer (pH 7) after the treatment at 375 MPa for 15 min. In another study, it has been reported that the treatment at 207 MPa for 10 min at 25°C, caused a 0.7 log (CFU/mL) reduction in population numbers of two strains of *L. monocytogenes* suspended in a peptone solution (pH 7.2) [Alpas *et al.*, 2000]. Stewart *et al.* [1997] described the effect of HHP on the injury and destruction of two strains of *L. monocytogenes* (Scott A and CA) in buffer suspensions. They observed complete sterility in buffer pH 4.0 in the case of the samples pressurized at 404 MPa for 10 min. However, under the same HHP conditions in buffer 6.0, both strains were reduced by 4.0 log (CFU/mL) and 6.0 log (CFU/mL), respectively. On the other hand, the number of cells of both strains decreased by 5.0 log (CFU/mL) in pH 4.0 and by 3.0 log (CFU/mL) in pH 6.0 upon pressurization at 300 MPa, 25°C for 10 min [Stewart *et al.*, 1997].

Sublethal injury to bacterial cells

Microorganisms are said to be sublethally injured if they survive an inactivation treatment. Some of the damages might be repaired, especially while microorganisms are stored under favorable conditions [Jofré *et al.*, 2010]. Pressure treatment at 300–600 MPa, at ambient temperature for a few minutes destroys pathogenic bacteria, such as *Listeria*, *Escherichia*, *Salmonella*, as well as causes sublethal injuries [Patterson *et al.*, 1995]. However, under these conditions some bacteria are sublethally injured. This phenomenon has been confirmed in our study (Table 1). As aforementioned, survivors may re-

TABLE 1. The level of sublethal injuries of *L. innocua* strains after HHP treatment.

Strains/HHP parameters	200 MPa			300 MPa			400 MPa		
	1 min	5 min	10 min	1 min	5 min	10 min	1 min	5 min	10 min
Sublethal injuries in beetroot juice (log CFU/mL)									
Listeria innocua CIP 80.11T	0.11±0.26^{a}	0.08±0.23^{a}	0.37±0.26^{a}	-0.03±0.27^{a}	0.72±0.26^{a}	0.58±0.69^{a}	Nd^{a}	Nd^{a}	Nd^{a}
Listeria innocua – wild type strain 23/13	-0.02±0.00^{a}	0.01±0.05^{a}	-0.09±0.09^{a}	0.13±0.04^{a}	0.10±0.00^{a}	2.34±0.26^{b}	0.83±0.13^{a,b}	-0.05±1.02^{a}	0.01±0.17^{a}
Sublethal injuries in McIlvain buffer pH 4.0 (log CFU/mL)									
Listeria innocua CIP 80.11T	-0.06±0.27^{a}	0.05±0.25^{a}	0.12±0.22^{a}	0.54±0.29^{a}	0.45±0.67^{a}	0.54±0.00^{a}	0.81±0.00^{a}	0.24±0.00^{a}	0.15±0.00^{a}
Listeria innocua – wild type strain 23/13	-0.19±0.22^{a}	-0.09±0.24^{a}	0.07±0.26^{a}	0.42±0.39^{a}	0.28±0.92^{a}	0.45±0.21^{a}	0.30±0.16^{a}	0.30±1.01^{a}	0.42±0.34^{a}
Sublethal injuries in McIlvain buffer pH 7.0 (log CFU/mL)									
Listeria innocua CIP 80.11T	0.10±0.21^{a}	0.09±0.28^{a}	0.17±0.25^{a}	0.14±0.24^{a}	0.17±0.26^{a}	0.31±0.27^{a}	0.67±0.25^{a,b}	2.64±0.34^{c}	3.83±0.32^{d}
Listeria innocua – wild type strain 23/13	0.04±0.28^{a}	0.08±0.20^{a}	-0.01±0.25^{a}	0.03±0.23^{a}	0.05±0.22^{a}	0.24±0.27^{a}	0.28±0.24^{a}	1.21±0.23^{b}	2.39±0.25^{c}

All data were the mean ± SD, n=2. Values in rows (a-d) denoted with different letter are significantly different at $p<0.05$. Nd: not detected.

veal increased sensitivity to inhibitors, such as sodium chloride [Mackey, 2000]. In our study, it has been observed that the maximum level of sublethal injury occurred when the cells were suspended in buffer pH 7.0 (Table 1) and exposed to the pressure of 400 MPa for 10 min. The level of sublethal injury was 3.83 log (CFU/mL) and 2.39 log (CFU/mL) for the collection strain and wild type strain, respectively. Pressure treatment at 200 MPa and 300 MPa in buffer pH 7.0 caused no significant changes ($p\geq0.05$) in the levels of sublethal injury of bacterial cells. The same observation was made in buffer pH 4.0 ($p\geq0.05$) (Table 1). In turn, beetroot juice samples exposure to 300 MPa for 10 min significantly ($p<0.05$) affected the level of sublethal injury of the wild type strain compared with the collection strain (Table 1). Sokolowska *et al.* [2014] confirmed that the pressure of 400 MPa triggered sublethal injury of *E. coli* cells in PBS. After 5 and 10 min of HHP treatment, 2.4 log (CFU/mL) and 2.7 log (CFU/mL) of sublethally injured cells were observed, while in beetroot juice the counts of injured cells reached 1.5 log (CFU/mL) and 0.8 log (CFU/mL), respectively. On the other hand, the application of 400 MPa for 10 min on five strains of *L. monocytogenes* suspended in a complex medium caused that the number of sublethally injured cells in population was less than 1 log (CFU/mL) [Jofré *et al.,* 2010]. The number of sublethally injured survivors in the population depends on high pressure treatment parameters, as well as on the type of microbiota and medium. In some cases, the level of sublethally injured cells might be almost 100%. The adequate identification and quantification of the sublethally injured population plays an important role in food safety.

TEM observations

The character of sublethal injuries of *L. innocua* triggered by high hydrostatic pressure, was illustrated by TEM microscopy. Changes in *L. innocua* cells morphology are shown in Figure 4. TEM images of untreated samples demonstrated intact, characteristic rod-shaped *L. innocua* cells, single or dividing. Cell membrane and walls were clearly defined with centrally located genome surrounded by the integrated cytoplasmic area and tickly packed ribosomes (Figure 4 a,b). The mechanisms of microbial inactivation by HHP have been mostly associated with the damage of cell membrane, as the major target of pressure treatment. Loss of membrane integrity and swelling leads to the leakage of cellular materials and nucleoid condensation [Hauben *et al.*, 1996; Mañas & Mackey, 2004]. According to plate count results, the reduction of both strains suspended in buffer pH 7.0 after HHP treatment at 400 MPa for 5 min was less than 1 log (CFU/mL). The level of sublethal injury was 2.64 log (CFU/mL) and 1.21 log (CFU/mL) for collection and wild type *L. innocua* strain, respectively (Figure 3). It was coherent with the results which we achieved using the transmission electron microscopy technique. Most of the cells in the population observed by TEM had an intact cell membrane. Only a few cells of *L. innocua* wild type strain have presented surface damage (Figure 4 f). TEM observations confirmed aggregation of cytoplasm. Disorganization of the genome area containing fibrillar regions was observed in all populations of both strains (Figure 4 c,d,e,f). Alterations in the appearance of the interior of the *Escherichia coli* cells were reported after HHP treatment at 300 and 600 MPa for 5 min [Prieto-Calvo *et al.*, 2014] and of *Listeria monocytogenes* cells interior after the treatment at 450 MPa for 5 min [Huang *et al*., 2015]. Monitoring of the cellular ultrastructure by TEM showed the cellular enlargement, disruption of cellular membranes, condensation of the cytoplasmic material and disorganization of the genome area [Huang *et al*., 2015; Prieto-Calvo *et al.*, 2014]. Mackey *et al.* [1994] observed that cells of *L. monocytogenes* treated under 250 MPa were characterized with unusual symmetrical areas in the cytoplasm. These changes were related to the deprivation of ribosomes, resembling gas bubbles, which could have been due to the osmotic effects or phase changes in the membrane. Under the same treatment conditions, amorphous compacted regions were

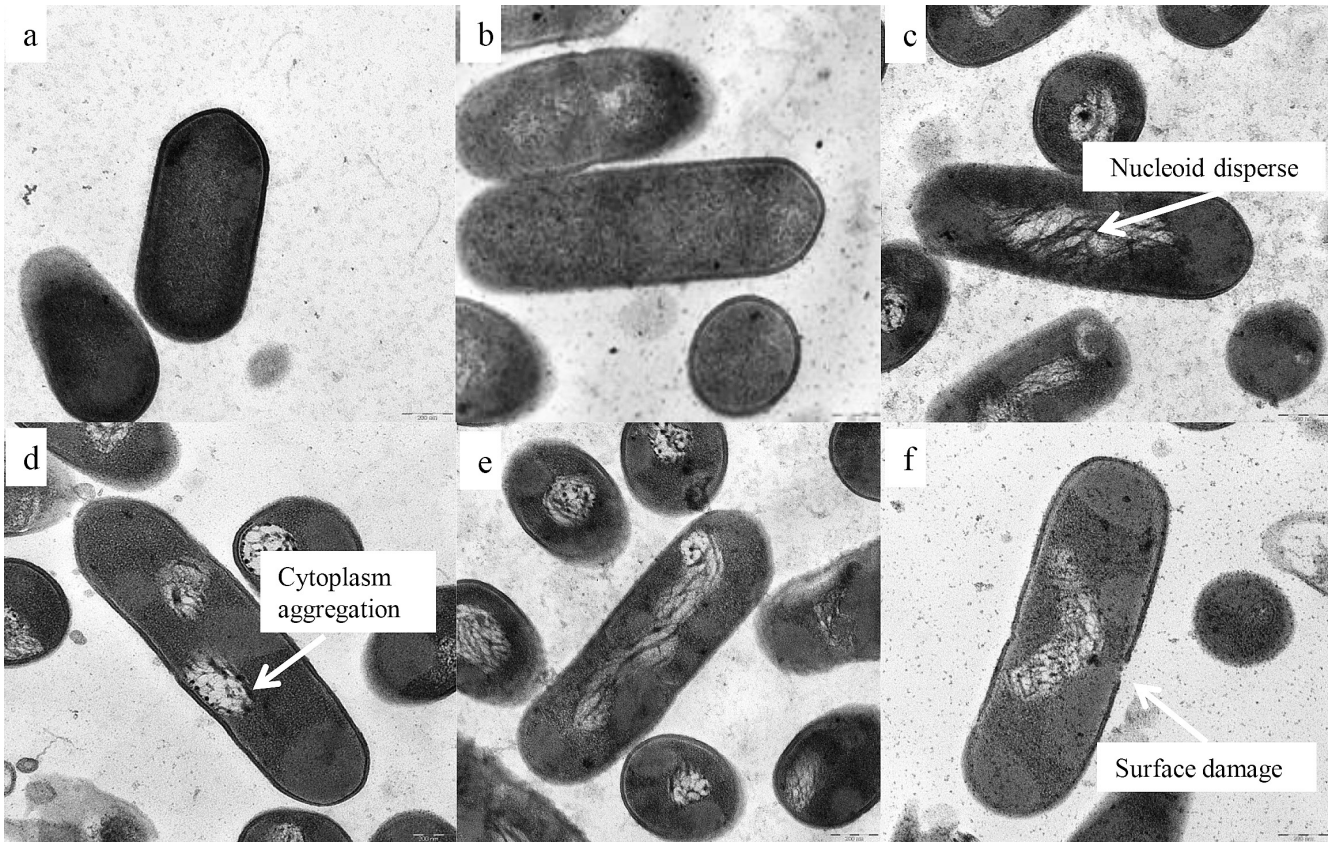


FIGURE 4. TEM images of untreated *L. innocua* strains (a) CIP80.11T and (b) wild type strain 23/2013 and after exposure to 400 MPa for 5 min (c-d) and (e-f) respectively. Scale bar, 200 nm. Representative images of the samples are shown.

noticed in *Salmonella* Thompson. It was probably induced by denaturation of cytoplasmic protein [Mackey *et al.*, 1994]. Increased pressure up to 500 MPa resulted in extreme condensation of the cytoplasm, whilst the outline of the cells was intact [Mackey *et al.*, 1994].

Variation in resistance to HHP

Numerous studies have demonstrated that variations in the resistance of microorganisms to high pressure occurred not only among the different species of bacteria, but also among the strains belonging to the same species [Alpas *et al.*, 1999; Jordan *et al.,* 2001; Boeijen *et al.*, 2010; Huang *et al.*, 2015]. It has been reported that some bacterial strains with very high pressure resistance were isolated from the natural environment. Because of biodiversity of microorganisms, the results that were obtained in different studies varied significantly [Alpas *et al.*, 2000]. The studies that we have conducted on *Listeria*, which was suspended in beetroot juice, showed a certain phenomenon. The collection strain was easier to inactivate than the strain isolated from the natural environment (Figure 1). Moreover, the wild type strain was not completely inactivated in an acidic medium, even being treated in a very harsh way (Figure 2). Alpas *et al.* [1999] studied the variation in pressure resistance among nine strains of *L. monocytogenes*. They observed that after pressure treatment at 345 MPa for 5 min at 25°C, some strains were more resistant to pressure than others. The viability loss of cells ranged from 0.9 to 3.5 log (CFU/mL). In another work, all the survivors of two strains of *L. monocytogenes* suspended in a peptone solution were completely injured after being exposed to the aforementioned factors. The diversity between 24 piezotolerant variants of *L. monocytogenes*, which were resistant to pressure treatment at 350 MPa was examined by Boeijen *et al.* [2010]. Those 24 strains were compared with the wild type strain. In most cases the wild type strain revealed greater sensitivity than the used variants. Most of them were also resistant to other stresses besides HHP, such as high temperature and low pH. Differences among the variants were observed in *e.g.* acid resistance, growth rate or motility. The authors suggested that this population diversity may be essential to the persistence of pathogens such as *L. monocytogenes* in a range of environments [Boeijen *et al.*, 2010].

CONCLUSIONS

It has been confirmed in our study that high pressure can result in the loss of viability of *L. innocua* cells. It was found that the level of reduction by HHP treatment at 20°C in beetroot juice and buffer solutions was strictly dependent on a couple of factors, including: the pressure applied, the duration of the process, as well as the origin of the strain. In spite of the fact that the pH of both media was similar, the survivability of both strains was greater in beetroot juice than in the buffer. It can be explained by the content of organic compounds which are known to be able to produce a protective layer for bacterial cells which could inhibit the effect of pressure treatment.

On an industrial scale, juices are exposed to pressures of 300–600 MPa for a few minutes at 20°C or below. This environment is sufficient to reduce the number of spoilage microorganisms such as: yeast, moulds, and lactic acid bacteria. However, the results of this study have proved that the mentioned above factors are not always sufficient enough to inactivate pathogens and ensure consumer safety.

To attain safe standards of high pressure processed foods, particular attention should be paid to the potential presence of sublethal injured cells. Moreover, baroresistance among microbial species and strains should also be taken into consideration. The conditions of pressure processing should be properly selected for the type of product as well as the expected conditions and duration of storage. It should be particularly taken into account that the possibility of recovery of sublethally injured cells may occur. Therefore, it is worth considering the coupling of HHP and other treatments to ensure microbiological stability and health safety of juices and beverages from beetroots or other root vegetables.

RESEARCH FUNDING

This work was funded from statutory funds of prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology, Warsaw, Poland.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B. (2000). Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure – resistant and pressure – sensitive strains of foodborne pathogens. *International Journal of Food Microbiology, 60*, 33–42.
2. Alpas, H., Kalchayanand, N., Bozoglu, F., Sikes, A., Dunne, C.P. (1999). Variation in resistance to hydrostatic pressure among strains of food – borne pathogens. *Applied and Environmental Microbiology, 65*(9), 4248–4251.
3. Boeijen, I.K.H. van, Chavaroche, A.A.E., Valderrama, W.B., Moezelaar, R., Zweitering, M.H., Abee, T. (2010). Population diversity of *Listeria monocytogenes* LO28: phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. *Applied and Environmental Microbiology, 76*(7), 2225–2233.
4. Clifford, T., Howatson, G., West, D.J., Stevenson, E.J. (2015). The potential benefits of red beetroot supplementation in health and disease. *Nutrients, 7*(4), 2801–2822
5. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs
6. Escolar, C., Gómez, D., Ruiz-Garcia, M.D.R., Conchello, P., Herrera, A. (2017) Antimicrobial resistance profiles of *Listeria monocytogenes* and *Listeria innocua* isolated from ready-to-eat products of animal origin in Spain. *Foodborne Pathogens and Diseases, 14*(6), 357–363.
7. Espina, L., Garcia–Gonzalo, D., Pagan, R. (2016). Detection of thermal sublethal injury in *Escherichia coli via* the selective medium plating technique: mechanisms and improvements. *Frontiers in Microbiology, 7,* art. no. 1376.
8. Goulet, V., Hedberg, C., Le Monnier, A., de Valk, H. (2008). Increasing incidence of listeriosis in France and other European countries. *Emerging Infectious Diseases, 14*(5), 734 – 740.
9. Hauben, K.J.A., Bartlett, D.H., Soontjens, C.C.F., Cornelis, K., Wuytack, E.Y., Michiels, C.W. (1997). *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Applied and Environmental Microbiology, 63(3), 945–950.
10. Hoover, D.G., Metrick, C., Papineau, A.M., Farkas, D.F., Knorr, D. (1989). Biological effects of high hydrostatic pressure on food microorganisms. *Food Technology, 43*(3), 99–107.
11. Huang, H.W., Lung, H.M., Chang, Y.H., Yang, B.B., Wang, Ch.Y. (2015). Inactivation of pathogenic *Listeria monocytogenes* in raw milk by high hydrostatic pressure. *Foodborne Pathogens and Diseases, 12*(2), 139–144.
12. Jofré, A., Aymerich, T., Bover-Cid, S., Garriga, M. (2010). Inactivation and recovery of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* after high hydrostatic treatments up to 900 MPa. *International Microbiology, 13*(3), 105–112.
13. Jordan, S.L., Pascual, C., Bracey, E., Mackey, B.M. (2001). Inactivation and injury of pressure – resistant strains of *Escherichia coli* O157 and *Listeria monocytogenes* in fruit juice. *Journal of Applied Microbiology, 91*(3), 463–469.
14. Mackey, B.M., Forestière, K., Issaacs, N.S., Stenning, R., Brooker, B. (1994). The effect of high hydrostatic pressure on *Salmonella thompson* and *Listeria monocytogenes* examined by electron microscopy. *Letters in Applied Microbiology, 19*(6), 429–432.
15. Mackey. B.M., Injured bacteria (2000). In B.M. Lund, T.C. Baird-Parker, G.W. Gould (eds). *The Microbial Safety and Quality of Food*, vol. 1. Aspen Publisher Inc., Gaithersburg, Maryland, USA, pp. 315–341.
16. Mañas, P., Mackey, B.M. (2004). Morphological and physiological changes induced by high hydrostatic pressure in exponential and stationary phase cells of *Escherichia coli*: relationship with cell death. *Applied and Environmental Microbiology, 70*(3), 1545–1554.
17. Patterson, M.F, Quinn, M., Simpson, R., Gilmour, A. (1995). Sensitivity of vegetative pathogens to high hydrostatic pressure treatments in phosphate – buffered saline and foods. *Journal of Food Protection,58*(5), 524–529.
18. Prieto-Calvo, M., Prieto, M, Lopez, M., Alvarez-Ordóñez, A. (2014). Effects of high hydrostatic pressure on *Escherichia coli* ultrastructure, membrane integrity and molecular composition as assessed by FTIR spectroscopy and microscopic imaging techniques. *Molecules, 19*, 21310–21323.
19. Reynolds, E.S. (1963). The use of lead citrate at high pH as electron-opaque stain for electron microscopy. *Journal of Cell Biology, 17*, 208–213.
20. Sapers, G.M. (2003). Washing and sanitizing raw materials for minimally processed fruit and vegetable products. In J.S. Novak, G. M. Sapers, V.K. Juneja (eds.). *Microbial Safety of Minimally Processed Foods*. CRC Press, Boca Raton, Florida, USA, pp. 221–253.
21. Sokołowska, B., Woźniak, Ł., Skąpska, S., Porębska, I., Nasiłowska, J., Rzoska, S.J. (2017). Evaluation of quality changes of beetroot juice after high hydrostatic pressure processing. *High Pressure Research, 37*(2), 214–222.

22. Sokołowska, B., Skąpska, S., Niezgoda, J., Rutkowska, M., Dekowska, A., Rzoska, S.J. (2014). Inactivation and sublethal injury of *Escherichia coli* and *Listeria innocua* by high hydrostatic pressure in model suspensions and beetroot juice. *High Pressure Research, 34*(1), 147–155.
23. Sokołowska, B., Skąpska, S., Fonberg-Broczek, M., Niezgoda, J., Rutkowska, M., Chotkiewicz, M., Dekowska, A., Rzoska, S.J. (2013). The effect of high hydrostatic pressure on the survival of *Saccharomyces cerevisiae* in model suspensions and beetroot juice. *High Pressure Research 33*(1), 165–171.
24. Sokołowska, B., Chotkiewicz, M., Niezgoda, J., Dekowska, A. (2011). Evaluation of microbial contamination of commercially available unpasteurized, freshly squeezed fruity and vegetable juice. *Zeszyty Problemowe Postępów Nauk Rolniczych, 569*, 219––228 (in Polish; English abstract).
25. Stewart, C.M., Jewett, F.F., Dunne, C.P., Hoover, D.G. (1997). Effect of concurrent high hydrostatic pressure, acidity and heat on the injury and destruction of *Listeria monocytogenes*. *Journal of Food Safety, 17*(1), 23–36.
26. USDA Food Composition Databases [https://ndb.nal.usda.gov/ndb/search/list_28.02.2018].
27. Wang, C.Y., Huang, H.W., Hsu, C.P., Yang, B.B. (2016). Recent advances in food processing using high hydrostatic pressure technology. *Critical Reviews in Food Science and Nutrition, 56*(4), 527–540.
28. Wesche, A.M., Gurtler, J.B., Marks, B.P., Ryser, E.T. (2009). Stress, sublethal injury, resuscitation and virulence of bacterial foodborne pathogens. *Journal of Food Protection, 72*(5), 1121–1138.
29. Yang, B.W., Shi, Y., Xia, X.D., Xi, M.L., Wang, X., Ji, B.Y., Meng, J.H. (2012). Inactivation of foodborne pathogens in raw milk using high hydrostatic pressure. *Food Control, 28*(2), 273–278
30. Yuste, J., Capellas, M., Fung, D.Y.C., Mor–Mur, M. (2004). Inactivation and sublethal injury of foodborne pathogens by high pressure processing: Evaluation with conventional media and thin agar layer method. *Food Research International, 37*(9), 861 – 866.
31. Zielińska-Przyjemska, M, Olejnik, A., Dobrowolska-Zachwieja, A., Grajek, W. (2009). *In vitro* effects of beetroot juice and chips on oxidative metabolism and apoptosis in neutrophils from obese individuals. *Phytotherapy Research, 23*(1), 49–55.

Submitted: 1 March 2018. Revised: 17 July 2018. Accepted: 17 October 2018. Published on-line: 22 January 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 53–61
DOI: 10.31883/pjfns-2019-0009
http://journal.pan.olsztyn.pl

Original research article
Section: Nutritional Research

Continuous Consumption of Reused Palm Oil Induced Hepatic Injury, Depletion of Glutathione Stores, and Modulation of Cytochrome P450 Profiles in Mice

Waranya Chatuphonprasert[1], Yollada Sriset[2], Kanokwan Jarukamjorn[2,]*

[1]Faculty of Medicine, Mahasarakham University, Mahasarakham, 44000 Thailand
[2]Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Mitrapaab Road, Khon Kaen, 40002,Thailand

Key words: cytochrome P450, glutathione stores, oxidative stress, histology, liver, reused palm oil

Cooking oil deteriorates with repeated thermal exposure, resulting in appearance change and formation of oxygen radicals. Consumption of the deteriorated oil causes oxidative stress related to lipid metabolism. This study evaluated the effects of palm (*Elaeis guineensis* Jacq.) oil on hepatic histology, redox status, and cytochrome P450 (CYP) profiles. Adult female mice were orally given purified water (control), fresh, or reused palm oil (4.5 g/kg/day) daily for 16, 24, and 36 weeks. The livers were then collected for histological examination and for the evaluation of the redox system and CYP expression profiles. Treatment with fresh oil for 36 weeks resulted in some pyknosis and karyorrhexis in hepatic tissues, while reused oil resulted in more injuries to the nuclei with hepatic fat accumulation from week 24 onwards. Depletion in reduced glutathione (GSH) stores, with a significant decrease in the GSH/GSSG ratio, was observed with the reused oil but not with the fresh oil. The expression profiles of drug-metabolizing CYPs were significantly modulated; *Cyp2c29*, *Cyp3a11*, and *Cyp3a13* were suppressed by both fresh and reused oil, while only the reused oil elevated *Cyp2e1*. The expression of *Cyp4a10* and *Cyp4a14*, the key enzymes in lipid metabolism, were expectedly up-regulated by both. These findings suggest reused oil has a deleterious effect on hepatic ultrastructure, induces an imbalance of redox state, and causes *Cyp2e1* activation-associated oxidative stress. It is therefore recommended that fresh rather than reused palm oil be used for cooking, and large-scale or long-term consumption be avoided to reduce the risk of liver damage and drug-interactions.

INTRODUCTION

Palm (*Elaeis guineensis* Jacq.) oil is a frequently used cooking oil for frying, and is normally reused for economic reasons [Carter *et al.*, 2007; Ku *et al*., 2014]. Exposure to high temperatures during the frying process causes deterioration of oil physical and chemical properties [Latha & Nasirullah, 2014]. Oxidative physical changes include an increase in viscosity and the development of a brown color and rancid smell [Bordin *et al*., 2013], while chemical changes result in the production of hazardous by-products such as peroxides [Leong *et al*., 2012], and *trans*-fat [Kemény *et al*., 2001]. Peroxides are hazardous because they can overwhelm an organism's oxidant-antioxidant system by the excessive formation of prooxidants [Coyle *et al*., 2006], while *trans*-fats have been shown to damage cell membranes and impede the activity of major lipid metabolizing enzymes, leading to oxidative stress [Totani & Ojiri, 2007]. Oxidative stress has been identified as a risk factor for metabolic diseases such as insulin resistance [Chao *et al*., 2007], hypertension [Jaarin *et al*., 2011], and atherosclerosis [Ng *et al*., 2014]. Also, a previous study with male ICR mice receiving a regular diet supplemented with 25% reused oil for 12 weeks showed that the test animals developed swelling and necrosis of their hepatic cells [Hashem & Salama, 2012].

Cytochrome P450 (CYP) is a superfamily of mono-oxygenase enzymes abundant in the liver, and plays a pivotal role in the metabolism of endogenous substances and detoxification of foreign compounds [Nebert *et al*., 2013]. Consumption of reused oil is associated with the induction of these CYP enzymes and also antioxidant enzymes in rats. Levels of CYP3A, CYP4A, and catalase proteins were all increased in rats fed with oxidized soybean oil for 6 weeks [Huang *et al*., 2009], while rats fed a regular diet plus 20% reused soybean oil showed up-regulated expression of *Cyp4a1, Cyp4a2*, and *Cyp4a3* mRNAs [Chao *et al*., 2001]. Finally, an increase in free radical levels was believed to be responsible for reduced NO levels and increased blood pressure in rats fed reused palm and soybean oils for 24 weeks [Jaarin *et al*., 2011]. Therefore, it is of interest to evaluate how reused palm oil influences hepatic histology, redox status, and expression of CYP enzymes, including xenobiotic metabolizing isoforms, *i.e. Cyp1a2, Cyp2c9, Cyp2e1, Cyp3a11,* and *Cyp3a13*, and lipid metabolizing isoforms, *i.e*. *Cyp4a10* and *Cyp4a14*.

* Corresponding Author: Tel/Fax: 043–202379; 043–202305;
E-mail: kanok_ja@kku.ac.th (Prof. K. Jarukamjorn)

MATERIALS AND METHODS

Chemicals and reagents

The lipid profiles of fresh palm oil used in this study, Morakot® (total fat content 94.96 g/100 g; Samut Prakan, Thailand), and reused palm oil (total fat content 94.89 g/100 g) collected from a deep-frying food (fish, squid, chicken, pork, meat, and seasoning sausages) stall in Khon Kaen (Thailand) in September 2014, were certified by the Food and Nutrition Laboratory, Institute of Nutrition, Mahidol University, Thailand (Table 1).

Bovine serum albumin (BSA), glutathione reductase, reduced glutathione (GSH), oxidized glutathione (GSSG), malondialdehyde (MDA), 4-vinyl pyridine (4-VP), and thiobarbituric acid were supplied by Sigma-Aldrich Chemicals (St. Louis, Missouri, USA). ReverTraAce® was a product of Toyobo Co., Ltd (Osaka, Japan). *Taq* DNA polymerase, RNase inhibitors, and random primers were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). SYBR® Green I was purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME, USA). Forward and reverse primers of *Cyp1a2, Cyp2c29, Cyp2e1, Cyp3a11, Cyp3a13, Cyp4a10, Cyp4a14,* and *Gapdh* genes were synthesized by Bio Basic, Inc. (Markham Ontario, Canada). The primers of each gene are shown in Table 2. Eosin Y and Mayer's hematoxylin (H&E) was obtained from Bio Optica (Milano, Italy). All other laboratory chemicals were of the highest available purity from commercial suppliers.

Animal treatment

Seven-week-old female ICR mice were supplied by the National Laboratory Animal Center, Mahidol University, and housed in the Animal Unit of Faculty of Pharmaceutical Sciences, Khon Kaen University (Thailand) under the supervision of a licensed laboratory veterinarian. The animal handling and research protocols were approved by the Animal Ethics Committee for Use and Care of Animals, Khon Kaen University (Approval No. ACUC-KKU 30/2557) in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. All mice were housed on wood chip bedding in polysulfone cages with water and commercial regular diet (SmartHeart® from Perfect Companion Pet Care Company, Bangkok, Thailand) supplied *ad libitum* with a 12-h dark/light cycle under a controlled temperature (23±2 °C) and humidity (45±2%), and acclimated for a week before dosing, and random division into 3 groups. Each group (n=15 each)

TABLE 1. Fatty acid composition (g/100 g total fatty acid) of the fresh and reused palm oil*.

Fatty acid**		Fresh	Reused
Caprylic acid	C 8:0	ND	0.02
Capric acid	C 10:0	ND	0.02
Lauric acid	C 12:0	0.24	0.37
Myristic acid	C 14:0	0.87	1.28
Palmitic acid	C 16:0	36.83	35.30
Palmitoleic acid	C 16:1	ND	1.01
Stearic acid	C 18:0	4.22	5.45
Elaidic	C 18:1 trans-9	ND	0.08
Oleic acid	C 18:1 n9 cis	47.10	43.94
Linoleic acid	C 18:2 c9 t12	ND	0.16
Linoleic acid	C 18:2 t9 c12	ND	0.15
Linoleic acid	C 18:2 n6 cis (c9 c12)	10.14	10.54
Linoleic acid	C 18:3 n3	0.60	0.06
Eicosenoic acid	C 20:1	ND	0.55
Eicosapentaenoic acid	C 20:5 n3	ND	0.26
Docosadienoic acid	C 22:2	ND	0.20
Docosahexaenoic acid	C 22:6, n-3	ND	0.63
Sum of *trans* fatty acids***		ND	0.38

*certified by the Food and Nutrition Laboratory, Institute of Nutrition, Mahidol University, Thailand; ND, not detected. **AOAC [2016a]. ***AOAC [2016b].

TABLE 2. Forward and reward primer sequences.

Genes	Accession No.	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)	Annealing temperature (°C)	Product size (bp)
Cyp1a2	NM_009993.3	CGT CAG CAA GCT TCA GAA GG	ACG ATG TTC AGC ATC TCC TCG	57.0	144
Cyp2c29	NM_007815	ATC TGG TCG TGT TCC TAG CG	AGT AGG CTT TGA GCC CAA ATA C	50.0	218
Cyp2e1	NM_021282.2	TCC CTA AGT ATC CTC CGT GA	GTA ATC GAA GCG TTT GTT GA	50.0	529
Cyp3a11	NM_007818.3	TTT GGT AAA GTA CTT GAG GCA GA	CTG GGT TGT TGA GGG AAT C	64.0	134
Cyp3a13	NM_007819.4	TGT GCT GGC TAT CAC AGA TCC	AAA TAC CCA CTG GAC CAA AGC	55.0	101
Cyp4a10	NM_010011.3	GTG CTG AGG TGG ACA CAT TCA T	TGT GGC CAG AGC ATA GAA GAT C	54.2	83
Cyp4a14	NM_007822.2	TGC AGA AGG CCA GGA AGA AG	CAC ATG GTG GTG TAG GGC AT	60.5	286
Gapdh	NM_008084.3	CCT CGT CCC GTA GAC AAA ATG	TGA AGG GGT CGT TGA TGG C	57.4	152

was orally given the fresh or reused palm oil (4.5 g/kg/day) daily for 16, 24, and 36 weeks. The control group (n=5) was given purified water (0.2 mL/mouse/day) for the same time periods. Mice were sacrificed 24 h after the last treatment and their livers were immediately excised; a portion was fixed for histological examination and the remains were stored at -80°C for further analysis.

Liver histological examination

Following excision, small pieces of liver tissue were washed immediately in phosphate buffered saline, fixed overnight in 10% neutral-buffered-formalin, dehydrated using gradient ethanol concentration, and embedded in paraffin. The paraffin embedded tissues were cut into 5 mm sections using a Microm HM315 machine (Thermo Scientific, Walldorf, Germany) and fixed on microslides. The microslides were stained with hematoxylin and eosin (H&E) and examined at 400× magnification on an Axiostar plus microscope (Carl Ziess, Oberkochen, Germany) coupled with EOS SDS digital camera and EOS software (Canon®, Tokyo, Japan) [Jearapong *et al*., 2015].

Determination of hepatic glutathione contents

Liver homogenate was deproteinized with 5% (w/v) 5-sulfosalicylic acid before centrifugation at 10,000 ×*g* at 4°C for 10 min. The supernatant was mixed with a freshly prepared reaction mixture containing 95 mM potassium phosphate buffer (pH 7.0), 0.95 mM ethylenediamine tetraacetic acid, 0.04 mg/mL 5,5'-dithiobis(2-nitrobenzoic acid), and 0.12 units/mL glutathione reductase. The reaction was started by adding 0.04 mg/mL NADPH, and absorbance at a wavelength of 405 nm was immediately measured every 60 s for 5 min. Total glutathione content was determined by comparing the absorbance of sample with that of GSH standard. The GSH (reduced form) content was calculated by subtracting the GSSG (oxidized form) content from the total glutathione content. To determine the GSSG content, 4-VP was added to the supernatant, and the sample was incubated for 60 min at room temperature. Other steps were done as described for the assay of the total glutathione content. The contents of total glutathione, GSH, and GSSG were expressed in units of nmol per mg of protein [Akerboom & Sies, 1981]. Protein content was quantified using the method of Bradford, with the protein-dye complex measured at a wavelength of 595 nm using BSA as a standard [Bradford, 1976].

Determination of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation was determined by measuring the formation of TBARS [Wasowicz *et al*., 1993]. In brief, a 50 μL aliquot of liver homogenate was incubated at 37°C for 1 h, and then a mixture (0.5 mL) of an equal volume of 10% (w/v) trichloroacetic acid (TCA) and 0.8% (w/v) 2-thiobarbituric acid (TBA, in 25% acetic acid) was added. The reaction mixture was boiled at 100°C for 15 min before immediately cooling in an ice bath. The reaction was stopped by the addition of 10% (w/v) TCA (0.5 mL) and the reaction mixture was centrifuged (Sartorius Model 2–16K, Göttingen, Germany) at 1,200 ×*g* at room temperature for 5 min. The supernatant was measured by a spectrofluorometer at an excitation wavelength of 520 nm and an emission wavelength of 590 nm. MDA was used as a standard in concentration ranging from 0.5 to 4 nmol. Results were expressed as nmol MDA per mg of protein. The protein content of the liver homogenate was determined using the method of Bradford [Bradford, 1976].

Assessment of RT-qPCR

Total RNA was reverse transcribed using ReverTraAce® under the conditions recommended by the supplier (Toyobo Co. Ltd., Osaka, Japan) at 25°C for 10 min, 42°C for 60 min, and 95°C for 5 min on a GeneAmp PCR system 2720 ThermalCycler (Applied Biosystem, Singapore). The CYP mRNAs were quantified by qPCR using SYBR® Green I with specific forward and reverse primers which were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Bio Basic Inc. (Markham ON, Canada) (Table 2). The reaction of qPCR was performed in a final volume of 20 μL containing 20 ng of cDNA, Thunderbird® SYBR® qPCR Mix (Toyobo Co. Ltd., Osaka, Japan), and 5 pmol of each primer. The following thermal program was applied: a single cycle of DNA polymerase activation for 10 min at 95°C followed by 40–50 amplification cycles of 20 s at 95°C (denaturing step) and 20 s at annealing step, and 30 s at 72°C for extension step. Subsequently, melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 60 to 95°C (± 0.5°C/20 s). The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and the threshold limit setting was performed in automatic mode, according to the iCycler Thermal Cycler (Hercules, CA, USA) and Biorad-iQ5 software (Hercules, CA, USA). The CYP gene mRNA levels were normalized to a reference gene, *Gapdh* [Jearapong *et al*., 2015].

Statistical analysis

A power analysis was performed with G*Power version 3.1 [Faul *et al.*, 2007] to determine what sample size would be needed to detect a significant difference in TBARS and glutathione contents, and relative CYP expression. The sample size was calculated using the power procedure for one-way analysis of variance (ANOVA), considering α probability of 0.05 with a power of 0.80. The power analysis determined a sample size of 5 mice per group. Statistical analyses were performed using one-way ANOVA followed by the Tukey *post hoc* test (SPSS version 17.0, SPSS Inc., Chicago, IL, USA). All data are expressed as mean±SD (n=5). A difference with $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Reused oil drew hepatic injury in the mice

Palm oil provides nearly 30% of the world edible vegetable oil, and is regularly used for frying food in Southeast Asia [Carter *et al*., 2007]. Palm oil becomes thermally oxidized after several rounds of heating to 150°C, generating oxygen-derived free radicals and hydroxylated products that are harmful to tissues [Frankel, 2014]. Wistar rats fed with either fresh or oxidized palm oil for 18 weeks had higher serum lev-

els of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) [Owu *et al.*, 1998]. Serum total cholesterol levels of rats fed hydrogenated oil containing palm oil for 4 weeks was significantly increased compared to controls too [Amini *et al*., 2017].

In this study, consumption of fresh or reused palm oil for 36 weeks did not alter mouse weight profiles between groups (data not shown), but did affect hepatocellular structures. H&E stained micrographs of hepatic tissues are shown in Figure 1. The hepatic tissues of mice that consumed fresh palm oil exhibited normal histology at week 24, with limited pyknosis and karyorrhexis of hepatocyte nuclei later observed at 36 weeks. The hepatic tissues of mice that consumed reused palm oil showed much more damage and extensive pyknosis, this being detectable at week 24. At week 36, pyknosis had developed into karyorrhexis and the hepatocytes had become swollen with extensive vacuolation and fat droplet accumulation. Hepatocyte degeneration, damage to sinusoid structure, and loss of hepatic architectural integrity were also observed at week 36 in the mice fed reused palm oil.

Ingestion of heated and oxygenated corn oil has been shown to induce liver injury in rats by cell membrane damage from active oxygen radicals contained in the heated and also oxygenated oil [Shibayama, 1992]. Heated palm oil increased blood pressure with necrosis of cardiac tissue while fresh palm oil did not show these deleterious effects in Sprague Dawley rats [Leong *et al*., 2008]. Hence, fresh palm oil, with a low oxidation value, appeared to be less injurious to liver than thermally oxidized palm oil. According to our results, fresh palm oil treatment for 36 weeks led to some pyknosis and karyorrhexis in hepatic tissues while reused palm oil induced more severity including fat accumulation in the hepatic tissues. Our previous study reported fat accumulation in the livers of mice after 8 weeks of a combination of high fat (containing *trans*-fats) and high fructose diet [Jearapong *et al*., 2015]. In this study, the administration of reused palm oil for 36 weeks induced fat accumulation in the liver tissues itself but the fresh palm oil did not produce a significant change in fat accumulation. From Table 1, several unsaturated fatty acids, particularly *trans*-fatty acid, that were not detectable in the fresh oil, were found in the reused palm oil. These unsaturated fatty acids may derive from the deep frying of food products. For example, elaidic was from deep fried food, *e.g.* French fried [Bansal *et al.*, 2009], whereas linoleic acid, eicosapentaenoic acid, and docosadienoic acid were from seafood, fish, pork, and meat [Chin *et al.*, 1992; Hornstein *et al*., 1961; Hu *et al*., 2002]. Moreover, re-heating of palm oil with food or meats might increase accumulation of *trans*-fatty acid and other fatty acids in reused palm oil. In this study, mice received reused palm oil at the dose of 4.5 g/kg/day which is equivalent to *trans*-fatty acid intake of 17 mg/kg/day. Dhibi *et al.* [2011] demonstrated that Wistar rats which received oxidized soybean oil diet (contained *trans*-fatty acid 123–172 mg/kg/day) and margarine diet (contained *trans*-fatty acid 240–336 mg/kg/day) for 4 weeks showed hepatic oxidative stress with an increase in AST, ALT, ALP, and LDH levels [Dhibi *et al.*,

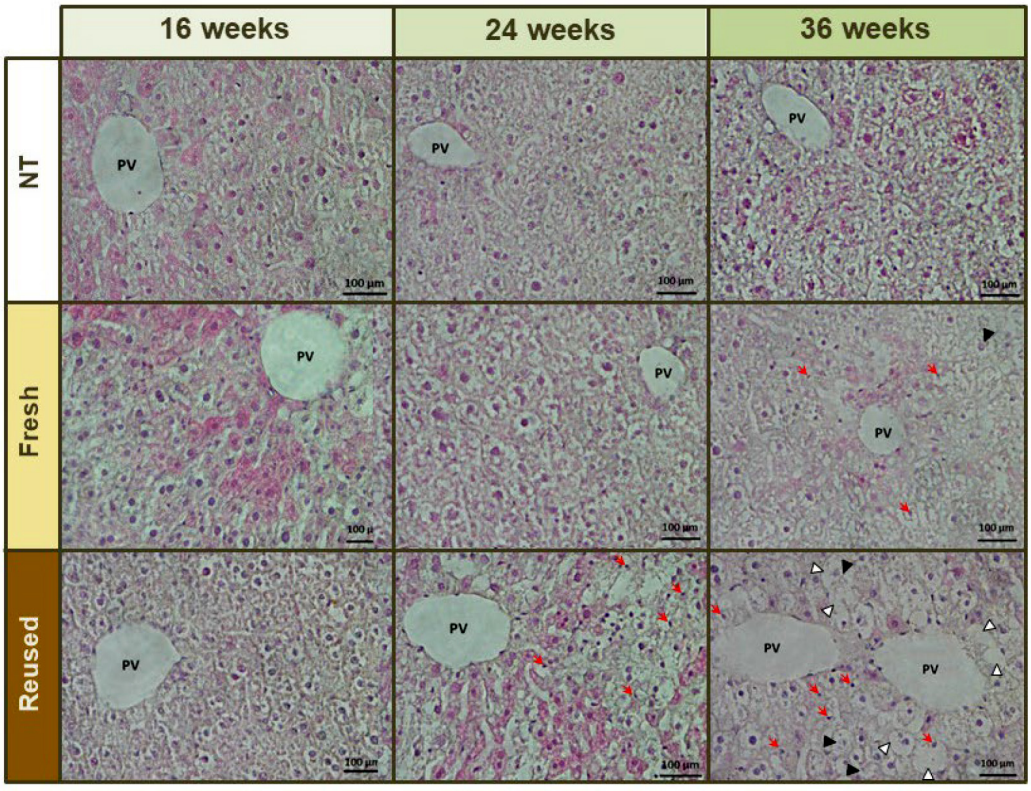


FIGURE 1. Hepatic histological changes in mice treated with fresh and reused palm oil.

Mice were orally given distilled water (NT), fresh or reused palm oil (4.5 g/kg/day) daily for 16, 24, and 36 weeks. Micrographs are shown at 400× magnification. PV, portal vein. Red arrows indicate pyknosis; black triangles indicate karyorrhexis; blank triangles indicate fat accumulation.

2011]. Therefore, *trans*-fat from the re-heating process might be an important factor for hepatic fat accumulation.

Effect of reused oil on oxidant-antioxidant system in the mouse livers

Total glutathione content and GSH sharply decreased and GSSG relatively increased, resulting in a significant decrease in the GSH/GSSG ratio in mice fed reused oil for 36 weeks (Table 3). The depletion of GSH stores corresponded with the level of lipid peroxidation, presented as excessively raised TBARS contents in the mouse livers during the 36-week-treatment (Figure 2). By contrast, fresh oil consumption did not significantly disrupt the GSH/GSSG ratio, though the TBARS level was augmented at 16 weeks of the treatment. These observations revealed that both the fresh and reused palm oil induced hepatic tissue damage, but that the fresh palm oil had a less deleterious effect than the reused palm oil.

The lipid peroxidation that occurs in high-fat diets results in the production of several aldehyde species. MDA is one of several low-molecular-weight end-products formed *via* the decomposition of certain primary and secondary lipid peroxidation products, and is used as a convenient biomarker of lipid peroxidation because its reaction with thiobarbituric acid generates an intensely colored and readily detectable chromogen [Janero, 1990]. Wistar rats receiving hydrogenated oil containing palm oil or pure palm olein oil for 4 weeks have been shown to have significantly higher serum TBARS levels [Amini *et al*., 2017], which agrees with our results that both fresh and reused palm oil elevated hepatic TBARS levels. These observations reveal that both fresh and reused palm oil induce hepatic tissue damage, with the fresh palm oil having a less deleterious effect than the reused palm oil.

Another active biochemical mediator from lipid peroxidation is *trans*-4-hydroxy-2-hexenal (HHE), a major α,β--unsaturated aldehyde product of *n*-3 poly-unsaturated fatty acid oxidation. HHE and other products of lipid peroxidation have been shown to deplete GSH content by binding to

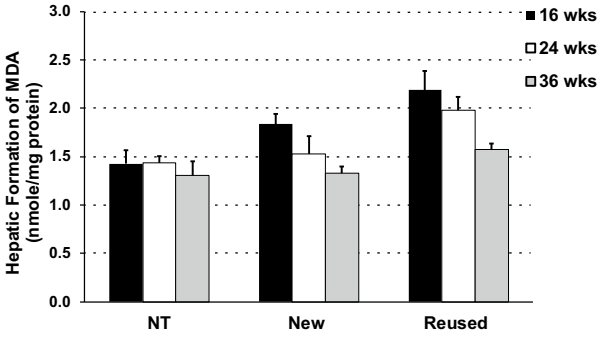


FIGURE 2. Effects of fresh and reused palm oil on the level of thiobarbituric acid reactive substances (TBARS) in mouse livers.

Mice were orally administered fresh or reused palm oil (4.5 g/kg/day) daily for 16 (black bar), 24 (blank bar), and 36 (grey bar) weeks. The control (NT) was given distilled water (0.2 mL/mouse/day) daily for the same period. The data are expressed as the mean±SD (n=5). $^{*}P<0.05$ *versus* NT.

TABLE 3. Glutathione content profiles in mouse livers following different treatments.

Treatment	Total glutathione (nmol/mg protein)			GSH (nmol/mg protein)			GSSG (nmol/mg protein)			Ratio of GSH/GSSG		
	16 wk	24 wk	36 wk	16 wk	24 wk	36 wk	16 wk	24 wk	36 wk	16 wk	24 wk	36 wk
Non-treatment	37.72±0.37	33.15±1.59	68.06±1.74	26.07±0.37	22.56±1.28	47.81±1.15	11.65±0.15	10.59±0.40	20.25±0.61	2.24±0.05	2.13±0.08	2.36±0.02
Fresh palm oil	48.73±1.73***	60.99±0.73***	55.60±2.55***	32.51±1.81**	40.24±1.07***	37.17±2.75***	16.98±0.31***	20.75±0.90***	18.43±0.59*	1.91±0.12	1.94±0.13	2.02±0.18
Reused palm oil	26.59±1.90***	52.79±2.17***	58.57±2.18**	15.13±1.39***	33.02±1.50***	36.50±0.85***	11.46±0.56	19.77±0.81***	22.07±1.51	1.32±0.07***	1.67±0.06***	1.66±0.09***

The data are expressed as mean±SD (n=8–10) from 3 independent experiments. GSH, reduced glutathione; GSSG, oxidized glutathione; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ *versus* Non-treatment in the same period.

complexes with GSH *via* the glutathione-*S*-transferase reaction [Long & Picklo, 2010]. According to our results, TBARS levels were elevated in the reused palm oil-treated mice with a decrease in the antioxidant glutathione capability detected by a lowering of the GSH/GSSG ratio.

A recent study described diet-induced nonalcoholic steatohepatitis (NASH) in C57BL/6J mice using a diet-rich in *trans*-fat (40%) and fructose (22%) for 26 weeks [Kristiansen *et al.*, 2016]. Pathogenesis of NASH begins with fat accumulation in hepatocytes, while oxidative stress, apoptosis, or mitochondrial dysfunction cause consequent development of inflammation and fibrosis [Day & James, 1998]. Although our study did not investigate inflammation and fibrosis, the fat accumulation and oxidative stress *via* an increase in the TBARS level and a depletion of the GSH stores might imply that the ingestion of reused palm oil over a long period leads to harmful effects in the liver.

Effect of reused oil on hepatic cytochrome P450 profiles in mice

The expression of *Cyp1a2* mRNA was not modified by either fresh or reused oil (Figure 3A), whereas both fresh and reused oil significantly lessened the expression of *Cyp2c29* (Figure 3B), *Cyp3a11* (Figure 3D), and *Cyp3a13* (Figure 3E) mRNAs. *Cyp2e1* mRNA expression (Figure 3C) was significantly induced by the reused oil, but remained almost unchanged in the control mice and mice consuming fresh oil. In accordance with expectations, both fresh and reused oil significantly elevated the expression of *Cyp4a10* (Figure 4A) and *Cyp4a14* (Figure 4B), isoforms responsible for lipid metabolism.

An increasing number of reports documenting the adverse effects associated with food-drug interactions have been noted. Measurement of CYP activities, including those of the major drug metabolizing isoforms, CYP1A2, CYP2C, and CYP3A4, has been employed to evaluate the possibility of food-drug interactions [Sasaki *et al.*, 2017]. In one study, levels of phase I enzymes and total CYPs were not significantly changed in rats fed a diet rich in red palm (*E. guineensis*) oil [Manorama *et al.*, 1993]. On the other hand, rabbits fed *ad libitum* with a diet supplemented with 1% cholesterol for 8 weeks exhibited a decreased total P450 content with suppressive effects on several CYP isoforms [Irizar & Ioannides, 1998]; activities of ethoxy- and methoxyresorufin *O*-dealkylation represented CYP1A [Namkung *et al.*, 1988], benzphetamine *N*-demethylation of CYP2C [Ryan *et al.*, 1984], *p*-nitrophenol hydroxylation of CYP2E1 [Koop & Tierney, 1990], erythromycin- and ethylmorphine-demethylation of CYP3A [Wrighton *et al.*, 1985], and lauric acid hydroxylation of CYP4A1 [Chaurasia *et al.*, 1995]. Our finding that fresh and reused palm oil significantly down-regulates the expression of *Cyp2c29*, *Cyp3a11*, and *Cyp3a13* mRNA, each encoding major drug metabolizing isoforms, correlates well with this previous study [Irizar & Ioannides, 1998]. Food-drug interactions may therefore arise during palm oil consumption due to CYP modulation.

High-fat diet-fed ICR mice also show a significant increase in levels of hepatic MDA, CYP2E1 protein and mRNA according to one study [Jian *et al.*, 2017], a finding which our results are in agreement with. Induction of *Cyp2e1* expression in the reused palm oil-treated mice might be explained by the oxidative stress pathway. CYP2E1 is a member of the oxidoreductase cytochrome family, which is responsible for oxidizing a variety of substances including fatty acids, xenobiotics, ethanol, and most organic solvents [Caro &

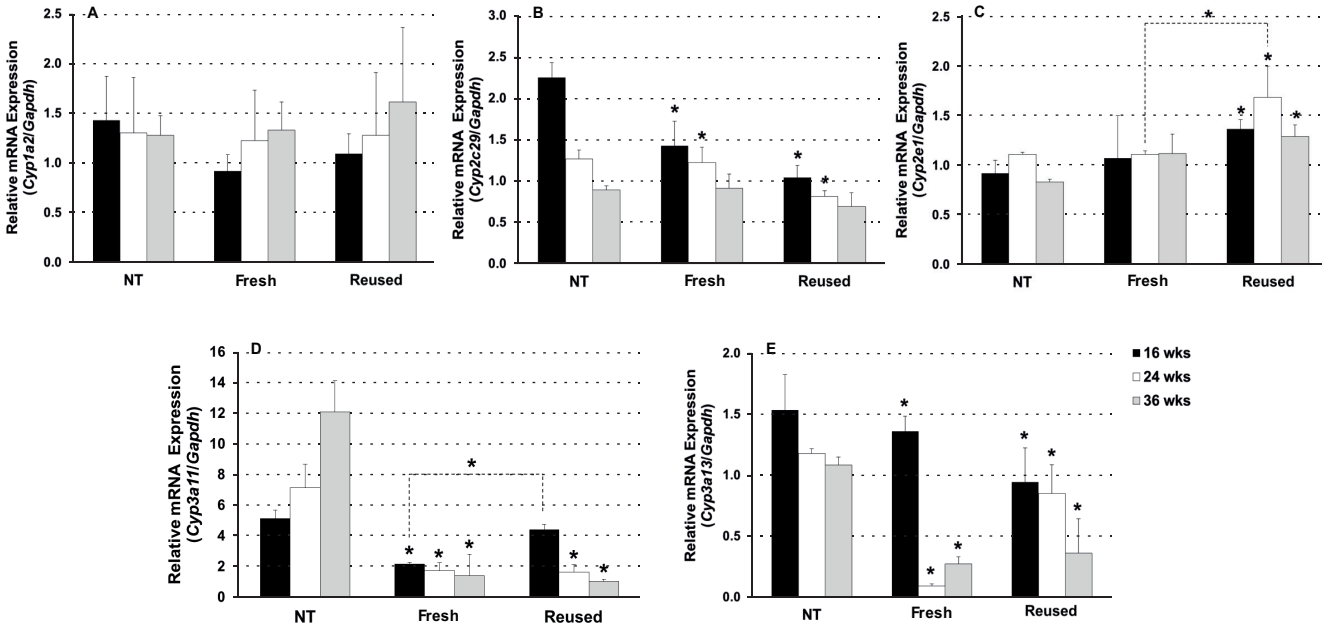


FIGURE 3. Effects of fresh and reused palm oil on the relative mRNA expression of drug metabolizing enzymes *Cyp1a2* (A), *Cyp2c29* (B), *Cyp2e1* (C), *Cyp3a11* (D), and *Cyp3a13* (E) in mouse livers.

Mice were orally administered fresh or reused palm oil (4.5 g/kg/day) daily for 16 (black bar), 24 (blank bar), and 36 (grey bar) weeks. The control (NT) was given distilled water (0.2 mL/mouse/day) daily for the same period. The data are expressed as the meanSD (n=5). $^{*}P<0.05$ *versus* NT.

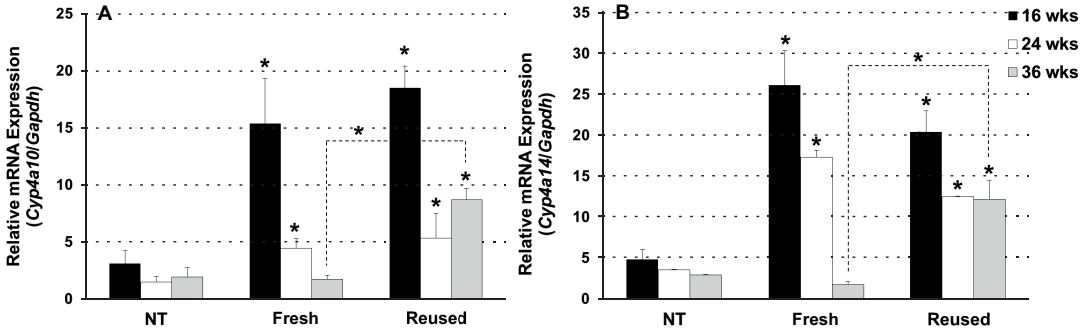


FIGURE 4. Effects of fresh and reused palm oil on the relative mRNA expression of lipid metabolizing enzymes *Cyp4a*10 (A) and *Cyp4a14* (B) in mouse livers.

Mice were orally administered fresh or reused palm oil (4.5 g/kg/day) daily for 16 (black bar), 24 (blank bar), and 36 (grey bar) weeks. The control (NT) was given distilled water (0.2 mL/mouse/day) daily for the same period. The data are expressed as the mean±SD (n=5). $^{*}P<0.05$ *versus* NT.

Cederbaum, 2004]. Moreover, CYP2E1 makes a significant contribution to the oxidative stress in non-alcoholic fatty liver disease (NAFLD) [Aubert *et al*., 2011]. The reused palm oil induced *Cyp2e1* mRNA expression while the fresh one did not, confirming that the reused palm oil stimulated more hepatic oxidative stress than the fresh palm oil did *via* the pathway of *Cyp2e1* metabolism.

In an induced steatohepatitis in mice, *Cyp4a10* and *Cyp4a14* are up-regulated and have been shown to be highly capable lipid peroxides [Leclercq *et al*., 2000]. Methionine and choline-deficient dietary-induced steatohepatitis in C57BL/6 mice increased *Cyp4a10* and *Cyp4a14* mRNA expression 2.7-fold and produced hepatic lipoperoxides [Ip *et al*., 2003]. However, an increase in *Cyp4a* expression by fresh or reused palm oil might cause the latter effect to remove excess fatty acid as a substrate for lipid peroxidation [Ip *et al*., 2003]. In our study, *Cyp4a10* and *Cyp4a14* expression was induced by both fresh and reused palm oil, whereas *Cyp2e1* activation was only induced by the reused palm oil. Hepatic tissue damage may therefore have occurred *via* the oxidative stress pathway.

CONCLUSIONS

Though fresh and reused palm oil both caused liver injury through mechanisms of oxidation and depletion of GHS stores, with alteration of major CYP isoforms, the fresh palm oil was less deleterious causing, for example, lesser damage to hepatocytes, and milder modulation of TBARS level and GSH stores, with no change in the level of *Cyp2e1* expression. Fresh palm oil is therefore preferable to reused oil, though care should be taken to limit its intake in terms of quantity, frequency, and duration of consumption.

RESEARCH FUNDING

This work was supported by the Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology, Khon Kaen University, Thailand under the Grant PANPB2558. We thank Dr. Tim Cushnie, Mahasarakham Univerisity, Thailand, for English-editing assistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Akerboom, T.P., Sies, H. (1981). Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods in Enzymology*, 77, 373–382.
2. Amini, S.A., Ghatreh-Samani, K., Habibi-Kohi, A., Jafari, L. (2017). Comparison of pure palm olein oil, hydrogenated oil-containing palm, and canola on serum lipids and lipid oxidation rate in rats fed with these oils. *Archives of Iranian Medicine, 20*(2), 96–100.
3. AOAC. Official Methods of Analysis of AOAC International, 20th Edition (2016a) 948.15 and 945.16
4. AOAC. Official Methods of Analysis of AOAC International, 20th Edition (2016b) 963.22 and 969.33
5. Aubert, J., Begriche, K., Knockaert, L., Robin, M.A., Fromenty, B. (2011). Increased expression of cytochrome P450 2E1 in non-alcoholic fatty liver disease: Mechanisms and pathophysiological role. *Clinics and Research in Hepatology and Gastroenterology, 35*(10), 630–637.
6. Bansal, G., Zhou, W., Tan, T.W., Neo, F.L., Lo, H.L. (2009). Analysis of trans fatty acids in deep frying oils by three different approaches. *Food Chemistry, 116*(2), 535–541.
7. Bordin, K., Kunitake, M.T., Aracava, K.K., Trindade, C.S.F. (2013). Changes in food caused by deep fat frying-a review. *Archivos Latinoamericanos de Nutricion, 63*(1), 5–13.
8. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.
9. Caro, A.A., Cederbaum, A.I. (2004). Oxidative stress, toxicology, and pharmacology of CYP2E1. *Annual Review of Pharmacology and Toxicology, 44*(1), 27–42.
10. Carter, C., Finley, W., Fry, J., Jackson, D., Willis, L. (2007). Palm oil markets and future supply. *European Journal of Lipid Science and Technology, 109*(4), 307–314.
11. Chao, P.M., Chao, C.Y., Lin, F.J., Huang, C. (2001). Oxidized frying oil up-regulates hepatic acyl-coa oxidase and cytochrome P-450 4 A1 genes in rats and activates PPARα. *The Journal of Nutrition, 131*(12), 3166–3174.

12. Chao, P.M., Huang, H.L., Liao, C.H., Huang, S.T., Huang, C. (2007). A high oxidised frying oil content diet is less adipogenic, but induces glucose intolerance in rodents. *British Journal of Nutrition, 98*(1), 63–71.
13. Chaurasia, C.S., Alterman, M.A., Lu, P., Hanzlik, R.P. (1995). Biochemical characterization of lauric acid ω-hydroxylation by a CYP4A1/NADPH-cytochrome P450 reductase fusion protein. *Archives of Biochemistry and Biophysics, 317*(1), 161–169.
14. Chin, S.F., Liu, W., Storkson, J.M., Ha, Y.L., Pariza, M.W. (1992). Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *Journal of Food Composition and Analysis, 5*(3), 185–197.
15. Coyle, C.H., Martinez, L.J., Coleman, M.C., Spitz, D.R., Weintraub, N.L., Kader, K.N. (2006). Mechanisms of H_2O_2-induced oxidative stress in endothelial cells. *Free Radical Biology & Medicine, 40*(12), 2206–2213.
16. Day, C.P., James, O.F.W. (1998). Steatohepatitis: A tale of two "hits"? *Gastroenterology, 114*(4), 842–845.
17. Dhibi, M., Brahmi, F., Mnari, A., Houas, Z., Chargui, I., Bchir, L., Hammami, M. (2011). The intake of high fat diet with different trans fatty acid levels differentially induces oxidative stress and non alcoholic fatty liver disease (NAFLD) in rats. *Nutrition & Metabolism, 8*(1), art.no. 65.
18. Faul, F., Erdfelder, E., Lang, A.G., Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods, 39*(2), 175–191.
19. Frankel, E.N. (2014). Research methods in lipid peroxidation In E.N. Frankel (ed). *Lipid Oxidation*. 2nd edition, Elsevier Science. s pp. 129–164.
20. Hashem, A.M., Salama, E.N. (2012). The ameliorative effect of olive oil against the histopathological lesion of the liver of mice fed on repeatedly heated fried oil. *The Egyptian Journal of Experimental Biology (Zoology), 8*(1), 105–111, art.code 14.01.12.
21. Hornstein, I., Crowe, P., Heimberg, M. (1961). Fatty acid composition of meat tissue lipids. *Journal of Food Science, 26*(6), 581–586.
22. Hu, F., Bronner, L., Al, E. (2002). Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *JAMA – Journal of the American Medical Association, 287*(14), 1815–1821. Retrieved from http://dx.doi.org/10.1001/jama.287.14.1815
23. Huang, W.C., Kang, Z.C., Li, Y.J., Shaw, H.M. (2009). Effects of oxidized frying oil on proteins related to alpha-tocopherol metabolism in rat liver. *Journal of Clinical Biochemistry and Nutrition, 45*(1), 20–28.
24. Ip, E., Farrell, G.C., Robertson, G., Hall, P., Kirsch, R., Leclercq, I. (2003). Central role of PPARα-dependent hepatic lipid turnover in dietary steatohepatitis in mice. *Hepatology, 38*(1), 123––132.
25. Irizar, A., Ioannides, C. (1998). Marked inhibition of hepatic cytochrome P450 activity in cholesterol-induced atherosclerosis in rabbits. *Toxicology, 126*(3), 179–193.
26. Jaarin, K., Mustafa, M. R., Leong, X.F. (2011). The effects of heated vegetable oils on blood pressure in rats. *Clinics, 66*(12), 2125–2132.
27. Janero, D.R. (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology and Medicine, 9*(6), 515–540.
28. Jearapong, N., Chatuphonprasert, W., Jarukamjorn, K. (2015). Effect of tetrahydrocurcumin on the profiles of drug-metabolizing enzymes induced by a high fat and high fructose diet in mice. *Chemico-Biological Interactions, 239*, 67–75.
29. Jian, T., Ao, X., Wu, Y., Lv, H., Ma, L., Zhao, L., Li, W. (2017). Total sesquiterpene glycosides from Loquat (*Eriobotrya japonica*) leaf alleviate high-fat diet induced non-alcoholic fatty liver disease through cytochrome P450 2E1 inhibition. *Biomedicine & Pharmacotherapy, 91*(Supplement C), 229–237.
30. Kemény, Z., Recseg, K., Hénon, G., Kővári, K., Zwobada, F. (2001). Deodorization of vegetable oils: Prediction of trans polyunsaturated fatty acid content. *Journal of the American Oil Chemists' Society, 78*(9), 973–979.
31. Koop, D.R., Tierney, D.J. (1990). Multiple mechanisms in the regulation of ethanol-inducible cytochrome P450IIE1. *BioEssays, 12*(9), 429–435.
32. Kristiansen, M.N.B., Veidal, S.S., Rigbolt, K.T.G., Tølbøl, K.S., Roth, J.D., Jelsing, J., Feigh, M. (2016). Obese diet-induced mouse models of nonalcoholic steatohepatitis-tracking disease by liver biopsy. *World Journal of Hepatology, 8*(16), 673–684.
33. Ku, S.K., Muhamad Ruhaifi, M.S., Fatin, S.S., Saffana, M., Taty Anna, K., Das, S., Kamsiah, J. (2014). The harmful effects of consumption of repeatedly heated edible oils: a short review. *La Clinica Terapeutica, 165*(4), 217–221.
34. Latha, R.B., Nasirullah, D.R. (2014). Physico-chemical changes in rice bran oil during heating at frying temperature. *Journal of Food Science and Technology – Mysore, 51*(2), 335–340.
35. Leclercq, I.A., Farrell, G.C., Field, J., Bell, D.R., Gonzalez, F.J., Robertson, G.R. (2000). CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. *Journal of Clinical Investigation, 105*(8), 1067–1075.
36. 34 Leong, X.F., Salimon, J., Mustafa, M. R., Jaarin, K. (2012). Effect of repeatedly heated palm olein on blood pressure-regulating enzymes activity and lipid peroxidation in rats. *Malaysian Journal of Medical Sciences, 19*(1), 20–29.
37. Leong, X.F., Aishah, A., Nor Aini, U., Das, S., Jaarin, K. (2008). Heated palm oil causes rise in blood pressure and cardiac changes in heart muscle in experimental rats. *Archives of Medical Research, 39*(6), 567–572.
38. Long, E.K., Picklo, M.J. (2010). *Trans*-4-hydroxy-2-hexenal, a product of *n*-3 fatty acid peroxidation: Make some room HNE. *Free Radical Biology and Medicine, 49*(1), 1–8.
39. Manorama, R., Chinnasamy, N., Rukmini, C. (1993). Effect of red palm oil on some hepatic drug-metabolizing enzymes in rats. *Food and Chemical Toxicology, 31*(8), 583–588.
40. Namkung, M.J., Yang, H.L., Hulla, J.E., Juchau, M.R. (1988). On the substrate specificity of cytochrome P450IIIA1. *Molecular Pharmacology, 34*(5), 628–637.
41. Nebert, D.W., Wikvall, K., Miller, W.L. (2013). Human cytochromes P450 in health and disease. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 368*(1612), SI, art.no. 20120431. https://doi.org/10.1098/rstb.2012.0431
42. Ng, C.Y., Leong, X.F., Masbah, N., Adam, S.K., Kamisah, Y., Jaarin, K. (2014). Heated vegetable oils and cardiovascular disease risk factors. *Vascular Pharmacology, 61*(1), 1–9.
43. Owu, D.U., Osim, E.E., Ebong, P.E. (1998). Serum liver enzymes profile of Wistar rats following chronic consumption of fresh or oxidized palm oil diets. *Acta Tropica, 69*(1), 65–73.

44. Ryan, D.E., Iida, S., Wood, A.W., Thomas, P.E., Lieber, C.S., Levin, W. (1984). Characterization of three highly purified cytochromes P-450 from hepatic microsomes of adult male rats. *Journal of Biological Chemistry, 259*(2), 1239–1250.
45. Sasaki, T., Sato, Y., Kumagai, T., Yoshinari, K., Nagata, K. (2017). Effect of health foods on cytochrome P450-mediated drug metabolism. *Journal of Pharmaceutical Health Care and Sciences, 3*(1), 14, art.no. UNSP 14. https://doi.org/10.1186/s40780–017–0083-x
46. Shibayama, Y. (1992). Hepatotoxicity of heated and oxygenated corn oil. *Experimental and Toxicologic Pathology, 44*(5), 255–258.
47. Totani, N., Ojiri, Y. (2007). Thermal deterioration of oil and frying food stuffs. *Journal of Oleo Science, 56*(10), 543–551.
48. Wasowicz, W., Nève, J., Peretz, A. (1993). Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. *Clinical Chemistry, 39*(12), 2522–2526.
49. Wrighton, S.A., Maurel, P., Schuetz, E.G., Watkins, P.B., Young, B., Guzelian, P.S. (1985). Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry, 24*(9), 2171–2178.

Submitted: 21 August 2018. Revised: 15 October and 5 December 2018. Accepted: 13 December 2018. Published on-line: 22 January 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 63–70
DOI: 10.31883/pjfns-2019-0006
http://journal.pan.olsztyn.pl

Original research article
Section: Food Quality and Functionality

Post-Harvest Warehouse Management of *Actinidia arguta* Fruits

Nicole Roberta Giuggioli, Rossella Briano, Claudio Baudino, Cristiana Peano*

Department of Agricultural Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, Grugliasco (TO) 10095, Italy

Key words: storage, wrap film, baby kiwifruit, cultivar

In the baby kiwifruit food chain, the extent of losses caused by high perishability of the edible skin is a barrier for these fruit's position in the fresh market. Thus, maintaining the overall quality in the warehouse is fundamental for the market channel. The wrapping technique with a stretch film represents a fine opportunity for maintaining fruit quality. Hortgem Rua® and Hortgem Tahi® cultivars of kiwifruits were evaluated during storage at 1±1°C for up to 60 days. The stretch wrap film used to protect the fruit has shown to limit the weight loss maintained by the pulp of both cultivars that were firmer (2.39 N and 2.13 N) and had lower gumminess values (280.92 and 213.57 for Hortgem Rua® and Hortgem Tahi®, respectively). Considering sensorial attributes, the wrapped samples also maintained overall quality, especially in terms of fruit colour (luminosity), consistency and taste.

INTRODUCTION

It is well-recognized that storage practices can widely influence the quality of fruits in the post-harvest chain [Wang *et al.,* 2015]. The research plays an important role, especially for new fruit cultivars, including *Actinidia arguta* (Siebold et Zucc.) Planch. ex Miq. Among fresh and perishable fruits, the baby kiwifruits are consumed without peeling, similarly to berries. Due to the rapid transformation to softening, skin wrinkling, water loss and fruit decay [Latocha *et al*., 2014], baby kiwifruits require efficient storage tools in the supply chain management [Giuggioli *et al*., 2017; White *et al.,* 2005]. When compared to other Actinidiaceae (*A. deliciosa*, and *A. chinensis)*, the baby kiwifruit can be stored for a shorter period of time, namely for 1–2 months at 0°C [Baudino *et al*., 2017; Lim *et al.,* 2016; Strik & Hummer, 2006]. Previous studies have attempted to identify the best method for increasing the shelf-life, by utilizing 1-methylcyclopropene (1-MCP) [Wang *et al.,* 2015], edible coating [Fisk *et al*., 2008; Kaya *et al*., 2016] or by identifying the optimal harvest date for improving storability [Oh *et al*., 2017]. According to the current social and economic scenarios, the driver of the innovation and the key point to improve the performance of all preserving techniques is the sustainability requirement to satisfy the competitiveness asset of the supply chain. In addition, it is reported that some storage methods involving the management of the atmosphere in the storage room (controlled atmosphere) are more expensive when compared to other tools [Wang *et al*., 2015]. The warehouse storage represents a critical point in the fresh fruit supply chain [Peano *et al*., 2017], but the use of packaging and pallet bags strategies has been reported just as useful to maintain the safety and quality of different species, such as blueberry, strawberry, raspberry, and plums [Peano *et al*., 2015, 2017; DeEll, 2002].

With the pallet bag, it is possible to store large quantities of products and facilitate their transfer from the warehouse to a truck or a container for shipment [Bouchery *et al.*, 2017]. Additionally, using pallet bags to store different kinds of fruits in the same cold-room helps to avoid possible cross-contaminations with other products. While the pallet bag approach has its benefits to the storage of kiwifruit, the stretch wrap film in the warehouse is easier to manage. The pallet bag system, for example, requires the use of a plastic bag, a vacuum system, and a CO_2 injection if the products need a modified atmosphere storage, hence the use of a stretch wrap film could be promising and more economical in terms of cost and time.

Considering the importance of packaging storage techniques and the limited studies about the effect of this practice in the post-harvest storage warehouse of *Actinidia arguta*, the goal of the present research was to evaluate the effects of the wrapping technique with a stretch film. This was based on an easy and economic tool used to preserve the quality traits, phenolics content, and textural parameters of two different baby kiwifruit cultivars (Hortgem Rua® and Hortgem Tahi®) stored for up to 60 days.

MATERIALS AND METHODS

Fruit source, experimental design and sampling procedure

The baby kiwifruits orchard is located in Revello (Cuneo, Piedmont, Italy). The fruits of *Actinidia arguta* (Siebold

* Corresponding Author: Tel.: +39.011.670.8646; Fax +39.011.670.8658; E-mail: nicole.giuggioli@unito.it

et Zucc.) were from two different cultivars: Hortgem Rua® and Hortgem Tahi® marketed with the NERGI® brand [Baudino *et al*., 2017]. They are newly patented cultivars originating from New Zealand [McNeilage *et al*., 2003, 2004]. All fruits from each cultivar were collected at the harvesting maturity stage and stored inside a plastic fruit box (270 x 370 x 165 cm) (CPR System, Bologna, Italy). The fruits were transported to the Agrifrutta Cooperative warehouse (Peveragno, Cuneo, Piedmont, Italy) for storage at 1°C which rapidly decreased the core temperature of the fruits. Selected fruits of medium weights: 10.1–15.0 g for Hortgem Tahi® and 13.1––15.0 g for Hortgem Rua®, were packed inside a plastic punnet with lids made from R-PET material (INFIA s.r.l., Cesena, Italy). Punnet dimensions were 17.2 x 13.2 x 4.0 cm and contained 0.125 kg of fruit; lid dimensions were 17.8 x 13.6 cm. The experimental storage unit was a mini pallet loaded with 20 stacked crates wrapped with a polyethylene stretch film (Retarder s.r.l., Cuneo, Italy). The stretch wrap film was used to create a barrier protection against the cool air forced into the room. It was not hermetically sealed and, for this reason, changes in the atmosphere were not expected. Control samples were maintained in a normal atmosphere in a controlled cold room (1±1°C; 95% humidity). Three replicates were considered for each control. All fruits were stored for up to 60 days. Quality control was performed at the day of package processing (day 0) and after 20, 40, and 60 days of storage. At each time-point, 20 punnets (control and stretch) were randomly selected and the following parameters were determined: weight loss, quality indicators (total soluble solids, total acidity, and dry matter), total color difference, textural indicators (firmness and gumminess), and total phenolics content. A sensory analysis was also performed to better judge the fruits.

Weight loss, quality parameters, and total colour difference

Weight loss (%) was determined using an electronic balance (model SE622, VWR Science Education, Radnor, Pennsylvania, USA), with a 0.01 g accuracy. The weight was monitored throughout the storage time and it was calculated as the difference between the initial and final punnet weights. Content of total soluble solids (TSS) was determined with a digital refractometer Atago® Pal-1 (Atago Co. Ltd., Tokyo, Japan) and expressed as °Brix. For each quality control, the instrument was calibrated with distilled water. The total acidity (TA) was measured using an automatic titrator (Titritino 702, Methrom, Herisav, Switzerland) and determined potentiometrically using 0.1N NaOH to the end point of 8.1 in 5 mL of juice diluted in 25 mL of distilled water. The raw juice obtained by squeezing fruits in a mixer (Moulinex-Ju2000) was centrifuged at 1000 rpm for 5 min using a Rotofix 32-A Centrifuge (VWR, Milan, Italy). The supernatants were used for analyses. Results were expressed as g of citric acid equivalents per 100 mL of juice (g CA/100 mL) [Allegra *et al*., 2017; Briano *et al*., 2015].

Dry matter (DM) content of baby kiwifruits was measured in 10 whole fruits. The fruits were placed in an oven at 70±2°C for 24 h. Their initial and final weights were measured using an electronic balance and the value was expressed as g/100 g, according to McGlone *et al*. [2003].

Color parameters were quantified in the L^*, a^*, b^* color space. L^* refers to the lightness and ranged from $L^* = 0$ (black) to $L^* = 100$ (white). Negative and positive values of a^* indicate green and red colour, respectively, while positive and negative b^* indicate yellow and blue color, respectively [McGuire, 1992]. Color was assessed for 20 fruits in the middle part of the fruit using a tristimulus colour analyser (model CR-400, Konica Minolta, Langenhagen, Germany).

Change in fruit colour during the storage period was reported by the total colour difference (ΔE^*) index (equation 1), based on the evaluation of colour changes from the beginning of day 0 (L0, a0, b0) [Alexandre *et al*., 2012]:

$$\Delta E^* = (\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2})^{1/2} \quad \text{(Equation 1)}$$

Textural parameters

A texture profile analysis (TPA) performed with a Texture Analyser TA.XT.PLUS (Stable Micro Systems, WINOPAL Forschungsbedarf GmbH, Steinheim, Austria) (30 Kilo Load Cell) was used to evaluate the firmness (N) and the gumminess (g*s) parameters. A compression test was performed with a 30-mm aluminium flat tipped probe (P/3) to a 10% strain, with a pre-test speed of 1 mm/s, test speed 1 mm/s, post-test speed 5 mm/s, and 5 g trigger force. In the case of gumminess, a 75 mm aluminium compression plate (P/75) was used and the following parameters were applied: strain (25%), pre-test speed (1 mm/s), test-speed (5 mm/s), and trigger force (5 g).

Total phenolics content

Total phenolics content (TPC) of extracts of kiwifruits was determined. Fruits (10 g) were added to 25 mL of an extraction mixture which contained 500 mL of methanol, 23.8 mL of de-ionized water, and 1.4 mL of 37% hydrochloric acid. The mixture was kept in dark at room temperature for 1 h and afterwards thoroughly homogenized for 2 min with an ULTRA TURRAX (IKA, Staufen, Germany) and centrifuged for 15 min at 3000 rpm. The supernatant was transferred into glass test-tubes and stored at −20°C until analyzed. The total phenolics content of samples was measured using the Folin & Ciocalteu phenol reagent (Merck KGaA, Darmstadt, Germany). The absorbance of the blue color developed was measured at 765 nm following the method of Slinkard & Singleton [1977]. The results were expressed as mg of gallic acid equivalents per 100 g of fresh fruits (mgGAE/100 g).

Sensory analysis

The quality of baby kiwifruit was evaluated by means of sensory analysis, involving 10 panellists previously trained using commercial samples. Panellists received 10 whole fruits for each sample and provided descriptions of their taste, appearance, overall acceptability, luminosity (one of the colour parameters), and consistency.

Evaluations took place in individual testing booths at room temperature. All the attributes were evaluated by using a 5-point scale (5 = excellent, 4 = good, 3 = fair, 2 = poor, and 1 = unusable) which was adopted from Meilgaard *et al*. [2006].

Statistical analysis

All statistical analyses were performed using SPSS Statistics 24 software package (2017, IBM, Milan, Italy) for Mac. All data sets (cultivar Hortgem Rua® and Hortgem Tahi®) for the quality analysis were subjected to the analysis of variance (ANOVA) with Tukey's post hoc test. A statistically significant difference was indicated by $P \leq 0.05$. A principal component analysis (PCA) with Varimax rotation with Kaiser normalization was entered to underline the relationships between the different qualitative parameters measured and results of the sensory analysis. The PCA was performed using standardized data due to the reduction of the dimensionality in the multivariate data. Two PCAs were obtained for each cultivar, *i.e.* Hortgem Rua® and Hortgem Tahi®.

RESULTS AND DISCUSSION

Weight loss

The baby kiwifruit weight loss is reported in Figure 1. The loss in weight was progressively increasing with the post-harvest time and statistically significant differences were observed for each sample during the storage time. Samples of both cultivars stored in the mini pallet wrapped with the stretch film had limited weight loss of the product when compared to the control samples. The control samples of both cultivars showed a similar trend of losing the maximum weight at the end of the storage time (9.03% and 9.64% for Hortgem Rua® and Hortgem Tahi®, respectively). The best performance of the stretch wrap film to control fruits management was observed for the Hortgem Tahi®. In fact, at the end of the 60--day storage period, the weight loss was only 3.57%, while at the same time point, the weight loss noted in fruits of cv. Hortgem Rua® reached 6.40%. Considering the limit of the commerciality at 6% weight loss [Briano *et al*., 2017; Almenar *et al*., 2007], the use of wrapped mini pallet had a positive effect upon fruit management and on reducing peel wrinkling already after 20 days and up to 60 days of storage in the warehouse.

Quality parameters

Changes in fruit quality parameters during storage were evaluated by measuring total acidity (TA) and determining contents of total soluble solids (TSS) and dry matter (DM) (Table 1). For the collected samples wrapped with stretch film, it was possible to observe an increase in the TSS concentration during the storage time due to the weight loss of fruits. The highest TSS values were observed for all fruits at the end of the 60-day storage period. Effects resulting from storage time and different management in the warehouse on TA were similar for both tested cultivars. Statistically significant differences ($P<0.05$) were observed during the storage time and a decrease of values was observed for all samples. For both cultivars, the lowest fruit acidity was reported at the end of the storage time for the samples maintained in the stretch wrap film.

The storability of the kiwifruits is well-recognized to be correlated with the DM content [Harker *et al*., 2009; Jordan *et al*., 2000]. Statistically significant differences ($P<0.05$) were observed during the storage time for fruits of both cultivars and for each sample. A general decrease in DM content was observed for all samples and the lowest DM content was achieved at the end of the storage time for all baby kiwifruits. Similar values were observed among wrapped fruits and control ones at each control time, suggesting the limited influence of the stretch film on maintaining high levels of DM content during storage

Total color difference

The external aspect and colour are important attributes for the edibility of these fruits that are eaten with the peel. The total colour difference (ΔE^*) index, which is a combination of L, a and b values, was used to evaluate the discoloration of fruits (Figure 2). The ΔE^* value increased statistically in the storage time, meaning a progressive ripening of the fruits for all the samples, but this trend was limited for all fruits wrapped with the stretch wrap film. At the end of storage time, the minimum ΔE^* value was achieved for the wrapped samples of the cv. Hortgem Rua® (11.80). Due to the best maintenance of weight loss (water content), these fruits actually maintained the highest luminosity (data not shown). Wrapped samples

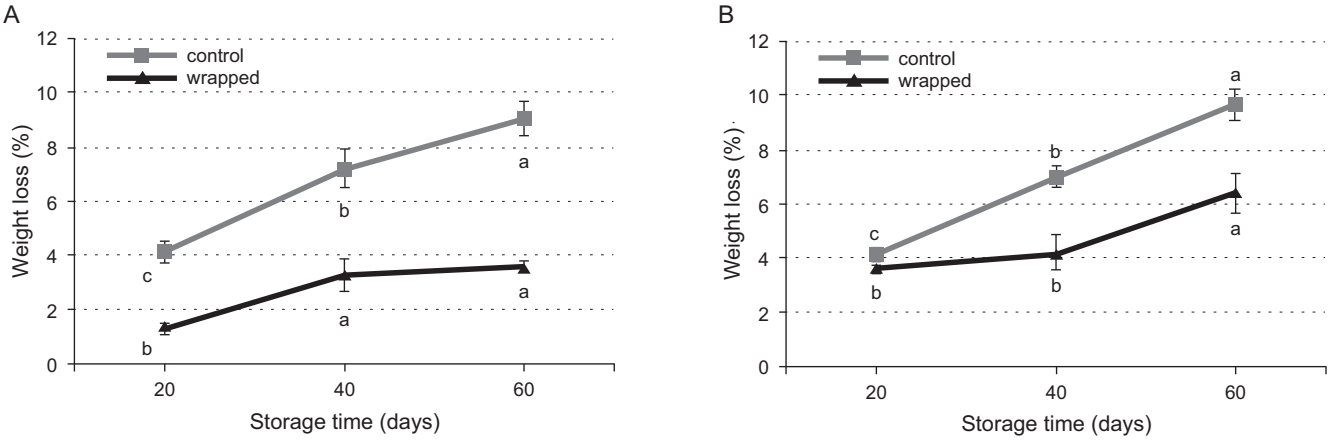


FIGURE 1. Weight loss of baby kiwifruits of Hortgem Rua® (A) and Hortgem Tahi® (B) cultivars during storage.

All data are expressed as average value and the standard error as bars. Different letters on the line indicate significant differences among every harvesting time ($P<0.05$).

TABLE 1. Total soluble solids (TSS), total acidity (TA), and dry matter (DM) of the kiwifruits of Hortgem Rua® and Hortgem Tahi® cultivars during storage.

Quality parameters	Cultivar	Samples	Storage time (days)			
			0	20	40	60
TSS (°Brix)	Rua®	Control	9.90±0.10^{c}	13.68±0.12^{b}	14.57±0.13^{a}	14.23±0.12^{a}
		Wrapped	9.90±0.10^{c}	13.90±0.21^{b}	13.73±0.13^{b}	14.96±0.12^{a}
	Thai®	Control	11.00±0.22^{c}	14.20±0.23^{b}	14.57±0.10^{b}	15.07±0.14^{a}
		Wrapped	11.00±0.22^{c}	14.87±0.14^{a}	13.73±0.31^{ab}	14.80±0.11^{a}
TA (g CA/100mL)	Rua®	Control	0.90±0.00^{a}	0.72±0.12^{b}	0.42±0.41^{c}	0.61±0.31^{c}
		Wrapped	0.90±0.00^{a}	0.64±0.22^{b}	0.58±0.01^{c}	0.59±0.11^{c}
	Thai®	Control	1.20±0.50^{a}	0.89±0.06^{b}	0.73±0.02^{c}	0.77±0.01^{c}
		Wrapped	1.20±0.50^{a}	0.92±0.52^{b}	0.92±0.55^{b}	0.59±0.12^{c}
DM (g/100g)	Rua®	Control	18.43±1.30^{a}	17.97±0,91^{a}	15.86±0.21^{b}	15.74±0.11^{b}
		Wrapped	18.43±1.30^{a}	17.75±0.81^{a}	15.58±0.01^{b}	15.67±0.01^{b}
	Thai®	Control	19.39±1.10^{a}	19.88±0.02^{a}	18.25±0.11^{b}	16.53±0.36^{c}
		Wrapped	19.39±1.10^{a}	18.11±0.20^{b}	17.15±0.62^{bc}	15.68±0.29^{d}

All data are expressed as average values and the standard deviation determined for different fruits. Different letters within the same line indicate significant differences among every harvesting time (Tukey test; $p<0.05$).

of the cv. Hortgem Tahi® have shown a higher loss in luminosity (ΔE^* value of 15.10) probably due to the higher development of anthocyanin during the ripening process, as suggested by previous studies [Montefiori *et al*., 2009].

Textural parameters

Although knowledge about the importance of the softening models of baby kiwifruits has increased in recent years [Giuggioli *et al*., 2017], the evolution of texture parameters under packaging conditions, such as the wrapping of mini pallet, has not been reported. Changes in firmness and gumminess values are reported in Table 2. As expected, a decrease in texture parameters of kiwifruits was observed in all samples of Hortgem Rua® and Hortgem Tahi® due to the dehydration associated with the storage time [Wang *et al*., 2015]. The best control of moisture loss was associated to the stretch wrap film that positively influenced the maintenance of fruit firmness. At each control time, the stretch film maintained a higher value of firmness for fruits of Hortgem Rua® than control samples. At the end of storage, 2.39 N of firmness was registered for the wrapped baby kiwifruits against 1.60 N of the unwrapped fruits. The same trend was observed for the cv. Hortgem Tahi® after 60 days of storage, where the average value of 2.13 N was observed against 1.79 N. The evolution of the dimensional gumminess (hardness x cohesiveness) parameters was similar to the firmness behaviour of unwrapped samples of both cultivars (control) showing the lowest gumminess values, suggesting a strong correlation with the moisture content. After 60 days of storage, gumminess

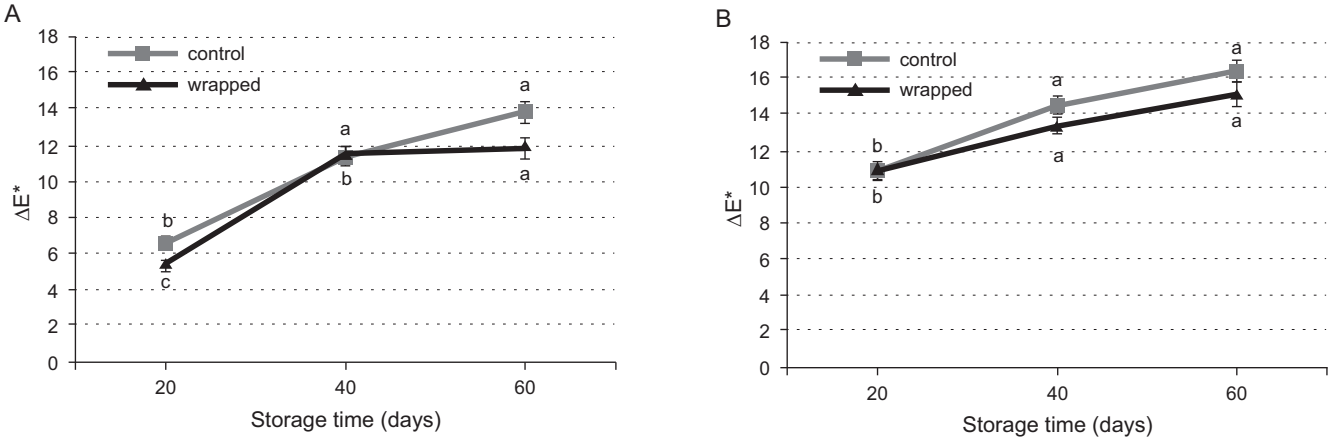


FIGURE 2. Evolution of the total colour difference (ΔE^*) of baby kiwifruits of Hortgem Rua® (A) and Hortgem Tahi® (B) cultivars during storage.
All data are expressed as average value and the standard error as bars. Different letters on the line indicate significant differences among every harvesting time ($P<0.05$).

TABLE 2. Textural parameters and total phenolic content (TPC) of the kiwifruits of Hortgem Rua® and Hortgem Tahi® cultivars during storage.

Quality parameters	Cultivar	Samples	Storage time (days)			
			0	20	40	60
Firmness (N)	Rua®	Control	6.21±0.50[a]	3.56±0.21[b]	1.21±0.48[c]	1.60±0.19[c]
		Wrapped	6.21±0.50[a]	3.84±0.13[b]	1.90±0.36[c]	2.39±0.19[c]
	Thai®	Control	7.93±0.30[a]	2.72±0.25[b]	2.44±0.26[c]	1.79±0.30[c]
		Wrapped	7.93±0.30[a]	2.91±0.06[b]	2.10±0.48[b]	2.13±0.48[b]
Gumminess	Rua®	Control	1152.90±1.71[a]	681.95±0.86[b]	502.10±0.46[c]	213.57±1.11[d]
		Wrapped	1152.90±1.71[a]	656.66±0.52[b]	531.77±0.96[c]	280.92±1.21[d]
	Thai®	Control	936.32±1.43[a]	800.27±1.01[b]	416.97±1.85[c]	211.08±0.91[d]
		Wrapped	936.32±1.43[a]	691.58±1.21[b]	432.83±1.24c	221.58±0.87[d]
TPC (mgGAE/100 g)	Rua®	Control	189.90±1.32[a]	190.90±0.91[b]	167.01±2.50[c]	154.88±0.92[d]
		Wrapped	189.90±1.32[b]	182.24±0.70[b]	191.52±0.50[b]	249.57±0.13[a]
	Thai®	Control	187.51±0.91[c]	225.13±0.11[b]	291.81±0.10[a]	298.79±0.57[a]
		Wrapped	187.51±0.91[b]	220.72±0.81[b]	280.50±0.20[a]	290.50±0.48[a]

All data are expressed as average values and the standard deviation determined for different fruits. Different letters within the same line indicate significant differences among every harvesting time (Tukey test; $p<0.05$).

of fruits of cv. Hortgem Rua® and Hortgem Tahi® wrapped with the stretch film was 280.92 and 213.57, respectively. The decrease in the gumminess for all the control samples depended also on the shrivelling of these fruits.

Total phenolics content

Fruits of *Actinidia arguta* are a rich source of phenolic compounds [Latocha *et al*., 2014]. Changes in total phenolics content are reported in Table 2. It is possible to observe the difference among the two cultivars. According with previous studies [Latocha *et al*., 2014; Krupa *et al*., 2011], the cv. Hortgem Tahi® showed a higher total phenolics content when compared to Hortgem Rua®. For both cultivars, the total phenolics content was increasing with the time of storage and it was not possible to observe the effect of the stretch wrap film on the regular evolution of these bioactive compounds in the samples.

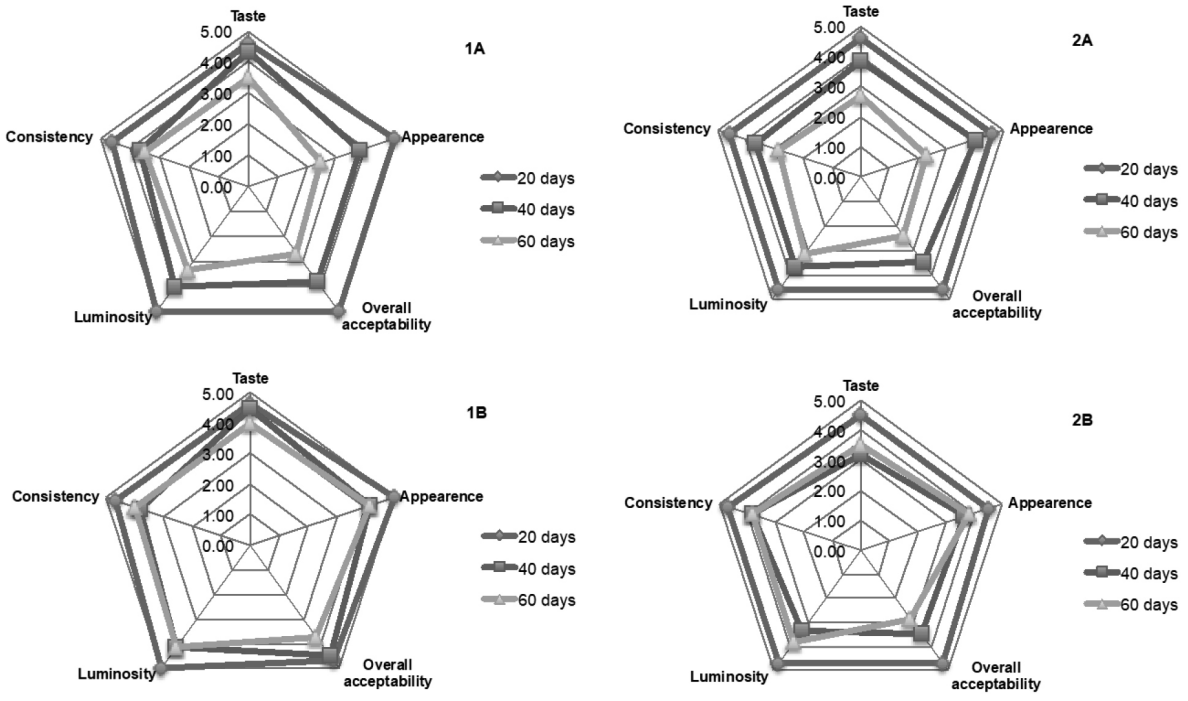


FIGURE 3. Sensory analysis of control and wrapped kiwifruits of Hortgem Rua® (respectively 1A and 2A) and Hortgem Tahi® (1B and 2B) cultivars stored up to 60 days in the warehouse.

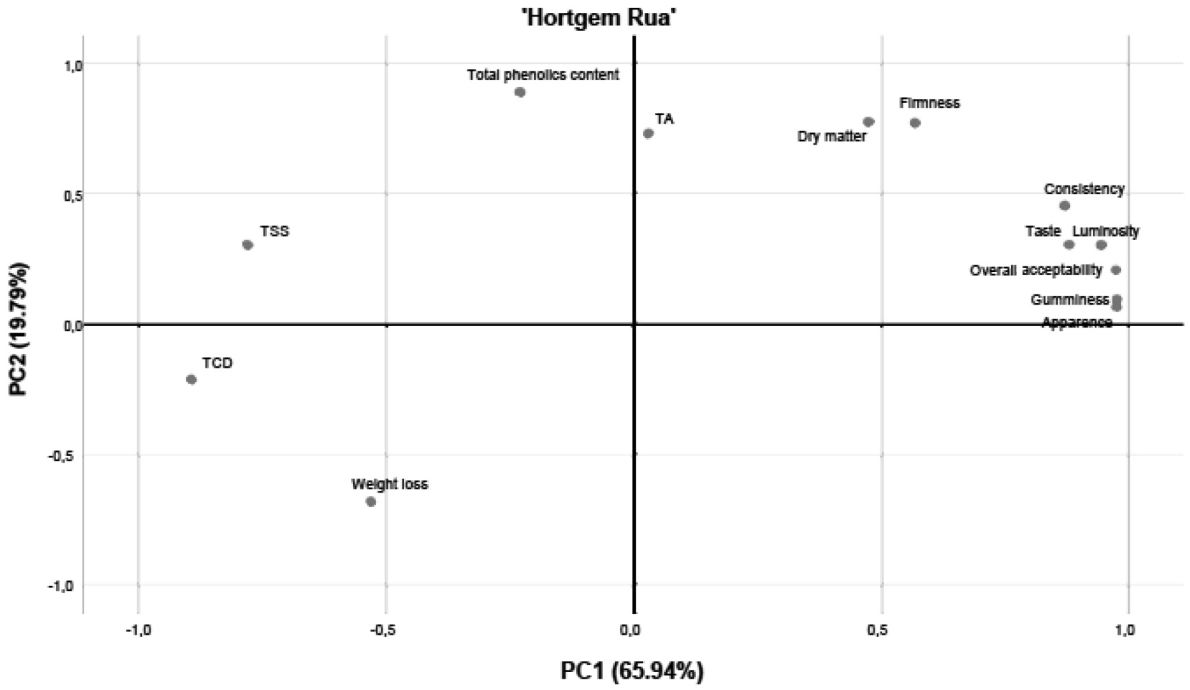


FIGURE 4. PCA of all parameters analyzed for kiwifruits of 'Hortgem Rua®' cultivar.

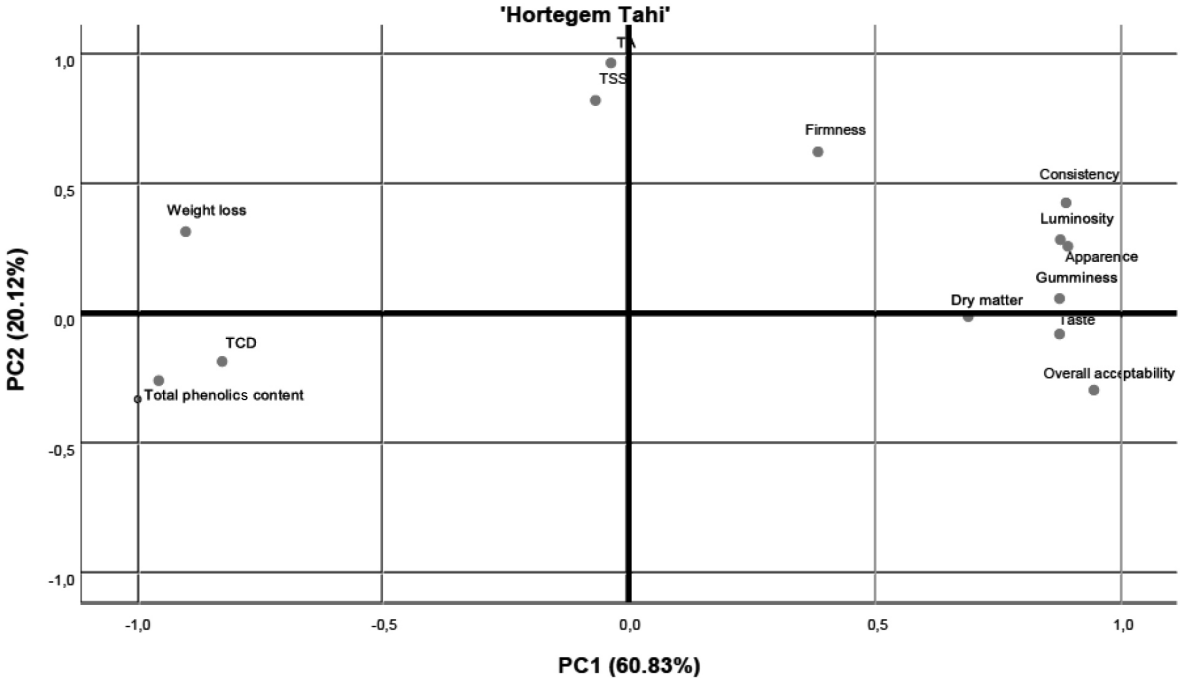


FIGURE 5. PCA of all parameters analyzed for 'Hortgem Tahi®'cultivar.

Sensory evaluation

To support the instrumental measurements of quality, the sensory evaluation was conducted by the panellists in order to judge the overall quality of baby kiwifruits in terms of consumers' point of view. The overall sensory quality scores of samples of Hortgem Rua® and Hortgem Tahi® fruits (Figure 3) decreased successively when the storage time elapsed, achieving the lowest score profile after 60 days. Fruits of both of cultivars wrapped with the stretch film maintained the best overall quality, especially in terms of fruit luminosity, consistency, and taste. The sensory quality of the control samples of both cultivars diminished (which reduced their marketability) due to the reduction in freshness as a consequence of severe water loss (Figure 1). According to Garcìa--Ramos *et al.* [2003], the cultivar Hortgem Tahi® has a strong flavor and is appreciated more for the taste when compared to Hortgem Rua®.

PCA

Principal component analysis (PCA) was carried out on the correlation matrix produced from the quality parameters and the scores of the sensory analysis of baby kiwifruits of both cultivars stored. The PCA plots for the cultivar Hortgem Rua® and Hortgem Tahi® are reported in Figures 4 and 5, respectively. The cumulative variance contribution of all the principal components was 85.73% for the cv. Hortgem Rua® which contributed most to the first (PC1) and the second (PC2) principal component (65.94% and 19.79% respectively). In the case of the cv. Hortgem Tahi®, the cumulative variance contribution of all the principal components was 81.04% (60.83% for the PC1 and 20.21% for the PC2, respectively). For Hortgem Rua®, the PC1 was described by the gumminess, the weight loss, and all the parameters considered in the sensory evaluation. For the Hortgem Tahi®, the dry matter, gumminess, total phenolics content, and all the sensory parameters explained the PC1. As can be seen, it is possible to affirm that the textural parameter and the sensory evaluation attributes of the two cultivars belonged to the PC1. This shows how the external judgement is the driver of the consumer acceptance of the baby kiwifruits according to previous studies on *Actinidia arguta* [Latocha *et al*., 2011].

CONCLUSION

The short storage life of berry fruits (*Actinidia arguta*) is a critical point for their marketability, but a simple storage tool in the warehouse can be employed to support the economic sustainability of the storage process. The post-harvest management of these fruits can follow the berries supply--chain, thus using a stretch wrap film for the pallet storage could be a promising solution to maintain the quality of fruits for a long time. The baby kiwifruits represent a new product in the commercial channel of distribution and are particularly appreciated for the edibility of the whole fruit. Because of the fruits external sensitivity, the storage management in the warehouse is fundamental. Samples of Hortgem Tahi® and Hortgem Rua® fruits stored for up to 60 days with the stretch wrap film have maintained good quality characteristics when compared to the control samples, especially in terms of weight loss and textural properties. The firmness, gumminess, and sensory attributes have the greatest impact on consumer acceptance of the baby kiwifruits. Improving the chain value of these fruits could be a promising choice for the storage in the mini pallet material from the green chemistry as experimentally made for other species.

ACKNOWLEDGEMENTS

We would like to thank all of the producers, professionals, operators, colleagues and collaborators who actively participated in the research project.

FUNDING SOURCE

Research developed into the program ALTA FORMAZIONE IN APPRENDISTATO 2011–2013. Regione Piemonte (Art. 50—D. Lgs. N. 276/2003 s.m.i.).

REFERENCES

1. Alexandre, E.M., Brandão, T.R., Silva, C.L. (2012). Efficacy of non-thermal technologies and sanitizer solutions on microbial load reduction and quality retention of strawberries. *Journal of Food Engineering, 108*(3), 417–426.
2. Allegra, A., Sortino, G., Inglese, P., Settanni, L., Todaro, A., Gallotta, A. (2017). The effectiveness of *Opuntia ficus-indica* mucilage edible coating on post-harvest maintenance of 'Dottato' fig (*Ficus carica* L.) fruit. *Food Packaging and Shelf Life, 12*, 135–141.
3. Almenar, E., Del-Valle, V., Hernández-Muñoz, P., Lagarón, J.M., Catalá, R., Gavara, R. (2007). Equilibrium modified atmosphere packaging of wild strawberries. *Journal of the Science and Food Agriculture, 87*(10), 1931–1939.
4. Baudino, C., Giuggioli, N.R., Briano, R., Massaglia, S., Peano, C. (2017). Integrated methodologies (SWOT, TOWS, LCA) for improving production chains and environmental sustainability of kiwifruit and baby kiwi in Italy. *Sustainability 9*, art.no. 1621.
5. Bouchery, Y., Corbett, C.J., Fransoo, J.C., Tan, T. (2017). Introduction. In Y. Bouchery, C.J. Corbett, J.C. Fransoo, T. Tan (Eds.) *Sustainable Supply Chains*. vol. 4. Springer Nature Switzerland AG, pp. 1–12.
6. Briano, R., Giuggioli, N.R., Girgenti, V., Peano, C. (2015). Biodegradable and compostable film and modified atmosphere packaging in postharvest supply chain of raspberry fruit (cv. Grandeur). *Journal of Food Processing and Preservation, 39*(6), 2061–2073.
7. Briano, R., Peano, C., Giuggioli, N.R. (2017). The effects of the "green passive MAP" on the evolution on the gaseous and quality parameters in strawberries. *Emirate Journal of Food and Agriculture, 29*(3), 1–8.
8. DeEll, J.R. (2002). Modified atmospheres for berry crops. *Ohio State University Extension Newsletter, 6*, 27.
9. Fisk, C.L., Silver, A.A., Strik, B.C., Zhao, Y.X. (2008). Postharvest quality of hardy kiwifruit (*Actinidia arguta* 'Ananasnaya') associated with packaging and storage conditions. *Postharvest Biology and Technology, 47*(3), 338–345.
10. Garcìa-Ramos, F.M, Ortiz-Caìavate, J., Ruiz-Altisent, M., Dez JFlores, L. Homer, I., Ch'vez, J.M. (2003). Development and implementation of an on-line impact sensor for firmness sensing of fruits. *Journal of Food Enginerring, 58*(1), 53–57.
11. Giuggioli, N.R., Girgenti, V., Baudino, C., Giacalone, G., Chiabrando, V. (2017). Prediction models for the softening of new commercial *Actinidia* cultivars. *Journal of Food Processing and Preservation, 41*(6), art no. e 13245.
12. Harker, F.R., Carr, B.T., Lenjo, M., Macrae, E.A., Wismer, W.V., Marsh, K.B., Williams, M., White, A., Lund, C.M., Walker, S.B., Gunson, F.A., Pereira, R.B. (2009). Consumer liking for kiwifruit flavour: A meta-analysis of five studies on fruit quality. *Food Quality Preferences, 20*(1), 30–41.
13. Jordan, R.B., Walton, E.F., Klages, K.U., Seelye, R.J. (2000). Postharvest fruit density as an indicator of dry matter and ripened soluble solids of kiwifruit. *Postharvest Biology and Technology, 20*, 163–173.
14. Kaya, M., Česonienė, L., Daubaras, R., Leskauskaitė, D., Zabulionė, D. (2016). Chitosan coating of red kiwifruit (*Actinidia melanandra*) for extending of the shelf life. *International Journal of Biological Macromolecules, 85*, 355–360.

15. Krupa, T., Latocha, P., Liwińska, A. (2011). Changes of physicochemical quality, phenolics and vitamin C content in hardy kiwifruit (*Actinidia arguta* and its hybrid) during storage. *Scientia Horticulturae, 130*(2), 410–417.
16. Latocha, P., Jankowski, P., Radzanowska, J. (2011). Genotypic difference in postharvest characteristics of hardy kiwifruit (*Actinidia arguta* and its hybrids), as a new commercial crop Part I. Sensory profiling and physicochemical differences. *Food Research International, 44*, 1936–1945.
17. Latocha, P., Krupa, T., Jankowski, P., Radzanowska, J. (2014). Changes in postharvest physicochemical and sensory characteristics of hardy kiwifruit (*Actinidia arguta* and its hybrid) after cold storage under normal versus controlled atmosphere. *Postharvest Biology and Technology, 88*, 21–33.
18. Latocha, P., Wołosiak, R., Worobiej, E., Krupa, T. (2013). Clonal differences in antioxidant activity and bioactive constituents of hardy kiwifruit (*Actinidia arguta*) and its year to year variability. *Journal of the Science and Food Agriculture, 93*(6), 1412–1419.
19. Lim, S., Han, S.H., Kim, J., Lee, H.J., Lee, J.G., Lee, E.J. (2016). Inhibition of hardy kiwifruit (*Actinidia aruguta*) ripening by 1-methylcyclopropene during cold storage and anticancer properties of the fruit extract. *Food Chemistry, 190*, 150–157.
20. McGlone, V.A., Jordan, R.B., Seelye, R. Clark, C.J. (2003). Dry matter – a better predictor of the post-storage soluble solids in apples? *Postharvest Biology and Technology, 28*, 431–435.
21. McNeilage, M.A., Beatson, R.A., Macrae, E.A. (2003). Kiwi plant named "Hortgem Tahi". Patent N° US PP13815P3 United States Plant Patent.
22. McNeilage, M.A., Beatson, R.A., Macrae, E.A. (2004). Kiwi plant named "Hortgem Rua". Patent N° US PP14625 P3 United States Plant Patent.
23. McGuire, R.G. (1992). Reporting of objective color measurements. *Horticultural Science, 27,* 1254–1255.
24. Meilgaard, M.C., Carr, B.T., Civille, G.V. (2006). *Sensory Evaluation Techniques*. CRC Press. Taylor & Francis Group, Parkway, NY, USA.
25. Montefiori, M., Comeskey, D.J., Wohlers, M., McGhie, T.K. (2009). Characterization and quantification of anthocyanins in red kiwifruit (*Actinidia* spp.). *Journal of Agricultural and Food Chemistry, 57*(15), 6856–6861.
26. Oh, S.B., Muneer, S., Kwack, Y.B., Shin, M.H., Kim, J.G. (2017). Characteristic of fruit development for optimal harvest date and postharvest storability in 'Skinny Green' baby kiwifruit. *Scientia Horticulturae, 222*, 57–61.
27. Peano, C., Briano, R., Giuggioli, N.R., Girgenti, V., Sottile, F. (2015). Evolution of qualitative characteristics during blueberry fruit storage in a modified atmosphere. *Acta Horticulturae, 1071*, 343–348.
28. Peano, C., Giuggioli, N.R., Girgenti, V., Palma, A., D'Aquino, S., Sottile, F. (2017). Effect of palletized MAP storage on the quality and nutritional compounds of the Japanese Plum cv. Angeleno (*Prunus salicina* Lindl.). *Journal of Food Processing and Preservation, 41*(2), art.no. e12786.
29. Slinkard, K., Singleton, V.L. (1977). Total phenol analysis: automation and comparison with manual methods. *American Journal of Enology and Viticulture, 28*, 49–55.
30. Strik, B., Hummer, K. (2006). 'Ananasnaya' hardy kiwifruit. *Journal of American Pomology Society, 60*, 106–112.
31. Wang, Y., Xu, F., Feng, X., MacArthur, R.L. (2015). Modulation of *Actinidia arguta* fruit ripening by three ethylene biosynthesis inhibitors. *Food Chemistry, 173*, 405–413.
32. White, A., de Salva, H.N., Requejo-Tapia, C., Harker, F.R. (2005). Evaluation of softening characteristics of fruits from 14 species of *Actinidia*. *Postharvest Biology and Technology, 35*, 143–151.

Submitted: 29 May 2018. Revised: 11 September and 25 October 2018. Accepted: 16 November 2018. Published on-line: 22 January 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 71–81
DOI: 10.31883/pjfns-2019-0001
http://journal.pan.olsztyn.pl

Original research article
Section: Food Chemistry

Nutritional Quality of Edible Marine Bivalves from the Southern Coast of Italy, Mediterranean Sea

Francesca Biandolino, Antonella Di Leo, Isabella Parlapiano, Loredana Papa, Santina Giandomenico, Lucia Spada, Ermelinda Prato*

CNR- IRSA Water Research Institute, UOS di Taranto, Via Roma 3, 74125 Taranto, Italy

Key words: edible molluscs, meat yield, proximate, minerals, fatty acids

Nutritional quality parameters of eight commercially important bivalve species (*Arca noae*, *Flexopecten glaber*, *Limaria tuberculata*, *Mimachlamys varia*, *Modiolus barbatus*, *Mytilus galloprovincialis*, *Ostrea edulis* and *Solen marginatus*) from the Ionian Sea (Southern Italy, Mediterranean Sea), were determined. The meat yield and lipid nutritional quality indices (atherogenic index, thrombogenicity index and hypocholesterolaemic/hypercholesterolaemic fatty acid ratio) have been also evaluated.

Meat yield values ranged from 31.4% in *F. glaber* to 44.5% in *M. varia*. The results showed that all species might be considered as food items with interesting dietetic properties due to high contents of proteins, minerals (Ca, K, Na, Fe, Zn, Cu), essential polyunsaturated fatty acids (PUFAs), and to low cholesterol content. Among PUFAs, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) exhibited the highest levels in *M. galloprovincialis* (11.74%) and in *M. varia* (14.41%), respectively. Elevated *n*-3/*n*-6 ratio characterized the fatty acids profile of all species ranging from 2.65 in *F. glaber* to 7.19 in *M. galloprovincialis*. The lipid nutritional quality indices showed that *M. varia*, *M. galloprovincialis*, *O. edulis*, *S. marginatus*, and *L. tuberculata* might have beneficial effects on the consumer's health. This paper will be of practical value from a health perspective for populations who consume shellfish and a powerful marketing tool for farmers of the bivalves.

INTRODUCTION

The marine molluscs are important for marine ecology and play an important role in human's diet, since they are a good source of nutrients. High quality of protein, minerals, low lipid content, and especially high proportion of polyunsaturated fatty acids (PUFAs) characterize the mollusc flesh, contributing to their nutritional value and organoleptic characteristics [Orban *et al.,* 2007].

PUFAs are considered as essential fatty acids that humans cannot synthesize and must be provided with food. Recent studies have clearly shown the importance of PUFAs and their nutritional value for human health. A particular emphasis is placed on the *n*-3 PUFAs, eicosapentaenoic (EPA) acid and docosahexaenoic (DHA) acid that can be associated with several health benefits. DHA plays a role in the development and function of the brain, the photoreception, and the reproductive system [Kris-Etherton *et al.,* 2003; Sidhu, 2003]. EPA is the precursor of a family of prostaglandins, which control blood clotting and other arterial functions. This may be important in reducing the risk of cardiovascular disease, decrease in mild hypertension, prevention of certain cardiac arrhythmias and sudden death [Kris-Etherton *et al.,* 2003]. EPA and DHA also lower blood triglyceride concentrations and are substrates for the synthesis of resolvins, which are believed to play a key role in terminating inflammatory processes [Kohli & Levy, 2009]; moreover, they have beneficial effects on other diseases, namely skin disease, asthma, arthritis, nephritis, lupus erythematous, multiple sclerosis, and certain types of cancers [Harris, 2010; Massaro *et al*., 2010]. PUFAs are influenced by taxonomic relations and environmental conditions and depend on species, nutrient habits, food availability and physiological conditions, so they differ for molluscs species that come from different geographical areas [Orban *et al*., 2007].

Minerals such as sodium, potassium, magnesium, calcium, iron, zinc and copper, are essential substances for organisms and for their vital functions and growth. In fact, minerals are important components of hormones and enzymes activated in human nutrition [Khan, 1992], and are involved in physiological processes in the body, the most important of which are pH maintenance, osmotic pressure, nerve conductance, muscle contraction and energy production. Zinc, for example, has different important biochemical functions, in particular as cofactor to more than 300 enzymes involved in DNA and RNA metabolism and stabilization of cell membranes [Beyersmann, 2002; Rink & Gabriel, 2000].

Unsuited dietary intakes of the minerals Ca, Mg, Na and K, either insufficient or excessive, can cause serious health issues such as cardiovascular disease, osteoporosis, and hypertension. In general, the seafoods are excellent sources of Ca, K, Na, Fe, Zn, Cu and in particular, oysters are good sources of Fe, Zn, and Cu.

* Corresponding Author: Via Roma 3, 74125 Taranto,
Tel: +39 099 4542210; fax +39 099 4542215
E-mail address: francesca.biandolino@iamc.cnr.it

Bivalve molluscs represent a significant proportion of the world's fishery and aquaculture. In Italy, the harvesting and commercialization of bivalves represents an important productive sector in the national economy (~111.000 t of marine molluscs), after Spain (~223.000 t) and France (~155.000 t). About 70% of the Italian shellfish production is consumed domestically, while the remaining 30% is exported to European countries, first among them Spain [FAO, 2016].

Despite the presence of a rich diversity of edible and commercial seafood species along the Mediterranean coast [Coll *et al*., 2010], little information is available with regard to the nutritional quality for most parts of the edible marine bivalves from this important coastal region.

In recent years, consumers have become more health conscious and interested in maintaining or improving their health through the diet [Hasler, 2002]. Thus, the knowledge on biochemical composition of any edible organisms is extremely important because it reflects their nutritional value [Periyasamy *et al*., 2011].

The ability to identify healthy components enable consumers to make healthy food choices. There is a strong connection between foods and health, therefore making healthier food choices can prevent diseases with simple changes that can contribute to your overall health and well-being. In addition, this information can be useful to producers as a powerful marketing tool, in the promotion of food of high economic and nutritional values.

For these reasons, the present work aimed to investigate the nutritional quality of eight commercially important bivalve species from Ionian Sea (Southern Italy, Mediterranean Sea), well appreciated in the Mediterranean diet. For some of them it is the first time that such information is given. Therefore, the proximate, essential minerals and fatty acids compositions, as well as meat yield and lipids nutritional quality indices (LNQI) were determined.

MATERIALS AND METHODS

Collection, samples preparation and meat yield of bivalves

The bivalves *Arca noae*, *Flexopecten glaber*, *Limaria tuberculata*, *Mimachlamys varia*, *Modiolus barbatus*, *Mytilus galloprovincialis*, *Ostrea edulis*, and *Solen marginatus* from the Gulf of Taranto (Southern Italy, Mediterranean sea), were purchased from a local market in January-February 2014. Samples (maximum 24–48 h after harvesting) were immediately iced and transported to the laboratory within 1 h to be brushed, washed, and processed. Upon arrival to the laboratory, each sample of about 30 individuals was split up in two sub-groups: one for biometric measurements and determination of meat yield (MY) and one for biochemical determinations. Adult specimens of commercial size of each species with homogenous shell length were selected to ensure that any biochemical differences were not size dependent. Length (maximum measure along the anterior-posterior axis) and width (maximum lateral axis), were measured using a 0.1mm precision calliper. The bivalves were weighed, opened by cutting the adductor muscle with a scalpel, and the wet meat and shells were weighed. MY was calculated as follows: (wet meat weight/whole mussel weight) × 100 [Okumus & Stirling, 1998]. The biometric measurements are shown in Table 1.

A minimum of ten individuals (ranging 40–200 g of wet meat weight) from each species was minced for biochemical analyses. The specimens were manually shucked by cutting the adductor muscle with a knife. The bivalves juice was removed and the edible portion was collected. The soft body was not separated into organs or body parts to avoid leakage of intracellular fluids. Three replicates of ten individuals each were obtained. Each sample was stored at -20°C (for a maximum 7 days) in polyethylene bags.

Proximate analysis

Moisture content was determined by drying the sample in an oven at 105°C overnight until a constant weight was obtained. Ash content was determined by burning the samples in the furnace at 550°C overnight. The crude protein content was measured by the Kjeldahl procedure (nitrogen to protein conversion factor = 6.25). The methods of the Association of Official Analytical Chemists [AOAC, 1995] were used. Carbohydrates were quantified according to the phenol–sulphuric acid method [Dubois *et al*., 1956], using glucose as the standard. Total lipid (TL) content was determined gravimetrically after chloroform--methanol extraction according to Folch *et al*. [1957].

All analyses were conducted in triplicate and results were expressed on wet weight basis.

Lipid classes

Triacylglycerols (TAGs), total cholesterol (CHL), and phospholipids (PLs) were measured by the colorimetric enzymatic method using a commercial kit (SGM, Rome, Italy) as reported in Prato *et al*. [2010]. The values are expressed as percentage (mean ± SD) of total lipids. All analyses were performed in triplicate.

Fatty acid analysis

Fatty acids of total lipids were transesterified to methyl ester (FAMEs) according to the procedure described by Prato *et al*. [2018].

Analysis of FAMEs was performed by gas chromatography (GC) using an HP 6890 series GC (Hewlett Packard, Wilmington, DE, USA), equipped with a flame ionization detector. FAMEs were separated with an Omegawax 250 capillary column (Supelco, Bellafonte, PA, USA) (30 m long, 0.25-mm internal diameter, and 0.25-mm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min. The column temperature program was as follows: 150 to 250°C at 4°C/min and then held at 250°C. Peaks were identified by their comparison to the relative retention times of standards (Supelco 37 component FAME Mix), and the results were expressed as the percentages of peak areas.

Mineral composition

Mineral analysis was carried out on a lyophilized sample with 9 mL of concentrated HNO_3 (70 % v/v) and 1 mL H_2O_2 (30 % v/v) [US EPA, 1996] using a microwave diges-

tion system (MARSX CEM Corporation, Matthews, NC). After mineralization, the digested samples were diluted to 50 mL with Milli-Q® water. A blank digest was performed in the same way.

Contents of K, Na, Mg, Ca, Zn, Cu, and Fe were determined by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), using a Perkin Elmer model Elan 6100 DRC Plus (PerkinElmer, Norwalk, CT, USA). Each sample was analyzed in three replicates and the relative standard deviation (RSD) was <5 %. Accuracy was verified using the Community Bureau of Reference (BCR) Certified Reference Material CRM 278R (Trace elements in mussel tissue) produced by Joint Research Centre, Geel, Belgium.

The recovery of CRM 278R ranged between 95% (Zn^{66}) and 105% (Na^{23}). Chemicals were of ultrapure grade (Merck Suprapur, Darmstadt, Germany), and all the glasswares were treated prior to the analysis with 10% v/v HNO_3 for 24 h and afterwards rinsed with Milli-Q® water. Standard solutions were obtained daily using ultrapure deionized water (<0.1 μs at 25°C).

Lipid nutritional quality indices (LNQI)

The data from fatty acid composition analysis were used to determine the nutritional quality of the lipid fraction by means of three indices using the following calculations:

Atherogenicity index [Ulbricht & Southgate, 1991]:

$$AI = \frac{(C14:0 + 4 \times C14:0 + C16:0)}{\Sigma MUFAs + \Sigma PUFAs}$$

Thrombogenicity index [Ulbricht & Southgate, 1991]:

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{[(0.5 \times \Sigma MUFAs + 0.5 \times \Sigma\ n6\ PUFAs + 3 \times \Sigma\ n3\ PUFAs + (n3/n6)]}$$

Fatty acid hypocholesterolemic/hypercholesterolemic ratios [Santos-Silva *et al*., 2002]:

$$HH = \frac{(C18:1cis9 + C18:2n6 + C20:4n6 + C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3)}{(C14:0 + C16:0)}$$

Statistical analysis

Analyses were performed in three replicates and each one was measured for three repetitions. Results were expressed as mean values ± standard deviation.

The normality of the data was evaluated by Kolmogorov-Smirnov test. The homogeneity of variances was assessed by Levene test. When either assumptions were met, all data (means of proximate composition, lipid class, minerals, fatty acids and LIQN) were examined by analysis of variance (one-way ANOVA) to verify whether there were differences among the analysed species. The multiple comparison (Tukey's test) was applied when the variance analysis indicated significant differences. The level of significance was set as 0.05.

A principal component analysis (PCA) based on Pearson's correlation matrix relationships between variables, was performed. Multivariate statistical treatment of the whole set of data was preformed after logarithmic transformation to reduce the variability of data.

All statistical analyses were performed with the software package Past version 1.0 and STATISTICA® (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Biometric parameters and meat yield

Table 1 shows results of the biometric measurements, as well as meat yield of eight commercial bivalves. In this study, bivalves exhibited meat yield values ranging from 31.4% in *F. glaber* to 44.5% and 41.2% in *M. varia* and *M. galloprovincialis*, respectively.

Previous studies demonstrated that the percent meat yield or edibility of bivalves as a good commercial quality indicator varies seasonally and geographically, depending on food availability and the timing of the gametogenic cycle [Anibal *et al.,* 2011; Celik *et al*., 2012; Okumus & Stirling, 1998; Orban *et al*., 2007].

The results obtained were comparable to those observed for *F. glaber*, in Lapseki Bay in Canakkale (Turkey), by Berik & Çankiriligil [2013] (MY: 34.68 %), in the same period. Mussels and oysters showed high MY compared with findings from other studies in the Mediterranean area; *e.g.* mussel from Adriatic Sea, Dardanelles (Turkey) and Black Sea showed values in the range of 18–25% [Bongiorno *et al*., 2015; Yildiz *et al*., 2006]. In turn, Fuentes *et al*. [2009] reported the MY values of 31% and 34% for *M. galloprovincialis* from Galicia and Valencia (Spain), respectively, during the summer period. Moreover, *Ostrea edulis* from Dardanelles displayed values of about 8% in February [Yildiz *et al*., 2011]. As regard the other species no literature data exist.

Proximate analysis

The proximate composition of bivalves meat is shown in Table 1. As expected, the main component of all studied species is the moisture, whose content indicates flesh freshness. The highest values were found in *M. galloprovincialis* and *F. glaber*, and *L. tuberculata* (84.10, 83.67, and 82.60 g/100 g, respectively). These values did not differ statistically between each other ($p \geq 0.05$), and the lowest was found in *M. barbatus* (79.84 g/100 g).

The highest ash content, which indicates the amount of inorganic compounds in the tissues of bivalves, was found in *L. tuberculate*, *A. noae* and *S. marginatus* (5.33 – 4.26 g/100 g; differences between values were insignificant, $p \geq 0.05$). Significantly lower ($p < 0.05$) ash content was noted in *M. galloprovincialis* (2.62 g/100 g), although four other species exhibited not statistically different values ($p \geq 0.05$). Despite these values, *A. noae* showed, for the most minerals investigated, values lower than those of *M. galloprovincialis,* probably due to the presence of other minerals not investigated herein.

High protein levels and low lipid contents characterized the overall proximate profile of bivalves studied herein. *A. noae* showed the highest protein content with 12.25g/100 g. *A. noae* and *S. marginatus* represent a good source of protein considering the WHO Daily Value (DV) recommendation of 0.80g/kg/day [WHO, 2007].

The total lipid amount, also, exhibited differences among the species, with the highest values determined in *M. barbatus, L. tuberculata*, and *O. edulis* (2.98, 2.76, and 2.70 g/100 g, respectively) and the lowest ones in *F. glaber*, *A. noae*, and *S. marginatus* (1.04, 1.18, and 1.25 g/100 g, respectively).

TABLE 1. Biometric measurements, meat yield, proximate composition, lipid classes and mineral content of eight commercial bivalves species on a wet weight basis.

Specification	*A. noae*	*F. glaber*	*L. tuberculata*	*M. varia*	*M. barbatus*	*M. galloprovincialis*	*O. edulis*	*S. marginatus*
Shell lenght (mm)	53.0±3.2	44.0±1.5	43.5±1.6	42.2±3.1	48.3±4.60	59.6±6.0	82.5±2.0	100.5±10.5
Shell width (mm)	29.0±1.5	41.4±3.6	33.4±3.5	37.4±5.0	25.5±2.6	27.8±3.3	61.2±2.0	18.5±0.5
Total wet weight (g)	10.8±0.5	12.5±1.1	13.4±0.7	7.84±0.7	9.7±0.9	11.6±0.8	57.2±1.5	21.3±1.3
Meat yield (%)	40.3±2.3	31.4±3.9	35.2±2.3	44.5±4.5	40.0±1.8	41.2±4.3	37.5±2.3	39.0±3.5
Moisture (g/ 100g)	80.74±0.8^{c,d}	83.67±0.6^{a,b}	82.60±1.0^{a,b,c}	81.33±1.9^{b,c,d}	79.84±2.7^{d}	84.10±0.6^{a}	81.47±1.2^{b,c,d}	80.56±1.7^{c,d}
Ash (g/ 100g)	4.81±1.2^{a,b}	3.16±0.2^{c,d}	5.33±0.8^{a}	3.39±0.7^{b,c,d}	3.77±0.9^{b,c,d}	2.62±0.2^{d}	3.72±1.4^{b,c,d}	4.26±0.5^{a,b,c}
Protein (g/100 g)	12.25±1.8^{a}	7.08±0.4^{c,d}	8.57±0.0^{b,c}	8.68±0.1^{b,c}	6.88±0.0^{c,d}	6.58±0.5^{d}	8.10±1.2^{c,d}	9.96±1.4^{b}
Lipid (g/100 g)	1.18±0.0^{d}	1.04±0.3^{d}	2.76±0.4^{a,b}	2.26±0.6^{b,c}	2.98±0.2^{a}	2.15±0.3^{c}	2.70±0.3^{a,b,c}	1.25±0.1^{d}
PL (%)	83.47±2.5^{a,b}	89.59±2.7^{a}	66.29±3.5^{c}	69.14±3.4^{c}	73.32±4.5^{c,b}	69.83±3.8^{c}	69.54±3.4^{c}	64.35±4.5^{c}
TAG (%)	10.79±3.6^{c}	7.20±3.7^{c}	22.97±1.2^{b}	21.29±1.0^{b}	21.60±1.1^{b}	24.35±1.2^{b}	21.56±1.3^{b}	31.57±1.0^{a}
CL (%)	5.74±1.4^{b}	3.21±1.1^{c}	10.74±3.5^{a}	9.57±1.9^{a}	5.08±1.7^{b,c}	5.82±1.2^{b}	8.90±1.8^{a}	4.08±1.4^{c}
Na (mg/100 g)	91.92±3.1^{e}	301.61±10.1^{a}	226.47±7.5^{c}	167.45±5.6^{d}	237.33±7.9^{b,c}	240.3±8.0^{b}	244.52±8.2^{b}	167.37±5.6^{d}
Mg (mg/100 g)	19.30±0.6^{f}	47.00±1.6^{a}	37.33±1.2^{c}	24.80±0.8^{e}	40.08±1.3^{b}	35.72±1.2^{c}	29.79±1.0^{d}	29.75±1.0^{d}
K (mg/100 g)	49.63±1.6^{b}	34.46±1.2^{d}	25.97±0.9^{e}	45.18±1.5^{c}	74.89±2.5^{a}	34.32±1.1^{d}	46.45±1.6^{c}	77.17±2.6^{a}
Ca (mg/100 g)	9.7±0.35^{e}	21.78±0.7^{c}	21.59±0.7^{c}	14.00±0.5^{d}	14.45±0.5^{d}	8.12±0.3^{f}	35.50±1.2^{a}	27.15±0.9^{b}
Fe (mg/100 g)	12.50±0.4^{a}	7.64±0.2^{d}	4.58±0.1^{g}	5.18±0.2^{f}	6.54±0.2^{e}	6.30±0.2^{e}	11.24±0.4^{b}	9.57±0.3^{c}
Zn (mg/100 g)	3.5±0.1^{d}	4.31±0.1^{d}	0.79±0.0^{f}	23.4±0. 8^{c}	25.42±0.8^{b}	2.49±0.1^{e}	30.67±1.0^{a}	1.95±0.1^{e}
Cu (mg/100 g)	0.32±0.0^{d}	0.52±0.0^{c}	0.80±0.0^{b}	5.00±0.1^{a}	0.55±0.0^{c}	0.22±0.0^{d}	4.83±0.2^{a}	0.19±0.0^{d}

Note: Data are the mean values of three replicates ± standard deviation. Moisture and ash are expressed as % of body mass; proteins and lipids as g/100 g. Mineral as mg/100 g. Phospholipids (PL), Triacylglycerols (TAG) and Cholesterol (CHL) as % of total lipid. Means within the same row without a common lowercase letter differ significantly ($p<0.05$).

To the best of our knowledge, no information is available on the nutritional quality for most of the studied species. Thus, we compared our findings with some other bivalve's species of commercial size from different geographical areas.

Protein content in *M. galloprovincialis* was comparable to that found in mussels from Bay of Biscay and Ebro Delta (Spain) [Azpeitia *et al*., 2016; Fuentes *et al*., 2009]. Higher values were found, in the same study period, by Bongiorno *et al*. [2015] for bivalvles from the North Adriatic Sea (8.9–10.9%), by Dernekbasi *et al*. [2015] for species from the Black Sea (10––11%), by Fuentes *et al*. [2009] for bivalves from two different Spanish areas, Galicia and Valencia (10%), and by Yildirim & Ercan [2016] for species from Gulluk Gulf, Turkey (10%).

Lipids exert important biological functions as energy storage compounds, structural components of the cell membranes and as signalling molecules [Zhukova, 2014]. Lipid contents in species studied herein were higher than most data reported in literature [Azpeitia *et al*., 2016; Bongiorno *et al*., 2015; Fuentes *et al*., 2009; Yildirim & Ercan 2016], but agreed well with those reported by Dernekbasi *et al*. [2015] and Fuentes *et al*. [2009] from Valencia (2.10%).

Asha *et al*. [2014] reported a slightly higher protein and lipid content for *Crassostrea madrasensis* compared with those found in *O. edulis* in this study (9.41 *vs* 8.10 g/100 g of protein and 3.25 *vs* 2.79 g/100 g of lipid, respectively). In addition, Berik *et al*. [2017] for *F. glaber* and Orban *et al*. [2007] for *Chamelea gallina* reported a higher protein and lipid content (10.75 g/100 g and 1.84 g/100 g, respectively for *F. glaber* and 10.8 g/100 g and 1.59 g/100 g, respectively, for *C. gallina*).

Lipid classes

Phospholipids (PL), the major lipid class found herein, differed among species with *F. glaber* and *A. noae* showing the highest proportion (89.59% and 83.47% of total lipids, respectively) ($p<0.05$) (Table 1). Generally, PLs in bivalves are stable with respect to lipid class proportions, irrespective of growth rates or diets. Such stability is linked to the structural role of the PL in cell membranes where they give the desired structure to the membrane and determine its permeability [Caers *et al.,* 2000].

The level of triacylglycerols (TAG) in bivalves species ranged from 7.20% to 31.57% of total lipids. The highest TAG content was noted in *S. marginatus*. In turn, *F. glaber* and *A. noae* showed the lowest TAG proportion. TAGs play important roles as energy reserves that are mainly influenced by reproduction and/or by nutrition. Low levels of TAGs have been considered an indicator of low nutritional status [Okumus & Stirling, 1998].

The cholesterol (CL) maintains both membrane structural integrity and fluidity; moreover, it plays a key role in lipid metabolism [Los & Murata, 2004].

Among the bivalves examined, *L. tuberculata* and *O. edulis* had the highest cholesterol content (8.90–10.74% of total lipids), while *F. glaber* and *S. marginatus* and *M. barbatus* had the lowest (3.21 -5.08% of total lipids) (Table 1). It is noticeable that people who are designing low cholesterol diets for health purposes must pay attention to these differences, even though the flesh of the examined bivalves exhibited a relatively low cholesterol content.

Minerals

Contents of minerals (Na, K, Ca, Mg, Cu, Zn, and Fe) in fresh soft tissue of the marine bivalves found in this study are shown in Table 1.

Significant differences ($p<0.05$) were found in the distribution of metals among the species analyzed, though the Na, K, Mg, and Ca were predominant in the bivalves. The only exceptions are represented by *A. noae* where Fe > Ca and by *M. barbatus* and *M. varia* where Zn > Ca. Na, in particular, was the most abundant element with contents up to 3 orders of magnitude higher than the other minerals. Fe, Zn, and Cu, instead, were found generally at lower levels and followed the order Fe > Zn > Cu except in *M. varia*, *M. barbatus*, and *O. edulis* where Zn was found at higher levels than Fe. As regards the minimum and maximum contents, the highest contents of Na and Mg were found in *F. glaber* (301.61 and 47.00 mg/100 g, respectively), while the lowest levels of Na and Mg were found in *A. noae* (91.92 and 19.30 mg/100 g, respectively). The highest Ca and Zn levels were found in *O. edulis* (35.50 and 30.67 mg/100 g, respectively), while the lowest values of Ca and Zn level were found in *M. galloprovincialis* (8.12 mg/100 g) and *L. tuberculata* (0.79 mg/100 g), respectively.

The highest levels of Cu were detected in *M. varia* (5.00 mg/100 g) and *O. edulis* (4.83 mg/100 g), while *S. marginatus*, *M. galloprovincialis*, and *A. noae* had the lowest level of Cu (Table 1).

Fe was found at higher contents in *A. noae* (12.50 mg/100 g) and *O. edulis* (11.24 mg/100 g), while in the other species was uniformly distributed.

Lastly, *S. marginatus* and *M. barbatus* had the highest contents of K (77.17 and 74.89 mg/100 g, respectively) while its level was the lowest in *L. tuberculata* (25.97 mg/100 g).

In comparison with the literature data, Na, Ca, Fe, Cu, and Zn contents found in the *M. galloprovincialis* were higher than those noted for bivalves from Trieste Gulf (162 mg/100 g for Na; 487 mg/100 g for Ca; 119 mg/100 g for Fe; 5.1 mg/100 g for Cu; and 59 mg/100 g for Zn) and from Galician waters (218 mg/100 g for Na; 40 mg/100 g for Ca; 1.0 mg/100 g for Fe; 0.15 mg/100 g for Cu; and 2.3 mg/100 g for Zn) [Bongiorno *et al*., 2015; Fuentes *et al*., 2009, respectively]. As regards K, its levels in the *M. galloprovincialis* were similar to those reported by Fuentes *et al*. [2009] (36 mg/100 g) and lower than those obtained by Bongiorno *et al*. [2015] (41 mg/100 g). Concerning Mg contents, Fuentes *et al*. [2009] reported for *M. galloprovincialis* a higher value (56 mg/100 g), while Bongiorno *et al*. [2015] a lower value (14 mg/100 g).

Na, Ca, K, Mg, and Zn contents measured in oysters were much lower than levels found in oysters of the Croatian coasts (715 mg/100 g for Na, 155 mg/100 g for Ca, 248 mg/100 g for K, 90 mg/100 g for Mg, and 68 mg/100 g for Zn) [Bilandžić *et al*., 2016]. On the other hand, Fe levels were higher (4.6 mg/100 g) and Cu contents were similar (5.4 mg/100 g) to those obtained by Bilandžić *et al*. [2016]. No data on the minerals and trace metals studied in fresh soft tissue of *L. tuberculata, S. marginatus, M. varia*, and *F. glaber* were found in the literature. Although, Berik *et al*. [2017] in a recent paper, reported data on the mineral content of *F. glaber*, they are referred to digestive gland and adductor muscle, thus a direct comparison with data of this study is impossible. Only a few papers report data on the trace metals in *M. barbatus* and in particular regarding the Cu and Zn levels herein were much higher than levels found in bivalves from Croatian coasts (7.1 mg/100 g for Zn and 0.31 mg/100 g for Cu) by Cuculic *et al*. [2010]. Differences found in mineral levels in bivalves could be due to the different physiological state, abiotic factors (size, age, and sex of organisms), biotic factors (salinity, temperature, pH, and dissolved oxygen), genetic characteristics, chemical forms of metals, and contamination of the area [Fuentes *et al*., 2009]. Anyway, considering their WHO Daily Value recommendation [WHO, 2004], these bivalves can be good sources of minerals especially for Fe that in all products analyzed constituted more than 20% of the DV.

Fatty acids

Twenty-eight fatty acids, including C12:0 to C22:6 *n*-3, that exceeded a minimum of 0.1% of total fatty acids in a minimum of one bivalve sample, were identified (Table 2).

Fatty acid composition differed significantly among some species ($p<0.05$), although, saturated fatty acids (SAFAs) were the most predominant fatty acids in all samples, followed by polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs). The content of SAFAs ranged from 40.73% in *M. varia* to 50.51% in *F. glaber* ($p<0.05$). The main SAFAs were palmitic acid (C16:0, from 22.52% in *S. marginatus* to 30.50% of total FAs in *O. edulis*), myristic acid (C14:0, from 4.21% to 11.8% of total FAs in *O. edulis* and *L. tuberculata*, respectively), and stearic acid (C18:0, from 4.85% to 10.51% of total FAs in *L. tuberculata* and *F. glaber*, respectively). These findings are in agreement with other studies on *M. galloprovincialis*, *A. noae*, and *M. barbatus* [Azpeitia *et al*., 2016; Ezgeta-Balić *et al*., 2012].

Palmitic acid is an important component of FAs, since its desaturation and elongation lead to biosynthesis of essential FAs (C18:2 *n*-6 and C18:3 *n*-3 in a first instance and later to C20:4 *n*-6, C20:5 *n*-3, and C22:6 *n*-3) [Angioni & Addis, 2014].

MUFAs are often referred to as "good" fats because they help in reducing both total and low density lipoprotein-LDL blood cholesterol levels and protect against heart disease [Siri-Tarino *et al.,* 2015]. In the present study, MUFAs represented about a quarter of the total fatty acids, with *S. marginatus* and *A. noae* containing significantly($p<0.05$) the highest level (24.35–26.63% of total FAs) and *M. galloprovincialis* the lowest (18.00% of total FAs) ($p<0.05$). The latter result was higher than that reported by Azpeitia *et al*. [2016] for

TABLE 2. Fatty acid composition (% of total fatty acids) of eight edible bivalves Mediterranean species.

Fatty acids	*A. noae*	*F. glaber*	*L. tuberculata*	*M. varia*	*M. barbatus*	*M. galloprovincialis*	*O. edulis*	*S. marginatus*
C12:0	0.33 ± 0.05	0.47 ± 0.02	0.37 ± 0.14	0.36 ± 0.15	0.69 ± 0.25	0.41 ± 0.11	0.22 ± 0.01	1.05 ± 0.04
C14:0	7.61 ± 0.71	8.94 ± 3.25	11.79 ± 1.41	7.56 ± 1.02	9.22 ± 1.11	6.94 ± 0.18	4.21 ± 0.97	9.65 ± 0.66
C15:0	nd	2.09 ± 0.14	0.95 ± 0.11	nd	1.80 ± 0.32	1.16 ± 0.07	1.52 ± 0.35	nd
C16:0	27.21 ± 2.22	24.72 ± 0.58	23.43± 2.23	23.91 ± 1.42	24.31 ± 0.22	27.77 ± 0.68	30.50 ± 6.04	22.52 ± 1.58
C17:0	2.74 ± 0.22	2.93 ± 0.53	1.44 ± 0.10	1.94 ± 0.16	2.56 ± .,31	1.82 ± 0.18	3.01 ± 0.02	3.89 ± 0.58
C18:0	7.37 ± 0.22	10.51 ± 0.87	4.85 ± 0.13	6.98 ± 0.73	5.14 ± 0.45	5.21 ± 0.00	8.08 ± 0.31	5.26 ± 0.58
C20:0	nd	nd	nd	nd	nd	0.32 ± 0.00	nd	nd
C21:0	nd	0.85 ± 0.25	0.78 ± 0.15	nd	nd	0.73 ± 0.17	0.56 ± 0.07	1.34 ± 0.00
∑SAFA	**45.26 ± 3.30^{a,b}**	**50.51 ± 2.14^{c}**	**43.62 ± 3.19^{a,b}**	**40.75 ± 2.78^{a}**	**43.72 ± 1.26^{a,b}**	**44.36 ± 1.07^{a,b}**	**48.11 ± 5.47^{b,c}**	**43.71 ± 1.61^{a,b}**
C14:1	1.07 ± 0.16	nd	0.21 ± 0.16	nd	nd	0.29 ± 0.04	nd	nd
C15:1	nd	0.31 ± 0.09	nd	nd	nd	nd	nd	nd
C16:1	10.06 ± 0.86	8.38 ± 0.79	6.06 ± 0.72	4.94 ± 0.34	11.53 ± 0.39	7.62 ± 0.07	2.78 ± 0.53	5.70 ± 0.11
C17:1			0.97 ± 0.11	1.05 ± 0.08	2.15 ± 0.29	1.13 ± 0.06		1.23 ± 0.30
C18:1n7	3.60 ± 0.60	3.99 ± 0.14	3.01 ± 0.45	3.15 ± 0.29	4.11 ± 0.09	2.08 ± 0.06	2.54 ± 0.36	2.07 ± 0.15
C18:1n9t	nd	nd	nd	nd	1.02 ± 0.15	nd	nd	2.94 ± 0.22
C18:1n9c	7.86 ± 0.29	10.54 ± 3.43	11.73 ± 1.72	11.38 ± 0.90	4.17 ± 0.40	5.64 ± 0.12	9.70 ± 0.58	12.13 ± 0.92
C20:1n9	1.51 ± 0.15	0.74 ± 0.21	0.92 ± 0.14	0.63 ± 0.05	0.69 ± 0.01	0.77 ± 0.08	3.55 ± 0.69	1.33 ± 0.65
C22:1n9	0.38 ± 0.03	nd	nd	nd	nd	0.47 ± 0.00	0.56 ± 0.11	1.23 ± 0.18
∑MUFA	**24.35 ± 0.96^{d,e}**	**23.85 ± 3.97^{d}**	**22.90 ± 0.62^{c,d}**	**21.15 ± 1.33^{b,c}**	**23.67 ± 0.05^{c,d}**	**18.00 ± 0.17^{a}**	**19.13 ± 0.27^{a,b}**	**26.63 ± 0.65^{e}**
C18:2n6c	3.19 ± 0.08	2.49 ± 0.42	2.45 ± 0.10	2.17 ± 0.11	2.48 ± 0.08	1.50 ± 0.01	2.57 ± 0.28	2.04 ± 0.69
C18:3n6		0.79 ± 0.20	0.43 ± 0.22	0.59 ± 0.18	1.08 ± 0.01	0.45 ± 0.02	0.36 ± 0.06	0.93 ± 0.50
C18:3n3	5.36 ± 1.14	2.94 ± 0.21	4.47 ± 0.28	3.97 ± 1.02	3.72 ± 0.97	5.17 ± 1.09	6.24 ± 1.48	3.66 ± 0.60
C18:4n3	4.27 ± 0.15	2.85 ± 0.11	5.88 ± 0.10	6.57 ± 0.52	3.99 ± 0.05	4.76 ± 0.04	3.09 ± 0.07	4.78 ± 0.42
C20:2	0.23 ± 0.10	nd	nd	nd	nd	nd	nd	nd
C22:0 + 20:3n6	0.47 ± 0.15	0.80 ± 0.13	0.14 ± 0.16	nd	0.82 ± 0.01	0.39 ± 0.05	0.41 ± 0.24	1.21 ± 0.24
C20:3n3 + 22:1	0.35 ± 0.09	nd	0.15 ± 0.05	nd	0.23 ± 0.03	0.28 ± 0.01	0.37 ± 0.13	
C20:4n6	2.30 ± 0.16	2.93 ± 0.96	1.92 ± 0.36	2.22 ± 0.17	3.79 ± 0.08	2.16 ± 0.12	1.85 ± 0.45	3.51 ± 0.25
C20:5n3	8.45 ± 0.97^{c,d}	6.53 ± 2.00^{a,b}	7.11± 0.83^{a,b,c}	8.16 ± 1.02^{b,c,d}	9.27 ± 0.15^{d}	11.74 ± 1.05^{e}	7.46 ± 1.45^{a,b,c,d}	6.10 ± 0.59^{a}
C22:5n3	0.74 ± 0.04	nd	nd	nd	nd	0.61 ± 0.12	1.21 ± 0.04	1.47 ± 0.40
C22:6n3	5.96 ± 1.72^{a}	6.30 ± 2.19^{a}	10.88 ± 2.67^{b,c}	14.41 ± 4.32^{c}	7.21 ± 0.03^{a}	10.58 ± 2.05^{b}	9.20 ± 4.40^{a,b}	5.95 ± 0.70^{a}
∑ PUFA	**30.38 ± 4.25^{a,b}**	**25.63 ± 6.07^{a}**	**33.46 ± 3.81^{b,c}**	**37.86 ± 6.15^{c}**	**32.59±.1.29^{b,c}**	**37.64 ± 1.25^{c}**	**32.76 ± 5.75^{b,c}**	**29.66 ± 2.26^{a,b}**

Note: Data are the mean values of three replicates ± standard deviation. Means within the same row without a common lowercase letter differ significantly ($p<0.05$); nd= not detected. SAFA = saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

M. galloprovincialis from Bay of Biscay (Spain), but similar to that found by Fernández *et al*. [2015] for *M. edulis* collected at various locations in Ireland, in the same period of the year.

The major MUFAs were palmitoleic (C16:1) and oleic (C18:1 *n*-9) acids, which is in agreement with literature data [Bongiorno *et al*., 2015; Fuentes *et al*., 2009]. However, the relative contents of these fatty acids differed widely among all species. The oleic acid (C18:1 *n*-9) showed the highest values, with 12.13% and 11.73% of total FAs, in *S. marginatus* and *L. tuberculata,* respectively, and the lowest ones in *M. barbatus* (4.17% of total FAs) and *M. galloprovincialis* (5.64% of total FAs) (Table 2). Ezgeta-Balic *et al*. [2012] reported values of palmitoleic and oleic acids in *A. noae*, *M. galloprovincialis*, *M. barbatus*, and *O. edulis* from Adriatic Sea lower than those observed in this study period. The predominance of these MUFAs in some examined species may have two origins: exogenous from the diets or endogenous by desaturation of C16:0 and C18:0 acids, respectively [Ekin & Başhan, 2010].

Considerable variation in the relative proportion of total PUFAs was found in the Ionian bivalve species, showing the highest level in *M. varia*, *M. galloprovincialis*, *L. tuberculata*, *O. edulis*, and *M. barbatus* (32.59–37.86% of total FAs; differences between values were insignificant, $p\geq0.05$) (Table 2). Other species showed to have the PUFAs percentage greater than 30% of total FAs, such as *L. tuberculata* (33.46%), *O. edulis* (32.76%), *M. barbatus* (32.59%), and *A. noae* (30.38%).

The composition of the seafood lipid fraction has widely been studied in terms of its PUFAs and highly unsaturated fatty acids (HUFAs), in both the *n*-3 and *n*-6 family, for its implications in reducing cardiovascular diseases and inflammations [Simopoulos, 2006; Wijendran & Hayes, 2004].

DHA (C22:6 *n*-3) and EPA (C20:5 *n*-3) were the main *n*-3 FAs found in this study, accounting for over 65% of the total *n*-3 PUFAs in most of the bivalves, followed by α-linolenic (C18:3 *n*-3) and stearidonic acids (C18:4 *n*-3). However, EPA and DHA proportions significantly ($p<0.05$) differed among the bivalve species. *M. galloprovincialis* showed the highest EPA level with 11.74% of total FAs, while the highest DHA was found in *M. varia* and *L. tuberculata* (10.88–14.41% of total FAs), which is in line with literature data [Bongiorno *et al*., 2015; Dernekbasi *et al*., 2015; Prato *et al*., 2010; Radić *et al*., 2014; Telahigue *et al*., 2010]. In turn, Azpeitia *et al*. [2016] in their study with mussels (*M. galloprovincialis*) cultured in the Bay of Biscay (northern Spain) found lower levels of EPA (8.77%-8.82% of total FAs) and higher levels of DHA (18.24%-22.49%) than those determined in our study.

Among *n*-6 PUFAs, the most abundant were linoleic acid (C18:2 *n*-6) with content ranging from 1.50% (*M. galloprovincialis*) to 3.19% (*A. noae*), and arachidonic acid (C20:4 *n*-6) with contents from 1.85% (*O. edulis*) to 3.79% (*M. barbatus*) of total FAs (Table 2). These data are in accordance with those reported in literature, in the same period of the year for

M. galloprovincialis, *A. noae*, *M. barbatus, F. glaber* and *Ruditapes philippinarum* [Azpeita *et al*., 2016; Dernekbasi *et al*., 2015; Ezgeta-Balic *et al*., 2012; Fernández-Reiriz *et al*., 2017; Prato *et al*., 2010; Radić *et al*., 2014; Telahigue *et al*., 2010].

Essential fatty acids, such as C18:2 *n*-6 and C18:3 *n*-3, are of great physiological importance since they are the precursors for the two families: *n*-3 and *n*-6.

They are converted into long-chain highly unsaturated fatty acids such as arachidonic acid (C20:4 *n*-6), EPA and DHA *via* fatty acid desaturation and elongation steps. However, the conversion efficiency is low in humans, so direct uptake appears to be significantly more effective [Brenna, 2002; Burdge *et al*., 2003].

Lipids nutritional quality indices (LNQI)

Due to different effects of fatty acids on health, LNQI, with consideration to the fatty acid profile and their biological functions, were estimated.

The consumption of foods containing relatively high levels of *n*-3 PUFAs and lower amounts of *n*-6 PUFAs provides a high *n*-3/*n*-6 ratio that is favourable for human health and can been used as an index for comparing the nutritional values of shellfish, such as for fish oil [Piggott & Tucker, 1990]. On the other hand, a very high intake of *n*-6 PUFAs was recognized as undesirable [Kinsella *et al*., 1990; Simopoulos, 2006] due to their associated negative health impacts such as an increased risk of cardiovascular disease and autoimmune diseases [Simopoulos, 2006].

A high content of the nutritionally important *n*-3 PUFAs (from 18.62% to 33.12%) and low levels of total *n*-6 PUFAs (from 4.60% to 8.17%) were obtained herein. Consequently, high *n*-3/*n*-6 ratio values characterized the FAs profile of all species from Ionian Sea examined (Table 3). They ranged from 2.65 in *F. glaber* to 7.19 in *M. galloprovincialis*.

The recommended *n*-3/*n*-6 ratio differs between authors but it is always superior to 1 [Chow, 2008]. The U.K. Department of Health recommends an ideal *n*-3/*n*-6 ratio to be 4.0, at maximum [HMSO, 1994]. A ratio higher than 4.0 is of great importance in order to diminish coronary heart diseases, plasma lipid levels and cancer risks [Kinsella *et al*., 1990]. However, due to the excessive use of vegetable oil, rich in linoleic acid (18:2 *n*-6), in the human food chain and to reduced intake of seafood, this ratio is now lower, in most Western diets [Simopoulos, 2008].

In our study, *M. varia*, *L. tuberculata* and *O. edulis* had the *n*-3/*n*-6 ratio above 5, suggesting that these species could be categorized as ideal to human health consumption. Dernekbasi *et al*. [2015] reported for *M. galloprovincialis* an *n*-3/*n*-6 ratio between 1.44 (Autumn) and 2.23 (Winter), which is much lower compared to that obtained in this study (7.19). On the other hand, Azpeitia *et al*. [2016] found a similar value of 6.85 in *M. galloprovincialis*, in the same study period.

Asha *et al*. [2014] reported an *n*-3/*n*-6 ratio of 4.66 for *C. madrasensis*, which is slightly lower than that found in *O. edulis* (5.31).

Another useful key factor for the evaluation of nutritional quality of seafood is the PUFA/SAFA ratio. A minimum value of PUFA/SAFA ratio recommended is 0.45 [HMSO, 1994], which is lower than those obtained from all bivalve species studied herein. The highest PUFA/SAFA ratio was obtained for *M. varia* (0.94) followed by *M. galloprovincialis* (0.85), *L. tuberculata* (0.77) and *M. barbatus* (0.75). These values did not differ statistically between each other ($p \geq 0.05$).

SAFAs have been cited as responsible for a minor increase of HDL-cholesterol; however, such a positive effect does not prevent the harmful increase of low-density lipoprotein (LDL) cholesterol [DiNicolantonio *et al*., 2016].

Atherogenic index (AI), thrombogenicity index (TI) and hypocholesterolaemic/hypercholesterolaemic fatty acid ratio (HH) provide indications on the dietetic quality of lipids and their potential effect on the development of coronary disease [Ulbricht & Southgate, 1991]. Low values of AI and TI show the better nutritional quality of fatty acids, reducing the potential risk of coronary heart disease (CHD). Literature data reported values of AI from 0.20 to 2.37 and TI from 0.01 to 1.18 for different seafoods, including bivalves [Ghribi *et al.,* 2017; Joy & Chakraborty, 2017; Turan *et al*., 2007]. In this study, AI and TI fell within the above-mentioned range (Table 3). The values of AI≤1 were observed in *M. varia*, *O. edulis* (0.93) and *M. galloprovincialis* (1.0), while the highest one in *L. tuberculata* and *F. glaber* (1.24–1.26).

TABLE 3. Nutritional quality indexes of eight edibles bivalves species of commercial interest.

Specification	*A. noae*	*F. glaber*	*L. tuberculata*	*M. varia*	*M. barbatus*	*M. galloprovincialis*	*O. edulis*	*S. marginatus*
$\sum n$-3	$25.13 \pm 4.02^{b,c}$	18.62 ± 4.43^{d}	$28.50 \pm 3.13^{a,b}$	33.12 ± 6.28^{a}	$24.42 \pm 1.66^{b,c,d}$	33.08 ± 2.08^{a}	$27.57 \pm 7.65^{a,b,c}$	$21.96 \pm 1.51^{c,d}$
$\sum n$-6	5.96 ± 0.28^{b}	7.01 ± 1.64^{a}	4.94 ± 0.48^{b}	4.98 ± 0.12^{b}	8.17 ± 0.16^{a}	4.60 ± 0.10^{b}	5.18 ± 4.84^{b}	7.70 ± 1.69^{a}
n-3/*n*-6	4.21 ± 0.65^{c}	2.65 ± 0.06^{d}	5.77 ± 0.21^{b}	6.65 ± 1.44^{a}	2.99 ± 0.14^{d}	7.19 ± 0.28^{a}	5.31 ± 0.98^{b}	2.86 ± 0.44^{d}
PUFA/SAFA	$0.68 \pm 0.13^{b,c}$	0.51 ± 0.14^{c}	$0.77 \pm 0.15^{a,b}$	0.94 ± 0.13^{a}	$0.75 \pm 0.07^{a,b}$	$0.85 \pm 0.07^{a,b}$	$0.68 \pm 0.28^{b,c}$	$0.68 \pm 0.11^{b,c}$
UNS/SAFA	$1.21 \pm 0.15^{b,c}$	0.98 ± 0.08^{c}	$1.30 \pm 0.17^{a,b}$	1.45 ± 0.17^{a}	$1.29 \pm 0.09^{a,b}$	$1.25 \pm 0.08^{a,b}$	$1.10 \pm 0.34^{b,c}$	$1.29 \pm 0.12^{a,b}$
AI	$1.06 \pm 0.16^{a,b}$	$1.24 \pm 0.33^{a,b}$	1.26 ± 0.20^{a}	0.93 ± 0.12^{b}	$1.10 \pm 0.08^{a,b}$	$1.00 \pm 0.04^{a,b}$	0.93 ± 0.23^{b}	$1.10 \pm 0.08^{a,b}$
TI	0.46 ± 0.09^{b}	0.61 ± 0.15^{a}	0.38 ± 0.07^{b}	0.33 ± 0.06^{b}	0.42 ± 0.02^{b}	0.34 ± 0.02^{b}	0.44 ± 0.12^{b}	0.44 ± 0.03^{b}
HH	0.98 ± 0.19^{b}	0.95 ± 0.16^{b}	$1.13 \pm 0.20^{a,b}$	1.34 ± 0.20^{a}	0.87 ± 0.06^{b}	$1.08 \pm 0.06^{a,b}$	$1.14 \pm 0.31^{a,b}$	$1.08 \pm 0.08^{a,b}$

Note: Values are mean (± SD). Means within the same row without a common lowercase letter differ significantly ($p<0.05$). SAFA = saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, UNS= unsaturated fatty acids, AI= Atherogenic Index, TI= Thrombogenicity Index and HH hypocholesterolaemic/hypercholesterolaemic fatty acid ratio.

As regards the TI value, there were no significant differences among species (0.33–0.46) except for *F. glaber* that showed a significantly higher TI value (0.61) ($p<0.05$) (Table 3).

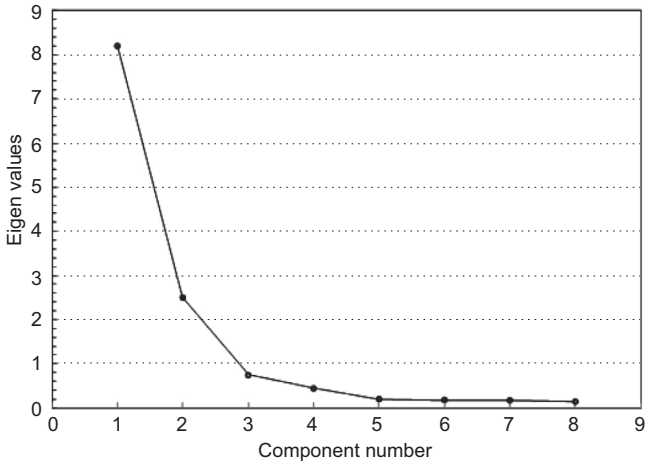


FIGURE 1. The scree plot of the eigenvalues for PCA.

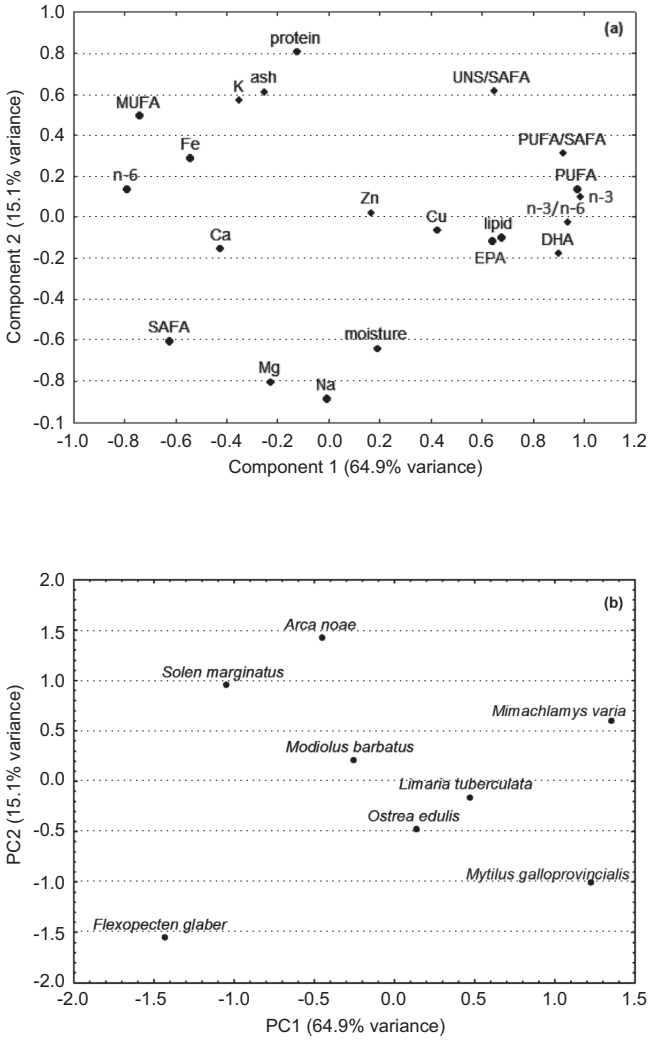


FIGURE 2. Loading of the variables on the first two principal components (a); scatter plot of different bivalve species (b).

As regards the hypocholesterolaemic/hypercholesterolaemic fatty acid ratio (HH), its higher values are desirable [Joy & Chakraborty, 2017; Ramos Filho *et al.,* 2008]. In this study, *M. varia* with 1.34 significantly differed from *A. noae*, *F. glaber* and *M. barbatus* that showed the lowest HH values (0.87–0.98) ($p<0.05$).

The similarities and differences among the nutritional parameters of bivalves species investigated were statistically assessed utilizing PCA on a data set of 8 cases (8 bivalves) and 21 variables (Na, Mg, K, Ca, Fe, Zn, Cu, ∑SAFA, ∑MUFA, ∑PUFA, *n*-3, *n*-6, *n*-3/*n*-6, UNS/SAFA, PUFA/SAFA, EPA, DHA, protein, lipid, moisture, ash).

Five principal components (PC) were extracted by covering 91% of the cumulative variance. The variance of the five principal components is 64.9%, 20.1%, 5.9%, 3.5% and 1.6%, respectively. Figure 1 reported the scree plot of the eingenvalues. In agreement to these results, the total variance explained by the two first components was 85%. Loading of variables on the first two principal components (Figure 2a) showed that moisture, Zn, Cu, EPA, lipid, UNS/SAFA, PUFA/SAFA, PUFA, *n*-3, *n*-3/*n*-6 and DHA had positive scores on the PC1, while K, Ca, Fe, Mg, Na, ash, protein, MUFA, *n*-6 and SAFA were characterized by negative scores on the same factorial component. Besides protein, K, ashes, UNS/SAFA, MUFA, Fe, PUFA/SAFA, *n*-6, PUFA, Zn and *n*-3 had positive scores on the PC2 while Na, Mg, SAFA, moisture, EPA, DHA, lipid, Ca, Cu and *n*-3/*n*-6 had negative scores on the PC2. The scatter plot of scores on the first two principal components PC1 and PC2 (Figure 2b) shows a separation among the bivalves. In fact, *M. varia, M. galloprovincialis, L. tuberculata* and *O. edulis*, had positive scores on the PC1, while *F. glaber*, *S. marginatus, A. noae* and *M. barbatus* were characterized by negative scores on the same principal component. Furthermore *A. noae, S. marginatus, M. varia* and *M. barbatus* had positive scores on the PC2 while *F. glaber*, *M. galloprovincialis, O. edulis* and *L. tuberculata* had negative scores on the PC2. PCA showed, therefore, that *F. glaber* was associated to higher Na, Mg, SAFA and Ca than other organisms; *M. galloprovincialis, O. edulis* and *L. tuberculata* were associated to higher moisture, EPA, DHA, lipid, *n*-3/*n*-6 and Cu, while *A. noae, S. marginatus* and *M. barbatus* were associated to higher protein, ash, K, MUFA, Fe and *n*-6. Lastly, *Mimachlamys varia* was associated to higher UNS/SAFA, PUFA/SAFA, PUFA, *n*-3.

CONCLUSION

The importance of the results of this study lies in the fact that, up to now, nutritional quality data are not available in the literature for half of the eight bivalves species. Therefore, it can provide information to consumers, nutritionists, food scientist and guide the farmers to promote the culture of these species.

Edible bivalves, from the Ionian coast of Italy may be considered as food item with interesting dietetic properties due to high contents of protein, Ca, K, Na, Fe, Zn, Cu, and low cholesterol content and to the interesting fatty acid composition. Both the amount of lipid (rather low) and the proportion of saturated, monounsaturated, and polyunsaturated fatty acids in bivalves contribute to a healthful diet.

One of the purposes of this study was just to obtain information on the profile of fatty acids in these bivalve species of commercial interest, widely appreciate in the markets.

The main result of the present study is the finding that many of them can satisfy the nutritional needs of consumers for valuable *n*-3 PUFAs, that represent the fraction of the seafood lipids that has the largest effect on human health.

All species showed elevated levels of *n*-3 PUFAs, especially EPA and DHA, plus a high *n*-3/*n*-6 ratio. *M. galloprovincialis* and *M. varia* showed the best values of these two FAs.

The *n*-3/*n*-6 ratio, PUFA/SAFA, HH, AI and TI, which are indicators of lipids nutritional quality in food, indicated that the consumption of these species could be beneficial to human health. Despite differences among bivalves investigated, all samples had the *n*-3/*n*-6 ratio within the recommended range and among them, *M. galloprovincialis*. *M. varia*, *L. tuberculata*, *O. edulis* had the best *n*-3/*n*-6 ratio. In terms of HH, AI and TI, *M. varia*, *O. edulis, M. galloprovincialis*, and *L. tuberculata* showed to have good potential as a nutritional food with beneficial effects for the consumer's health. Moreover, it must be emphasized that *L. tuberculata* is not very commercialized; it is considered a niche product well appreciated for its organoleptic qualities by estimators, in particular in Southern Italy. At present, no literature data of this species in the wild exist, but our results encourage further studies.

This study has shown that differences exist in fatty acid composition among specific Ionian bivalve species and this is particularly important when a nutritional evaluation of seafood is made and when recommendations of human consumption levels follow for specific health benefits. Considering the importance of exogenous (season, temperature, salinity) and endogenous factors related to the nutritional quality of bivalves, future research will extend the study period to one year.

RESEARCH FUNDING

The CNR- IRSA Water Research Institute, UOS di Taranto supported this research.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

REFERENCES

1. Angioni, A., & Addis, P. (2014). Characterization of the lipid fraction of wild sea urchin from the Sardinian Sea (western Mediterranean). *Journal of Food Science, 79*(2), C155–C162.
2. Anibal, J., Esteves, E., & Rocha, C. (2011). Seasonal variations in gross biochemical composition, percentage edibility and condition index of the clam Ruditapes decussatus cultivated in the Ria Formosa (South Portugal). *Journal of Shellfish Research, 30*(1), 17–23.
3. AOAC, Official Methods of Analysis (16th ed.) Association of Official Analytical Chemists. Washington DC USA (1995).
4. Asha, K. K., Anandan, R., Mathew, S., & Lakshmanan, P. T. (2014). Biochemical profile of oyster *Crassostrea madrasensis* and its nutritional attributes. *Egyptian Journal of Aquatic Research,, 40*(1), 35–41.
5. Azpeitia, K., Ferrer, L., Revilla, M., Pagaldai, J., & Mendiola, D. (2016). Growth, biochemical profile, and fatty acid composition of mussel (*Mytilus galloprovincialis* Lmk.) cultured in the open ocean of the Bay of Biscay (northern Spain). *Aquaculture (Amsterdam, Netherlands), 454*, 95–108.
6. Berik, N., & Çankiriligil, C. (2013). Determination of proximate composition and sensory attributes of scallop (*Flexopecten glaber*) gonads. *Marine Science and Technology Bulletin, 3*, 5–8.
7. Berik, N., Çankırılıgil, E. C., & Gül, G. (2017). Mineral content of smooth scallop (Flexopecten glaber) caught Canakkale, Turkey and evaluation in terms of food safety. *Journal of Trace Elements in Medicine and Biology, 42*, 97–102.
8. Beyersmann, D. (2002). Homeostasis and cellular functions of zinc. *Materialwissenschaft und Werkstofftechnik, 33*(12), 764–769.
9. Bilandžić, N., Sedak, M., Čalopek, B., Zrnčić, S., Oraić, D., Benić, M., & Ujević, I. (2016). Element differences and evaluation of the dietary intake from farmed oysters and mussels collected at different sites along the Croatian coast of the Adriatic Sea. *Journal of Food Composition and Analysis, 45*, 39–49.
10. Bongiorno, T., Iacumin, L., Tubaro, F., Marcuzzo, E., Sensidoni, A., & Tulli, F. (2015). Seasonal changes in technological and nutritional quality *of Mytilus galloprovincialis* from suspended culture in the Gulf of Trieste (North Adriatic Sea). *Food Chemistry, 173*, 355–362.
11. Brenna, J. T. (2002). Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Current Opinion in Clinical Nutrition and Metabolic Care, 5*(2), 127–132.
12. Burdge, G. C., Finnegan, Y. E., Minihane, A. M., Williams, C. M., & Wootton, S. A. (2003). Effect of altered dietary n-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [13C]alpha-linolenic acid to longer-chain fatty acids and partitioning towards beta-oxidation in older men. *British Journal of Nutrition, 90*(2), 311–321.
13. Caers, M., Coutteau, P., & Sorgeloos, P. (2000). Impact of starvation and of feeding algal and artificial diets on the lipid content and composition of juvenile oysters (*Crassostrea gigas*) and clams (Tapes philippinarum). *Marine Biology, 136*(5), 891–899.
14. Çelik, M. Y., Karayücel, S., Karayücel, I., Öztürk, R., & Eyüboglu, B. (2012). Meat yield, condition index, and biochemical composition of mussels (Mytilus galloprovincialis Lamarck, 1819) in Sinop, South of the Black Sea. *Journal of Aquatic Food Product Technology, 21*(3), 198–205.
15. Certified Reference Material 278R, Community Bureau of Reference (BCR), (Trace elements in mussel tissue) produced by Joint Research Centre, Geel (Belgium).
16. Chow, C. K. (2008). *Fatty Acids in Foods and their Health Implications* (3th ed.) CRC Press Inc., Boca Raton, Florida: C.K. Chow, p. 1296
17. Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Ben Rais Lasram, F., Aguzzi, J., & Voultsiadou, E. (2010). The biodiversity of the Mediterranean Sea: Estimates, patterns, and threats. *PLoS One, 5*(8), e11842.
18. Cuculic, V., Cukrov, N., Kwokal, Z., & Mlakar, M. (2010). Trace metals in bivalves' soft tissue from Mljet National Park Aquatorium, Croatia. *Rapports et procés-verbaux des réunions Commission internationale pour l'exploration scientifique de la Mer Méditerranée., 39*, 237.
19. Dernekbasi, S., Öksüz, A., Çelik, M. Y., Karayücel, I., & Karayücel, S. (2015). The Fatty acid composition of cultured mussels

(*Mytilus galloprovincialis* Lamarck 1819) in offshore longline system in the Black Sea. *Journal of Aquaculture and Marine Biology, 2*(6), 00049.
20. DiNicolantonio, J.J., Lucan, S.C., & O'Keefe, J.H. (2016). The evidence for saturated fat and for sugar related to coronary heart disease. *Progress in Cardiovascular Diseases, 58*(5), 464–472.
21. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric methods for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356.
22. Ekin, I., & Bashan, M. (2010). Fatty acid composition of selected tissues of Unio elongatulus (Bourguignat, 1860) (Mollusca: Bivalvia) collected from Tigris River, Turkey. *Turkish Journal of Fisheries and Aquatic Sciences, 10*(4), 445–451.
23. Ezgeta-Balic, D., Najdek, M., Peharda, M., & Blažina, M. (2012). Seasonal fatty acid profile analysis to trace origin of food sources of four commercially important bivalves. *Aquaculture (Amsterdam, Netherlands), 334–337*, 89–100.
24. FAO (2016). The state of World Fishery and Aquaculture. Contributing to food security and nutrition for all. Rome, p. 200.
25. Fernández, A., Grienke, U., Soler-Vila, A., Guihéneuf, F., Stengel, D.B., & Tasdemir, D. (2015). Seasonal and geographical variations in the biochemical composition of the blue mussel (*Mytilus edulis* L.) from Ireland. *Food Chemistry, 177*, 43–52.
26. Fernández-Reiriz, M. J., Irisarri, J., & Labart, U. (2017). Fatty acid composition of female and male clams (*Ruditapes philippinarum*): Energy intake and temperature reliance. *Aquaculture Nutrition, 23*(1), 119–128.
27. Folch, J., Lees, M., & Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry, 226*(1), 497–509.
28. Fuentes, A., Fernández-Segovia, I., Escriche, I., & Serra, J.A. (2009). Comparison of physico-chemical parameters and composition of mussels (*Mytilus galloprovincialis* Lmk.) from different Spanish origins. *Food Chemistry, 112*(2), 295–302.
29. Ghribi, F., Bejaoui, S., Rabeh, I., Aouini, F., Chetoui, I., & El Cafsi, M. (2017). Effects of culinary methods on nutritional characteristics of the edible shellfish Noah 's Ark (*Arca noae* L., 1758) from Tunisian Coasts. *Journal of Aquatic Food Product Technology, 26*(10), 1324–1336.
30. Harris, W. S. (2010). Marine omega-3 Fatty acids and plaque stabilization. *Current Atherosclerosis Reports, 12*(6), 357–358.
31. Hasler, C. M. (2002). Functional foods: Benefits, concerns and challenges-a position paper from the american council on science and health. *The Journal of Nutrition, 132*(12), 3772–3781.
32. HMSO UK (1994). Nutritional aspects of cardiovascular disease: report of the Cardiovascular Review Group, Committee on Medical Aspects of Food Policy. Series: Report on health and social subjects, Department of Health, No. 46, London: HMSO, p. 186.
33. Joy, M., & Chakraborty, K. (2017). Nutritional qualities of the low-value bivalve mollusks *Paphia malabarica* and *Villorita cyprinoides* at the estuarine waters of the Southwestern coast of India. *Journal of Aquatic Food Product Technology, 26*(1), 54–70.
34. Kinsella, J. E., Lokesh, B., & Stone, R. A. (1990). Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: Possible mechanisms. *The American Journal of Clinical Nutrition, 52*(1), 1–28.
35. Kohli, P., & Levy, B. D. (2009). Resolvins and protectins: Mediating solutions to inflammation. *British Journal of Pharmacology, 158*(4), 960–971.
36. Kris-Etherton, P. M., Harris, W. S., & Appel, L. J., & the AHA Nutrition Committee. American Heart Association. (2003). Omega-3 fatty acids and cardiovascular disease: New recommendations from the American Heart Association. *Arteriosclerosis, Thrombosis, and Vascular Biology, 23*(2), 151–152.
37. Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et Biophysica Acta, 1666*(1–2), 142–157.
38. Massaro, M., Scoditti, E., Carluccio, M. A., & De Caterina, R. (2010). Nutraceuticals and prevention of atherosclerosis: Focus on omega-3 polyunsaturated fatty acids and Mediterranean diet polyphenols. *Cardiovascular Therapeutics, 28*(4), e13–e19.
39. Okumus, I., & Stirling, H. P. (1998). Seasonal variations in the weight, condition index and biochemical composition of mussels (*Mytilus edulis* L.) in suspended culture in two Scottish sea lochs. *Aquaculture (Amsterdam, Netherlands), 159*(3–4), 249–261.
40. Orban, E., Di Lena, G., Nevigato, T., Casini, I., Caproni, R., Santaroni, G., & Giulini, G. (2007). Nutritional and commercial quality of the striped venus clam, Chamelea gallina, from the Adriatic sea. *Food Chemistry, 101*(3), 1063–1070.
41. Periyasamy, N., Srinivasan, M., Devanathan, K., & Balakrishnan, S. (2007). Nutritional value of gastropod *Babylonia spirata* (Linnaeus, 1758) from Thazhanguda, Southeast coast of India. *Asian Pacific Journal of Tropical Biomedicine, 1*(2), 249–252.
42. Piggott, G. M., & Tucker, B. W. (1990). *Food from the sea. in: Seafood: Effects of Technology on Nutrition,* Marcel Dekker, Inc., New York, USA, (eds. G.M. Piggot, B.W. Tucker). pp. 1–30.
43. Prato, E., Chiantore, M., Kelly, M. S., Hughes, A. D., James, P., Ferranti, M. P., & Fanelli, G. (2018). Effect of formulated diets on the proximate composition and fatty acid profiles of sea urchin *Paracentrotus lividus* gonad. *Aquaculture International, 26*(1), 185–202.
44. Prato, E., Danieli, A., Maffia, M., & Biandolino, F. (2010). Lipid and fatty acid compositions of *Mytilus galloprovincialis* cultured in the Mar Grande of Taranto (Southern Italy): Feeding strategies and trophic relationships. *Zoological Studies (Taipei, Taiwan), 49*(2), 211–219.
45. Radić, I. D., Carić, M., Najdek, M., Jasprica, N., Bolotin, J., Peharda, M., & Bratoš Cetinić, A. (2014). Biochemical and fatty acid composition of *Arca noae* (Bivalvia: Arcidae) from the Mali Ston Bay, Adriatic Sea. *Mediterranean Marine Science, 15*(3), 520–531.
46. Ramos Filho, M. M., Ramos, M. I. L., Hiane, P. A., & Souza, E. M. T. (2008). Lipid profile of four fish species from the Pantanal region, Mato Grosso. *Food Science and Technology (Campinas), 28*, 361–365.
47. Rink, L., & Gabriel, P. (2000). Zinc and the immune system. *The Proceedings of the Nutrition Society, 59*(4), 541–552.
48. Santos-Silva, J., Bessa, R. J. B., & Santos-Silva, F. (2002). Effects of genotype, feeding system and slaughter weight on the quality of light lambs. II. Fatty acid composition of meat. *Livestock Production Science, 77*, 187–194.
49. Sidhu, K. S. (2003). Health benefits and potential risks related to consumption of fish or fish oil. *Regulatory Toxicology and Pharmacology, 38,* 336–344.
50. Simopoulos, A. P. (2006). Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implica-

tions for chronic diseases. *Biomedicine. Pharmacotherapy, 60,* 502–507.
51. Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine, 233*, 674–688.
52. Siri-Tarino, P. W., Chiu S., Bergeron N., & Krauss R. M. (2015). Saturated fats versus polyunsaturated fats versus carbohydrates for cardiovascular disease prevention and treatment. *Annual Review of Nutrition, 35,* 517–543.
53. Telahigue, K., Chetoui, I., Rabeh, I., Romdhane, M. S., & El Cafsi, M. (2010). Comparative fatty acid profiles in edible parts of wild scallops from the Tunisian Coast. *Food Chemistry, 122*(3), 744–746.
54. Turan, H., Sonmez, G., & Kaya, Y. (2007). Fatty acid profile and proximate composition of the thornback ray (*Raja clavata*, L. 1758) from the Sinop coast in the Black Sea. *Journal of FisheriesSciences.com, 1,* 97–103.
55. Ulbricht, T. L., & Southgate, D. A. T. (1991). Coronary Heart Disease: seven dietary factors. *Lancet, 338,* 985–992.
56. US EPA, Method 3052 (1996). Microwave assisted acid digestion of siliceous and organically based matrices, in: *Test Methods for Evaluating Solid Waste*, 3rd Edition, 3rd Update, US Environmental Protection Agency, Washington DC.
57. WHO (World Health Organization) (2004). Vitamin and mineral requirements in human nutrition: report of a joint FAO/WHO expert consultation, 2nd ed., p. 341.
58. WHO (World Health Organization) (2007). Protein and amino acid requirements in human nutrition: Report of a joint FAO/WHO/UNU Expert Consultation. (WHO technical report series; no. 935), p. 265.
59. Wijendran, V., & Hayes, K. C. (2004). Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annual Review of Nutrition, 24,* 597–615.
60. Yildirim, P., & Ercan, E. (2016). Determination of seasonal differences in the composition of protein and lipids of *Mytilus galloprovincialis* (Lamarck, 1819) in Gulluk Gulf, Turkey. *Scientific Works of UFT, 63*(1), 24–29.
61. Yildiz, H., Berber, S., Acarli, S., & Vural, P. (2011). Seasonal variation in the condition index, meat yield and biochemical composition of the flat oyster *Ostrea edulis* (Linnaeus, 1758) from the Dardanelles, Turkey. *Italian Journal of Animal Science, 10*(1), 22–26.
62. Yildiz, H., Palaz, M., & Bulut, M. (2006). Condition indices of Mediterranean mussels (*Mytilus galloprovincialis* L. 1819) growing on suspended ropes in Dardanelles. *Journal of Food Technology, 4*(3), 221–224.
63. Zhukova, N. V. (2014). Lipids and fatty acids of Nudibranch Mollusks: Potential sources of bioactive compounds. *Marine Drugs, 12*(8), 4578–4592.

Submitted: 6 November 2017. Revised: 4 April and 11 June 2018. Accepted: 18 July 2018. Published on-line: 22 January 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 83–99
DOI: 10.31883/pjfns-2019-0008
http://journal.pan.olsztyn.pl

Review article
Section: Nutritional Research

Importance of Cheese Whey Processing: Supplements for Sports Activities – a Review

*Patricia Fassina[1,2], Gabriela Quadros Nunes[1,2], Fernanda Scherer Adami[2], Márcia Inês Goettert[3], Claucia Fernanda Volken de Souza[1]**

[1]Laboratory of Food Biotechnology, Postgraduate Program in Biotechnology, University of Vale do Taquari – Univates, Av. Avelino Tallini, 171, ZC 95914–014, Lajeado, RS, Brazil
[2]Center of Biological and Health Sciences, Nutrition Course, University of Vale do Taquari – Univates, Lajeado, RS, Brazil
[3]Cell Culture Laboratory, Postgraduate Program in Biotechnology, University of Vale do Taquari – Univates, Lajeado, RS, Brazil

Key words: dietary supplements, athletes, branched-chain amino acids, exercise

Whey protein (WP) is a highly nutritious, commercially available alternative food source that is used primarily as a food supplement by athletes and physically active individuals to provide them with essential amino acids and bioactive peptides, and additional benefits have been attributed to WP consumption. In this context, the objective of this review was to explore current evidence regarding the consumption of different WP supplements in sports nutrition to elucidate their efficiency in affecting muscle hypertrophy, physical performance, response to muscle injury, weight loss, and body composition changes. Furthermore, these effects were assessed by comparing whey protein hydrolysate (WPH), whey protein concentrate (WPC), and whey protein isolate (WPI) supplementation. Supplementation with WPI or WPC was related to increased muscle protein synthesis (MPS), and WPH caused muscle hypertrophy and improved physical performance. Compared to WPC and WPI, WPH improved peak torque associated with strength training without reducing the creatine kinase (CK) and tumor necrosis factor alpha (TNF-α) levels in this type of physical activity, and the decreases in CK and lactate dehydrogenase (LDH) associated with aerobic exercise were significant. Supplementation with WPC resulted in weight loss, satiety, and improved body composition, without compromising whole-body lean mass loss. WPH was more effective than WPC and WPI regarding improved peak torque and muscle hypertrophy associated with strength training, and WPH reduced muscle damage associated with aerobic exercise *via* decreased CK levels.

LIST OF ABBREVIATIONS

ALA: α-lactoalbumin; BCAAs: branched-chain amino acids; BF: body fat; BLG: β-lactoglobulin; BMI: body mass index; CBDdiet: carbohydrate breakfast diet; CHO: carbohydrates; CK: creatine kinase; CONC: concentric exercise; DRI: dietary references intakes; EAA: essential amino acids; ECC: eccentric exercise; EIMD: exercise-induced muscle damage; FAO: Food and Agriculture Organization; IOM: Institute of Medicine; IPT: isometric peak torque; LBM: lean body mass; LDH: lactate dehydrogenase; LIP: lipids; MP: milk protein; MPS: muscle protein synthesis; NWP: native whey protein; PBdiet: protein breakfast diet; PTN: proteins; RDA: recommended dietary allowance; REE: rest energy expenditure; RNS: reactive nitrogen species; ROS: reactive oxygen species; SPI: soy protein isolate; TNF-α: tumor necrosis factor alpha; WBdiet: whey protein breakfast diet; WC: waist circumference; WP: whey protein; WPC: whey protein concentrate; WPC-L: high-lactoferrin WPC; WPH: whey protein hydrolysate; WPI: whey protein isolate.

INTRODUCTION

The search for nutrients in alternative food sources has been widely explored [Oliveira *et al*., 2012]. Accordingly, whey, a product of cheese manufacture, has a significant commercial value due to its wide availability, low production cost, and a high nutritional value [Kankanamge *et al*., 2015]. Its use in the production of fruit-flavored dairy drinks, with fruit pulps such as acerola [Cappato *et al*., 2018] and chocolate [Monteiro *et al.*, 2018] has increased, constituting an alternative to yogurt. It has also increased in the processing of dairy foods such as whey-grape juice [Amaral *et al.*, 2018] and even in probiotic dairy drink formulations, added with inulin [Guimarães *et al.*, 2018]. Whey accounts for approximately 20 to 30% of the total protein content of bovine milk [Devries & Phillips, 2015]; β-lactoglobulin (BLG) (45–57%) is the most abundant protein, followed by α-lactoalbumin (ALA) (15–25%), immunoglobulins (10–15%), glycomacropeptide (10–15%), bovine serum albumin (10%), lactoferrin (~ 1%), and lactoperoxidase (<1%) [Bendtsen *et al*., 2013].

BLG is composed of approximately 26% of branched-chain amino acids (BCAAs) (L-leucine, L-valine, and L-isoleucine) [Pal & Radavelli-Bagatini, 2013], which are among

* Corresponding Author: Phone: +55 51 3714 7000; Fax: +55 51 3714 7001; E-mail address: claucia@univates.br (C.F. Volken de Souza)

the nine essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and histidine) [Waitzberg & Logullo, 2006], which are essential for muscle protein synthesis (MPS) [Burd *et al*., 2012] and, therefore, for muscle hypertrophy [Aoi *et al*., 2011]. Hypertrophy, increased lean body mass [Volek *et al*., 2013], and reduced muscle fatigue [Babault *et al*., 2014] are observed when strength training is followed by BCAA supplement ingestion because BCAAs minimize lean body mass loss and promote muscle regeneration [Aoi *et al*., 2011] after injuries caused by exhaustive exercise [Burd *et al*., 2012]. However, these processes occur as long as they are combined with a positive nitrogen balance [McArdle *et al.*, 2014]; although all 20 different types of amino acids are essential to protein synthesis, the organism needs a dietary intake of the nine essential amino acids, including BCAAs. The absence or inadequate intake of any of these amino acids leads to a negative nitrogen balance, and might cause weight loss and hinder growth, which does not benefit the MPS process and muscle hypertrophy [Mahan & Escott-Stump, 1998].

Using different technologies for production [Biocatalysts, 2014], whey proteins are sold as sports supplements termed whey protein (WP) [Haraguchi *et al*., 2006]. These products are available as whey protein concentrate (WPC), isolate (WPI), and hydrolysate (WPH) varieties [Pal & Radavelli-Bagatini, 2013]. WPC contains approximately 25–89% protein, with or without lactose; WPI has approximately 90–95% protein, usually without carbohydrates; and WPH has higher peptide fractions [Marshall, 2004].

WP is a complete protein containing all the essential amino acids [Aoi *et al*., 2011] required for MPS [Stark *et al*., 2012], enabling muscle hypertrophy [Devries & Phillips, 2015] and improved strength performance [Chen *et al*., 2014]. Furthermore, essential amino acids improve body composition [Devries & Phillips, 2015], reduce immunosuppression and inflammation caused by muscle injury during physical training, and attenuate oxidative stress induced by exercise because they have antioxidant effects, and comprise a nutritional supplement also referred to as immune-nutrient [Cruzat *et al*., 2014]. In addition, other amino acids such as L-glutamine and L-arginine might also play these roles. Therefore, protein and amino acid supply is required for optimal synthesis and concentrations of immune-related proteins, including cytokines and antibodies. Amino acids will help regulate the main metabolic pathways of immune cells, from cell response to oxidative stress, and anti-inflammatory response, all of which are essential for an optimal immune function and for recovery from intense periods of physical training [Cruzat *et al*., 2014]. For athletes, WPH provides greater advantages over WPC and WPI [Biocatalysts, 2014] (Figure 1) because WP hydrolysis releases biologically active amino acids and peptides [Madureira *et al*., 2010].

However, for athletes and physically active people, the issue of whether to supplement remains controversial due to inconsistent data on WP varieties. Several factors, including dosage, mode of administration, intake time(s), duration of use, and integration into a specific physical training program, affect protein supplementation effects [Naclerio & Larumbe-Zabala, 2016].

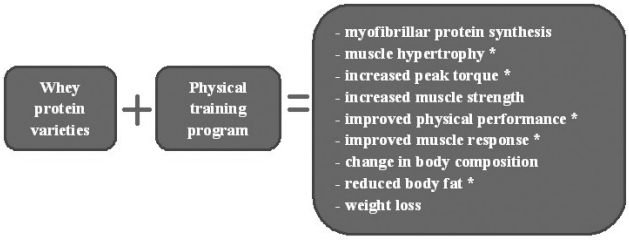


FIGURE 1. Effects of supplementation with WPC, WPI, and WPH combined with a physical activity program. *Greater advantage of WPH over WPC and WPI.

These factors highlight the need to review studies related to WP to elucidate its effects on hypertrophy, physical performance, response to muscle injury, weight loss, and change in body composition and to compare the effects of WPH, WPC, and WPI supplementation.

EFFECTS OF WHEY PROTEIN CONCENTRATE, WHEY PROTEIN ISOLATE, AND WHEY PROTEIN HYDROLYSATE CONSUMPTION ON MUSCLE HYPERTROPHY AND PHYSICAL PERFORMANCE

Muscle hypertrophy is the main objective of individuals seeking improved athletic performance. Muscle mass gain requires adequate nutrient intake and primarily requires protein [Rigon & Rossi, 2012]. The recommended dietary allowance (RDA) of protein established by the Dietary References Intakes (DRI) of the Food and Nutrition Board of the Institute of Medicine (IOM) of the United States is 0.8 g/kg weight/day [IOM, 2002/2005]. However, protein intake greater than the RDA is required for individuals to optimize their adaptation to strength training [Chen *et al*., 2014]. These individuals should maintain moderate protein consumption ranging from 1.4 to 2.0 g protein/kg body weight/day [Jäger *et al*., 2017; Roberts *et al*., 2017].

Accordingly, the high-quality proteins and essential amino acids found in WP make this supplement one of the most widely used for MPS, ensuring good physical performance [Monteyne *et al*., 2018; Morton *et al*., 2018]. Table 1 outlines the results on muscle hypertrophy and physical performance from studies of supplementation with WPC, WPI, and WPH.

Muscle hypertrophy associated with WP supplementation can improve the performance of strength athletes and athletes who perform aerobic sports, such as marathons, cycling, and swimming [Chen *et al*., 2014]. Several studies on WPH supplementation before and immediately after each workout involving different physical exercises showed muscle hypertrophy [Farup *et al*., 2014b; Rahbek *et al*., 2014; Stefanetti *et al*., 2014].

Athletes who supplement with WPH before and after each running session in a combined daily intake of 1.8 g protein/kg weight per day showed improved functional strength and physical performance [Hansen *et al*., 2015]. Conversely, supplementation with WPH or WPC immediately before and after the daily workout in a combined daily intake of 1.3 g protein/kg weight per day failed to improve physical performance, and only WPC consumption increased muscle mass

TABLE 1. Effects of supplementation with WPC, WPI, and WPH on muscle hypertrophy and physical performance.

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
Hansen *et al.* [2015]	18 elite runners (men and women) divided into 2 similar groups in number, age, height, weight, and %BF	2 groups: WPH (n=9) and CHO (n=9)	randomized, controlled intervention	0.3 g/kg weight WPH 10 minutes before and 0.3 g WPH/kg weight + 1 g CHO/kg weight 15 minutes after each workout; 1.3 g/kg weight CHO 10 minutes before and 15 minutes after each workout diluted in non-caloric iced tea, ingesting anything except water up to 2 h after each workout	with controlled diet: 15% PTN (1.8 g/kg weight), 63% CHO and 22% LIP	1 week	13 aerobic workouts with a 4-km run	WPH increased physical performance
Lollo *et al.* [2014]	24 elite soccer players (18 ± 0.8 years, 73.95 ± 4.87 kg, and 178.5 ± 0.48 cm)	3 groups: WPH (n=8), WPC (n=8), and CHO (n=8)	double-blind regarding dietary supplementation	0.5 g WPH, WPC, or CHO/kg weight, immediately before and immediately after the daily training or soccer league matches, including rest days	with controlled diet by a nutritionist: 2.3 g/kg weight PTN (1.3 g food and 1.0 g supplement), totaling 10 to 15% total energy (diet + supplement)	12 weeks	aerobic activity between training and soccer league matches	WPC increased LBM
Macnaughton *et al.* [2016]	30 men subjected to strength training allocated to 2 groups: lower LBM (≤65 kg) (21.3 ± 2.2 years, 1.78 ± 0.05 m, 76.8 ± 4.8 kg, 18.8 ± 3.7% BF) and higher LBM (≥ 70 kg) (23.2 ± 3.5 years, 1.84 ± 0.05 m, 98.8 ± 7.8 kg, 17.3 ± 4.9% BF)	2 groups: lower LBM (≤65 kg) (n=15) and higher LBM (≥ 70 kg) (n=15)	randomized, double-blind, cross-sectional	20 g WPI or 40 g WPI; diluted in 500 mL water; immediately after the workout	without controlled diet	2 weeks	2 strength training interventions on upper and lower limbs	WPI caused MPS 3 h after ingesting 40 g
Mitchell *et al.* [2017]	16 healthy, physically active men divided into 2 groups: WPC (52.6 ± 3.9 years, 26.7 ± 2.5 kg m^2) or mWPC (51.0 ± 3.5 years, 26.5 ± 3.1 kg /m^2)	2 groups: WPC (n=8) and mWPC (n=8)	randomized double-blind, parallel	20 g WPC or 20 g mWPC and containing less than 2 g LIP and approximately 2 g CHO; dissolved in 350 mL of water	without controlled diet	8 h	without physical exercise intervention	mWPC and WPC caused MPS 1.0 h after their ingestion
Atherton et al. [2010]	8 healthy, untrained but physically active men (21 ± 2 years; 22.9 ± 0.9 kg/m^2) BMI)	1 group: WPI (n=8)	controlled intervention	0.7 mg/kg weight/h leucine in continuous infusion + 48 g WPI diluted in 500 mL water, equivalent to 20 g EAA; after 2.5 h intravenous administration of leucine	without controlled diet	8.5 h	72 h after the last high-intensity strength training session (usual)	WPI caused MPS 45–90 minutes after ingesting 48 g WPI
Areta *et al.* [2013]	24 trained healthy men (minimum of 2 years of high-intensity resistance training more than 2 times per week) matched by body mass	3 groups: PULSO (n=8), INT (n=8), and BOLUS (n=8)	controlled intervention	80 g WPI over 12 h of recovery according to the following protocols: 8 × 10 g every 1.5 h (PULSE), 4 × 20 g every 3 h (INT), or 2 × 40 g in 6-h intervals (BOLUS)	with controlled diet 3 days before the experimental test: 45 kcal/kg LBM, 1.5 g PTN/kg weight and 4 g CHO/kg weight, LIP (remaining energy)	12 h	single strength training session: bilateral leg extension	PULSO, INT and BOLUS increased MPS between 1 and 12 h, but INT caused higher MPS than PULSO and BOLUS

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
West *et al.* [2011]	8 healthy, untrained but physically active men (21.5 ± 1 years, 1.81 ± 0.02 m, 80.1 ± 3.5 kg, 24.3 ± 0.8 kg /m^2 BMI)	2 groups: PULSO (n=8) and BOLUS (n=8) for both sessions	randomized, cross-sectional	25 g WPC (BOLUS) or 10 drinks of 2.5 g WPC every 20 min (PULSO); diluted in water, after the workout	without controlled diet	5 h per intervention	2 strength training interventions: bilateral leg extension; 30-day interval between interventions	BOLUS improved MPS and anabolic signaling and increased the concentration of EAA 60 min, 1 to 3 h and 3 to 5 h after the workout
Volek *et al.* [2013]	63 healthy men divided into 3 groups: WPC (22.8 ± 3.7 years, 171.8 ± 10.3 cm, 74.1 ± 15.7 kg and 25.3 ± 12.0% BF); CHO (22.3 ± 3.1 years, 172.0 ± 8.7 cm, 72.4 ± 14.9 kg and 26.4 ± 8.7% BF); and SOY (24.0 ± 2.9 years, 170.5 ± 2.9 cm, 72.0 ± 8.4 kg and 27.3 ± 11.0% BF)	3 groups: WPC (n=19), isocaloric CHO (n=22) and isonitrogenous soy protein isolate, free isoflavone (SOY) (n=22)	double-blind, prospective, parallel	1.4, 1.1, and 1.4 g/kg weight WPC, CHO, and SOY, respectively; CHO contained 0.8 g PTN, WPC 21.6 g PTN, and SOY 20.0 g PTN; diluted in 240 mL water; ingested after training and on non-training days, in the morning with breakfast	with diet controlled by a nutritionist	9 months	strength training program with 96 exercises for upper and lower muscles from 30 to 75 minutes	WPC increased LBM
Stefanetti *et al.* [2014]	24 healthy, untrained men (181.5 ± 1.5 cm; 78.1 ± 1.8 kg; 23.9 ± 0.8 years and 16 ± 0.9% BF)	2 groups: WPH (n=12) and CHO (n=12)	randomized, double-blind	0.3 g WPH + 0.3 g CHO/kg LBM, totaling 19.5 g WPH + 19.5 g CHO, or 0.6 g isocaloric CHO/kg LBM, totaling 39 g CHO, diluted in artificially flavored water; half of the solution ingested before and the other half immediately after each workout, without ingesting calories 1½ h before and 1 h after the workout	without controlled diet	12 weeks	strength training: ECC and CONC unilateral leg contraction	WPH combined with ECC and CONC increased LBM
Farup *et al.* [2014b]	22 healthy men (23.9 ± 0.8 years, 181.5 ± 1.5 cm, 78.1 ± 1.8 kg; 16.0 ± 0.9% BF)	2 groups: WPH + CHO (n=11) and CHO (placebo) (n=11), subdivided into 4 subgroups: WPH-ECC, Placebo-ECC, WPH-CONC, and Placebo-CONC	double-blinded regarding dietary supplementation	solution with 19.5 g WPH + 19.5 g CHO or 39 g CHO on workout days, ingesting half of the supplement immediately before the workout and the other half immediately after	without controlled diet	12 weeks	strength training, including ECC and CONC lower-limb strength	WPH-CONC showed greater muscle hypertrophy
Rahbek *et al.* [2014]	24 healthy men (23.9 ± 0.8 years, 1.82 ± 0.015 m, 78.1 ± 1.8 kg and 16 ± 0.9% BF)	2 groups: WPH + CHO (n=12) and CHO (n=12), subdivided into 4 subgroups: WPH + CHO-ecc, CHO-ecc, WPH + CHO-conc, and CHO-conc	double-blinded regarding dietary supplementation	500 mL drink containing 0.30 g WPH + 0.30 g CHO/kg weight, totaling 19.5 g WPH and 19.5 g CHO or 500 mL with 0.60 g CHO/ kg weight, totaling 39 g CHO on workout days, ingesting half immediately before the workout and the other half immediately after	without controlled diet	12 weeks	strength training, including ECC and CONC lower-limb strength	WPH + CHO showed higher muscle hypertrophy than CHO; the mode of muscle contraction had a weaker effect on muscle hypertrophy

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
Hamarsland et al. [2017]	22 trained men and women allocated to 2 groups: WPC or NWP (25 ± 2 years; 70.0 ± 11.6 kg; 52.9 ± 9.6 kg MM; 21.5 ± 6.4% BF) and MP (25 ± 5 years; 72.8 ± 12.4 kg; 57.1 ± 13.5 kg MM; 19.1 ± 7.2% BF)	2 groups: WPC or NWP (n = 10) and MP (n = 12)	double-blind, randomized, placebo-controlled, partial crossover trial	20 g of WPC or NWP diluted in water and 20 g of MP (20% whey and 80% casein) immediately and 2 hours after strength training	with diet guided by a nutritionist on the day before the experiment and during the rest of the trial period (2.5 days in total): 40 kcal/kg and 1.5 PTN g/kg/day. breakfast: 23 kJ, 0.11 g PTN, 0.30 g LIP and 0.58 g CHO/kg	2.5 days	lower-limb strength training	NWP increased the plasma leucine concentration in relation to WPC and MP. WPC increased MPS faster (within 1–3 hours post-workout) than NWP (within 1–5 hours post-workout). NWP increased MPS (1–5 h post-workout) more than MP.

BF: body fat; CHO: carbohydrates; PTN: protein; LIP: lipids; LBM: lean body mass; MPS: muscle protein synthesis; PULSO: supplemented group name; INT: intermediate (supplemented group name); BOLUS: supplemented group name; EAA: essential amino acids; ECC: eccentric exercise; CONC: concentric exercise; NWP: native whey protein; MP: milk protein.

in soccer players [Lollo *et al*., 2014]. However, responses such as improved resistance and physical performance seem to indicate that a higher intake of proteins (1.8 *vs* 1.3 g protein/kg weight per day) from a diet that includes several types of protein sources maximizes the beneficial effects on the skeletal muscle when combined to WPH supplementation.

The combination of strength training and post-workout protein supplementation may enhance muscle protein synthesis, particularly in young people [Nogiec & Kasif, 2013; Monteyne *et al*., 2018], as in these individuals protein supplementation after exercising might maximize MPS more easily than in older people, as the latter show more anabolic resistance of MPS to resistance exercise [Kumar *et al*., 2009]. However, a recent study by Jäger *et al*. [2017] concluded that protein intake before sleep increases overnight MPS and metabolism the next morning while improving muscle size and strength over 12 weeks of strength training, emphasizing that WP supplementation can also augment overnight MPS, during sleep, as well as after physical exercise, causing beneficial effects on the muscles of the organism [Jäger *et al*., 2017]. After ingestion, WP proteins are quickly digested and absorbed [West *et al*., 2011], thereby increasing the blood concentration of amino acids, especially leucine, resulting in rapid hyperaminoacidemia and particularly hyperleucinemia, which are crucial for the optimal stimulation of MPS, when it is not already initiating its maximal response [Devries & Phillips, 2015]. Thus, protein supplements which are quickly ingested and that contain essential amino acids, mostly leucine, are more effective in stimulating MPS in active individuals, decreasing protein degradation, and possibly, helping in the recovery after exercising, provided they are combined with food with complete protein sources containing all essential amino acids [Jäger *et al*., 2017].

The RDA of leucine has not been established, and the optimal level varies with age and level of physical activity, as well as with energy consumption by the individual [Devries & Phillips, 2015]. The post-workout intake of up to 3 g leucine in young, trained individuals [Moore *et al*., 2009; Burke *et al*., 2012] or intake combined with strength training may maximize MPS [Farup *et al*., 2014a]. However, the need for higher intake was shown by Churchward-Venne *et al*. [2014], who found that adding 5 g leucine to 6.25 g WPI stimulates MPS. Despite these results, there are studies proving that leucine supplementation associated or not to WP supplement does not exert additional effects on MPS [Grala *et al*., 2017; Teixeira *et al*., 2018]. However, studies are still required to elucidate the influence of leucine on MPS as a potential nutritional ergogenic resource [Grala *et al*., 2017].

The rapid intake of WP, the hyperaminoacidemia caused by ingesting a large quantity of the same amino acid, the duration that the amino acids remain in the plasma, and the number of doses are some of the factors that enhance MPS [Cruzat *et al*., 2014] and generate different responses after WP consumption. West *et al*. [2011] noted that the MPS rates were highest 1–5 h after the strength training session. This time period is associated with peak aminoacidemia caused by the ingestion of 25 g WPC in healthy men; the study compared this single ingestion with the intake of 2.5 g WPC every 20 min and concluded that the rapid aminoacidemia in the post-

workout period improved MPS and anabolic signaling when the protein was administered more frequently and in smaller quantities. On the other hand, Areta *et al*. [2013], who administered WPI gradually, 20 g every 3 h after strength training for a 12-h period, suggest that more frequent and smaller quantity supplementation may be an optimal intervention. These results suggest that increased WP intake, *e.g.* 20 to 25 g, in one single ingestion stimulates MPS more than repeated protein supplementation in small portions [West *et al*., 2011] and that the effect of modulating the distribution of protein intake on anabolic responses in the skeletal muscle has the potential to maximize training results, reaching the peak of muscle mass [Areta *et al*., 2013].

Furthermore, leucine consumption through WP varieties helps MPS. In a recent study by Mitchell *et al*. [2017], improved muscle synthesis was observed 1 h after consuming WPC microparticles (mWPC) or WPC, both with similar leucine concentrations, in physically active, healthy young men. Similarly, supplementation with WPI and leucine shortens MPS compared with supplementation exclusively with WPI [Macnaughton *et al*., 2016]. Atherton *et al*. [2010] observed increased MPS after 45–90 min when WPI intake was combined with the intravenous injection of 0.7 mg leucine/kg weight/h for 8.5 h.

Furthermore, native WP, produced by raw, unprocessed milk filtration, which is a production method that maintains milk proteins intact, has a higher leucine content than WPC, which is derived from whey [Hamarsland *et al*., 2017]. These authors also compared MPS in response to two 20-g doses of WPC or native WP ingested immediately and two hours after strength training and observed that native WP increased blood leucine concentrations more than WPC, although the MPS rates increased faster with WPC (within 1–3 hours post-workout), whereas native WP increased MPS rates within 1–5 hours post-workout. These results suggest that WP supplementation with leucine maximizes its effect on MPS and that leucine in connection with exercise plays a unique role in the regulation of MPS [Moberg *et al*., 2014]. Previous studies also suggest that leucine, composition of the protein, and rapidly ingested amino acids in the bloodstream are the major factors that determine MPS response [West *et al*., 2011].

According to the Food and Agriculture Organization [FAO, 2013], protein quality varies according to its amino acid content, digestibility, and bioavailability. Plant protein sources are frequently inferior in one or more essential amino acids and thus fail to meet the requirements of complete proteins, such as those contained in whey [Joy *et al*., 2013]. Therefore, WP quality is related to its high biological value and the content of essential amino acids [Aoi *et al*., 2011; Atherton *et al*., 2017]. Increased gains in lean body mass were observed in individuals who consumed daily isocaloric WPC supplements compared with those who consumed soy protein isolate and carbohydrates, thus highlighting the importance of protein quality in strength training [Volek *et al*., 2013]. This discrepancy occurs because strength training combined with WP supplementation is associated with increased lean body mass compared with other isoenergetic supplements containing carbohydrates or other sources of protein [Naclerio & Larumbe-Zabala, 2016].

However, in addition to type, the effects of protein supplementation on the concentration of blood amino acids will depend on quantity [Mitchell *et al*., 2015] and state (whether solid or liquid) required to increase the concentration of blood amino acids, *i.e.*, cause aminoacidemia [Baer *et al*., 2011], as well as absorption time and kinetics of plasma amino acids [Burd *et al*., 2012]. That is why consuming protein supplementation within 1 h after strength training is ideal for promoting hypertrophy and gains in muscle strength [Stark *et al*., 2012].

As mentioned above, protein intake regulates muscle protein synthesis in response to repeated anabolic stimuli to maintain and promote MPS, which should be considered in the development of nutritional strategies [Areta *et al*., 2013]. According to Devries & Phillips [2015], although protein intake is the strongest predictor of muscle hypertrophy after strength training, diets with a 20 to 35% higher protein content of total daily energy consumption maximize muscle hypertrophy compared with protein intake only a few hours before and/or after strength training. Furthermore, the quantity of muscle mass may also affect MPS, given the increased demand for amino acids, which may be met by exogenous sources [Macnaughton *et al*., 2016]. However, a protein intake higher than 2.0 g/kg weight/day is unlikely to provide additional gains in lean body mass [Pasiakos *et al*., 2013].

Protein supplements, essential amino acids, and leucine increase MPS rates while decreasing muscle protein degradation and possibly enhancing recovery after exercise [Jäger *et al*., 2017], although whether protein supplementation promotes hypertrophy and increases muscle strength gain remains unclear [Erskine *et al*., 2012] because studies have limitations, including small sample size, inaccurate measures of muscle size and strength, lack of control over previous training programs or regular protein intake, and issues with the study period or number of study variables [Nogiec & Kasif, 2013]. Furthermore, the individual response to strength training may vary between subjects [Erskine *et al*., 2010], and this effect may be reduced by the increased experimental control of physical activity and protein intake [Erskine *et al*., 2012]. The lack of studies measuring MPS also prevents the establishment of an RDA of protein during the muscle recovery period [Areta *et al*., 2013].

EFFECT OF SUPPLEMENTATION WITH WHEY PROTEIN CONCENTRATE, WHEY PROTEIN ISOLATE, OR WHEY PROTEIN HYDROLYSATE ON MUSCLE INJURY

The inflammation that occurs in response to muscle damage is induced and aggravated by the increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [Cruzat & Tirapegui, 2009]. Although physical training stimulates ROS production, the intracellular ROS concentration may not reach harmful levels due to the increase in and adaptations of antioxidant system responses [Cruzat *et al*., 2014]. However, inadequate physical training and a single, high-intensity training session or excessive physical training may cause changes in the redox states, oxidative stress [Petry *et al*., 2014], muscle fatigue, and muscle injury [Cruzat *et al*., 2010].

A significant release of hemoglobin-derived Fe^{2+} ions occurs during specific types of exercise, particularly those involving eccentric contractions [Welch *et al*., 2002]. Therefore, damage to erythrocyte membranes [Koury & Donangelo, 2003] may enhance oxidative stress, leading to fatigue and muscle damage [Niess & Simon, 2007] and thereby impairing athletic performance [Koury & Donangelo, 2003]. Moreover, ROS and RNS production may induce an increase in intracellular Ca^{2+} concentrations and inactivate several enzymes involved in anaerobic and aerobic metabolism, leading to muscle fatigue [Duhamel *et al*., 2005].

Muscle injury, neutrophil infiltration, and ROS generation are directly related to the initiation of the inflammatory response and depend on the intensity, type, and duration of physical exercise [Niess & Simon, 2007; Petry *et al*., 2014]. Furthermore, during the recovery period, immunity may become compromised, thereby contributing to chronic inflammation [Cruzat *et al*., 2014] through considerable changes in the immune function.

The effects of exercise on the immune system are mediated by changes in the production of hormones, such as adrenaline, cortisol, and growth hormone, and pro- and anti-inflammatory cytokines [Cannon, 2000]. The increase in circulating proinflammatory cytokines induces edema and pain and worsens inflammation until this increase is reversed and recovery from muscle injury is established [Cruzat & Tirapegui, 2009].

Among the pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) stands out [Rohde *et al*., 1997]. TNF-α induces inflammatory responses involving the release of cytokines that persist for up to 5 days, resulting in the pronounced accumulation of neutrophils and macrophages in the damaged tissue, where these cytokines perform key functions for damaged tissue repair [Tsivitse *et al*., 2003]. Circulating TNF-α levels increase one- to two-fold after strenuous and prolonged training [Ostrowski *et al*., 1999], and their pro-inflammatory effect decreases during moderate exercise due to the increase in anti-inflammatory cytokines [Cruzat *et al*., 2014], as the effects of exercise-induced cytokines depend on the type of mediator involved and on the balance between pro-inflammatory cytokines (IL-1, TNF-α, IFNα, IFNγ, TNF-β, IL-2, IL-12 e MCP-1) and anti-inflammatory cytokines (IL-4, IL-10, IL-13, IL-12p40, IL-lra). While performing moderate exercises, the production of pro-inflammatory cytokines is negatively regulated and that of anti-inflammatory cytokines is positively regulated [Rohde *et al*., 1997]. During strenuous and prolonged exercises, there is an increase in the levels of circulating pro-inflammatory cytokines, such as TNF-α, counterbalanced by the production of the anti-inflammatory cytokine IL-10 [Ostrowski *et al*., 1999]. Therefore, plasma IL-1 and TNF-α concentrations increase one- to two-fold, while IL-6 might increase over 100 times in the skeletal muscle after the practice of prolonged physical exercises, causing IL-6 to stimulate the circulation of anti-inflammatory cytokines IL-1ra and IL-10, thus inhibiting the production of the pro-inflammatory cytokine TNF-α [Ostrowski *et al*., 1999].

Intense and prolonged physical training [Brancaccio *et al*., 2008] also increases the serum levels of creatine kinase (CK) [Brancaccio *et al*., 2007] due to cell membrane rupture [Wallace, 2011], which damages the sarcomere of skeletal muscle cells [Brancaccio *et al*., 2007]. The CK levels tend to peak 16–24 h after the workout and remain high for approximately 72 h [Uchida *et al.*, 2009]. Increased lactate dehydrogenase (LDH) activity, indicative of muscle cell injury, is also seen when mechanical tension is applied to muscle fibers during exercises due to the increased sarcolemma permeability, which releases LDH into the bloodstream and increases its concentration [Van De Vyver & Myburgh, 2012].

Nutritional supplements that contain proteins and amino acids, such as WP, may contribute to reduced immunosuppression and excessive inflammation [Cruzat *et al*., 2014], accelerating muscle function recovery after strength training, although the evidence for this assumption remains unclear [Kim *et al*., 2017]. A systematic review made by Davies *et al*. [2018] demonstrated a mild-to-moderate ergogenic effect of WP use on the acceleration of muscle function recovery after strength training. Table 2 outlines the effects of supplementation with WPC, WPI, or WPH on the response to muscle injury induced by strenuous physical training.

The repair of damaged muscle tissue can be shown by assessing the activity levels of LDH [Brancaccio *et al*., 2008] and CK because they are indicators of muscle injury [Soares *et al*., 2012]. Significant decreases in CK and LDH were observed by Lollo *et al*. [2014] when WPH was provided to soccer players before and after aerobic training. The study by Brown *et al*. [2018] found that in physically active women, WPH supplementation immediately and 2 h after muscle damage induced by repeated sprint exercises also promoted greater reductions in CK. Similarly, WPH intake before and after each aerobic workout, as proposed by Hansen *et al*. [2015], attenuated the increase in CK, thus indicating that protein supplementation facilitated recovery from strenuous training among elite runners, despite the increase in LDH and the failure to alter TNF-α levels.

The reductions in muscle damage and acceleration of recovery from muscle injury caused by strenuous physical exercise may be related to the use of WPH supplementation [Brown *et al*., 2018]. These findings are in line with the study by Lollo *et al*. [2014], in which supplementation with WPC immediately before and after daily training sessions or soccer league matches showed a trend toward increased CK and LDH levels, and with the study by Hamarsland *et al*. [2017], in which CK levels increased upon intake of two 20-g doses of WPC-80, native WP or milk protein (20% whey and 80% casein), both immediately and two hours after strength training. Furthermore, other authors found no improvement in muscle damage markers (CK and TNF-α) or muscle pain in individuals supplemented with a 0.4 g/kg mixture of WPC with WPI 30 min before and after exercise, both with the short-term diet with high protein content (2.9 g protein/kg/day) and with diet with moderate protein content (1.8 g protein/kg/day), after repeated days of intensive training [Roberts *et al*., 2017].

Supplementation with WPH decreased the muscle damage indicators CK and LDH but failed to improve physical performance, increase muscle mass, or decrease body fat compared with supplementation with WPC among soccer players undergoing aerobic training [Lollo *et al*., 2014].

TABLE 2. Effects of supplementation with WPC, WPI, and WPH on response to muscle injury caused by strenuous physical training.

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
Buckley *et al.* [2010]	28 sedentary men (18 and 30 year)	WPH (n= 6), WPI (n=11), and placebo (n=11)	randomized, double-blind, parallel	25 g WPH, WPI (supplemented with 3.75 g vanilla scent + 1.25 g skim milk power) or placebo (7.5 g vanilla scent + 2.5 g skim milk powder) diluted in 250 mL flavored water at 3 different times: 2 minutes and 6 h after the ECC assessments and 2 h before the ECC assessments at 24 h	without controlled diet	24 h	single session of 100 maximum ECC knee extensor contractions, in fasting, in the morning	WPH improved IPT; WPH, WPI and placebo failed to decrease CK or TNF-α
Blacker *et al.* [2010]	10 healthy, physically active men, experienced in backpack load carriage (28 ± 9 years, 81.5 ± 10.5 kg, 1.82 ± 0.07 m; 16.4 ± 3.2% BF)	3 groups: placebo (n=10), CHO (n=10) and WPI + WPH (n=10)	Three-way randomized, cross-sectional	500 mL (490 mL water + 10 mL sugar-free orange flavor) for placebo; 500 mL (34 g CHO) (490 mL water + 10 mL sugar-free orange flavor) for CHO; 500 mL water + 44 g (WPI + WPH) (3 g CHO; 36 g PTN; 3 g LIP) orange flavor; 250 mL at the beginning and 250 mL after a 60-min walk	without controlled diet	5.9 ± 4.1 weeks	Mixed exercise program (aerobic + strength training): 3 treadmill walking tests (2 h at 6.5 km/h), carrying a 25-kg backpack; 2-week recovery period between each test	WPI + WPH recovered the isometric strength within 48 h and placebo within 72 h; placebo, CHO, and WPI + WPH increased IPT similarly
Lollo *et al.* [2014]	24 elite soccer players (18 ± 0.8 years, 73.95 ± 4.87 kg and 178.5 ± 0.48 cm)	3 groups: WPH (n=8), WPC (n=8) and CHO (n=8)	double-blinded regarding dietary supplementation	0.5 g WPH, WPC, or CHO/ kg weight, immediately before and immediate after the daily workout or soccer league matches, including rest days	with diet controlled by a nutritionist: 2.3 g/kg weight PTN (1.3 g food and 1.0 g supplement), totaling 10 to 15% total energy (diet + supplement)	12 weeks	aerobic activity between training and soccer league matches	WPH decreased CK and LDH; WPC increased CK and LDH, albeit nonsignificantly
Hansen *et al.* [2015]	18 elite runners (men and women) divided into 2 number-, age-, height-, weight-, and %BF-matched groups	2 groups: WPH (n=9) and CHO (n=9)	randomized, controlled intervention	0.3 g/kg weight WPH 10 minutes before and 0.3 g after WPH/ kg weight + 1 g CHO/kg weight 15 minutes after each workout; 1.3 g/kg weight CHO 10 minutes before and 15 minutes after each workout diluted in non-caloric iced tea, ingesting no food until 2 h after each workout, except water	with controlled diet: 15% PTN, 63% CHO and 22% LIP	1 week	13 aerobic workouts with a 4-km run	WPH reduced the increase in CK; WPH and CHO increased LDH; WPH and CHO caused no changes in TNF-α levels
Burnley *et al.* [2010]	21 healthy, untrained, young men (23 ± 2 years, 79.2 ± 10.1 kg, 179.6 ± 5.4 cm, 24.6 ± 3.0 BMI, 16.2 ± 5.0 BF)	3 groups: WPI (n=21), CHO (n=21) and placebo (n=21)	double-blind, cross-sectional	0.4 g/kg WPI with cherry flavoring, 0.4 g/kg sugar (CHO) with cherry flavoring or 0.0485 g/ kg weight artificial sweetener with cherry flavoring (placebo) dissolved in 240 mL water, immediately after training; without ingesting calories after 45 min	without controlled diet	3 days	10 sets of 10 repetitions of ECC knee extensions every day	WPI and CHO failed to decrease CK

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
Lockwood *et al.* [2017]	56 trained men (21.4 ± 0.4 years, 179 ± 1 cm, 79.5 ± 1 kg, 18.9% ± 0.7 BF)	4 groups: CHO (n=15), WPC (n=13), WPC-L (n=15) and WPH (n=13)	double-blind	30 g CHO, 30 g 80% WPC, 30 g WPC containing high lactoferrin (WPC-L) or 30 g WPH; diluted in 500 mL water; immediately before and after training and between mean on non-training days	without controlled diet	8 weeks	upper- and lower-limb strength training	WPH increased BF loss but not CK
Brown *et al.* [2018]	20 physically active women (20 ± 1 years, 165.9 ± 5.6 cm, 61.8 ± 7.9 kg)	2 groups: WPH (n=10) and CHO (n=10)	randomized, double-blind	2 doses of 20 g WPH or 20 g CHO per day: on exercise days immediately and 2 hours after exercise; two days after exercise in the morning, 30 to 60 minutes prior to laboratory visits, and before the evening meal; on the third day in the morning, fasting	with controlled diet: 5 to 7 g / kg of weight CHO and 1.2 to 1.7 g / kg of weight PTN, in all the experimental periods; standardized meal offer 24 h before the initial test and fasting for ≥10 h, except for water, which was consumed ad libitum	4 days	repeated sprint exercise	WPH promoted greater reduction of CK 48 h after EIMD
Hamarsland *et al.* [2017]	22 trained men and women allocated to 2 groups: WPC or NWP (25 ± 2 years; 70.0 ± 11.6 kg; 52.9 ± 9.6 kg MM; 21.5 ± 6.4% BF) and MP (25 ± 5 years; 72.8 ± 12.4 kg; 57.1 ± 13.5 kg MM; 19.1 ± 7.2% BF)	2 groups: WPC or NWP (n = 10) and MP (n = 12)	double-blind, randomized, placebo-controlled, partial crossover trial	20 g of WPC or NWP diluted in water and 20 g of MP (20% whey and 80% casein) immediately and 2 h after strength training	Diet guided by a nutritionist on the day before and during the trial period (2.5 days in total): 40 kcal/kg and 1.5 PTN g/ kg/day. breakfast: 23 kJ, 0.11 g PTN, 0.30 g LIP and 0.58 g CHO/ kg	2.5 days	lower limb strength training	WPC or NWP and MP increased the CK levels at 180 min, 300 min and 24 h, without significant differences
Roberts *et al.* [2017]	14 trained men and women (31 ± 6 years; 1.71 ± 0.12 m; 78.45 ± 24.72 kg; 64.32 ± 18.42 MM; 17.47 ± 3.99% BF)	2 groups: PROMOD (1.8 g PTN/kg/day) and PROHIGH (2.9 g PTN/kg/day)	randomized crossover trial	0.4 g/kg of a mixture of WPC + WPI diluted in water 30 min before and after exercise sessions	With controlled diet: PROMOD (1.8 g PTN/ kg/day) and PROHIGH (2.9 g PTN/ kg/day)	10 days	strength training	PROMOD and PROHIGH with WPI increased the pre- and post-workout CK levels with no significant differences in TNF-α or muscle pain

ECC: eccentric exercise; IPT: isometric peak torque; BF: body fat; CHO: carbohydrates; PTN: protein; LIP: lipids; BMI: body mass index; CK: creatine kinase; LDH: lactate dehydrogenase; TNF-α: tumor necrosis factor; EIMD: exercise-induced muscle damage; NWP: native whey protein; MP: milk protein.

When comparing WPH and WPI intake, Buckley *et al*. [2010] noted that WPH intake accelerated the recovery of strength capacity and muscle regeneration after a single session of eccentric exercise, although no significant differences in the serum activity of CK or in the plasma levels of TNF-α were found. This rapid recovery of strength capacity and muscle regeneration during bodybuilding exercises results from the increased rate of MPS and increased strength gains induced by WP compared with other dietary proteins because WP proteins in WPH are rapidly digested and absorbed due to the hydrolysis process [Burd *et al*., 2012].

In contrast, Burnley *et al*. [2010] determined that WPI supplementation immediately after moderate eccentric exercise had no significant effect on muscle recovery in young men, most likely because the muscle injury was light and the supplement dose was low. The authors concluded that a small quantity of protein supplementation might be insufficient to affect muscle injury recovery. The TNF-α and CK levels also remained unchanged in the study by Buckley *et al*. [2010], which may be explained by the short study period because serum CK activity can take up to 48 h to increase. Additionally, the absence of any effect on the plasma levels of TNF-α after maximal eccentric contractions may have resulted from the short duration of the exercise protocol used.

Strength generation capacity and accelerated recovery from muscle injury were observed 6 h after exercise when supplementation with WPH was performed 2 min after exercise, and the same improvements were also observed at 24 h after 2 additional administrations at 6 h and 22 h in experiments involving the induction of fatigue and injury in sedentary men [Buckley *et al*., 2010]. Isometric strength, as assessed by treadmill walking with a 25-kg load on the back, was recovered within 48 h when a WPI + WPH combination was administered. Protein supplementation favored the maintenance of an anabolic environment, which may have improved the repair of structural muscle proteins damaged during prolonged exercise with load, leading to the recovery of isometric muscle function [Blacker *et al*., 2010].

Therefore, for athletes, WPH has greater advantages than WPC or WPI [Biocatalysts, 2014] because WPH is digested and absorbed more rapidly [Burd *et al*., 2012] and shortens the recovery from muscle injury caused by strenuous physical exercise from days to hours [Biocatalysts, 2014]. It is likely that an increase in the supply of amino acids through WPH supplementation is responsible for accelerating repair of damaged skeletal muscle and its capacity to generate force [Brown *et al.*, 2018].

EFFECT OF SUPPLEMENTATION WITH WHEY PROTEIN CONCENTRATE, WHEY PROTEIN ISOLATE, OR WHEY PROTEIN HYDROLYSATE ON WEIGHT LOSS AND CHANGE IN BODY COMPOSITION

Protein intake has been related to satiety and reduced energy intake compared with other macronutrients [Monteyne *et al*., 2018]. However, this satiety effect is dependent on the protein source [Chungchunlam *et al*., 2017]. WP supplements may be a good choice for a weight loss diet due to the presence of proteins BLG and ALA [Bendtsen *et al*., 2013]. ALA is considered to be easily and quickly ingested, having the highest content of tryptophan (6%) among all sources of food proteins [Markus *et al*., 2002], thus being considered a source of tryptophan, a precursor of serotonin, which is known to suppress food intake and satiety [Halford *et al*., 2011]. This makes the intake of ALA-enriched proteins, such as WP, a precursor of increased levels of tryptophan and serotonin in the brain [Markus *et al*., 2002].

Weight loss and increased satiety were observed upon consumption of an energetic breakfast with a high-protein content from WPC relative to other sources of protein (eggs, tuna and soy) [Jakubowicz *et al*., 2017]. The results also showed that the satiating effect of WPI was similar to that of BLG or ALA due to rapid protein digestion and absorption, high concentrations of amino acids in the bloodstream [Bendtsen *et al*., 2013] and the release of gastrointestinal hormones related to satiety, including cholecystokinin, glucagon-like peptide-1, and peptide tyrosine [Jakubowicz & Froy, 2013]. A rapid whey protein intake is more satiating than that of intact proteins, such as casein, slowly digested in the short term and quickly digested in the long term, which might be partially explained by the difference in blood amino acid rates and postprandial secretion of gastrointestinal hormones [Bendtsen *et al*., 2013].

In addition to the satiating effect and weight loss, WPH may help modulate weight and reduce body fat [Bendtsen *et al*., 2013]. Supplementation with WPH immediately before and after strength training and between meals on non-training days reduced body fat to a greater extent than supplementation with WPC (80%) or high-lactoferrin WPC (WPC-L) [Lockwood *et al*., 2017]. However, according to the authors, further research is required to elucidate in which form WPH affects adipose tissue physiology.

In addition to regular physical training, high-protein diets (Table 3) may have beneficial effects on anthropometry and body composition. Supplementation with WPC 30 minutes before lunch has beneficial effects on the appetite, caloric intake, anthropometry, and body composition of overweight and obese male workers [Tahavorgar *et al*., 2014]. Conversely, Chungchunlam *et al*. [2017] found no significant decrease in hunger, satiation, or energy intake in "*ad libitum*" meals.

The decrease in body fat mass is related to the intake of different protein varieties. For example, a study assessing the intake of a commercial WPI + WPC + WPH supplement 20 minutes before a high-intensity strength training session in trained men and women showed an increase in resting energy expenditure, which is an effective strategy to reduce body fat mass without decreasing muscle mass [Hackney *et al*., 2010]. Weight loss without affecting muscle mass occurred in young men with WPI intake 5 minutes after the strength training session when followed by a full meal 60 minutes after supplementation [Monteyne *et al*., 2018]. According to Devries & Phillips [2015], the increase in protein intake increases the gains in lean body mass during strength training and enhances high-quality weight loss.

Supplementation with WPC decreased total body and abdominal fat and increased muscle mass when administered to untrained men after strength training [Hulmi *et al*., 2015]

TABLE 3. Effects of supplementation with WPC, WPI, and WPH on weight loss and change in body composition.

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
Hackney *et al.* [2010]	8 trained participants; men (n=5; 23.0 ± 3.8 years, 178 ± 6.4 cm, 85.6 ± 11.4 kg, 12.6% ± 7.5% BF) and women (n=3, 24.0 ± 1.5 years, 162 ± 6.4 cm, 65.1 ± 7.3 kg, 26.5% ± 6.7% BF)	Designer Whey® (WPI + WPC + WPH) (n=8) and CHO (n=8)	double-blind, cross-sectional	18 g WPI + WPC + WPH, 2 g CHO and 1.5 LIP or CHO (1 g PTN, 19 g CHO and 1 g LIP); 20 min before the workout	without controlled diet	8 days	2 4-day TRP lower-limb and abdominal workouts	WPI + WPC + WPH increased REE 24 h after TRP, indicating increased BF oxidation at rest
Monteyne *et al.* [2018]	15 untrained but physically active men who included resistance exercise in their exercise routine (21 ± 1 years, 78.0 ± 11.9 kg, 1.78 ± 0.07 m)	2 groups: WPI and CHO	randomized, double-blind	0.3 g/kg weight (23.9 ± 3.6 g) WPI or 26.5 ± 3.8 g CHO (dextrose) diluted in 400 mL water; 5 min after the workout; full meal (400 g pasta, 400 g bolognese sauce, and 32 mL olive oil = 12% PTN, 69% CHO, 19% LIP) 60 min after the workout	without controlled diet, only standard breakfast (15% VET=125 mL milk and 30 g cereal)	5 days	2 lower-limb strength workouts	WPI reduced energy intake without impairing muscle hypertrophy
Tahavorgar *et al.* [2014]	45 male workers of a company; WPC (39.4 ± 6.0 years, 171.1 ± 7.4 cm, 93.9 ± 11.5 kg, 32.1 ± 3.2 kg/m^2 BMI) and SPI (38.8 ± 8.8 years, 171.8 ± 8.5 cm, 95.2 ± 12.9 kg, 32.1 ± 2.7 kg/m^2 BMI)	2 groups: WPC (n=26) or SPI (n=19)	randomized double-blind	65 g WPC or 60 g SPI with strawberry flavoring (0.2 g for WPC and 0.1 g for SPI) and sucralose, dissolved in 500 mL water; 30 minutes before lunch	without controlled diet	12 weeks	usual physical activity	WPC showed a greater decrease in appetite, energy intake, anthropometry (weight, BMI, and WC) and body composition (BF loss and LM gain) than SPI
Chungchunlam *et al.* [2017]	20 normoweight, adult women (24.2 ± 0.8 years, 22.7 ± 0.4 kg /m^2 BMI)	3 groups: WPI (n=20), BLG (n=20) or ALA (n=20)	single-blind, controlled	60 g WPI, 54 g BLG, or 55 g ALA mixed with 190 g, 196 g, and 195 g marmalade, respectively, spread in 45 g bread with 100 mL water 2 h before eating the test meal: fried rice (white rice, chopped chicken, eggs, peas, corn, carrots, chicken broth, sugar, salt, and vegetable oil) "ad libitum" within 15 minutes	with controlled diet "ad libitum"	3 days, separated by at least 3 days	without excessive physical exercise the night before the study day	WPI, BLG, and ALA failed to reduce the energy intake in the test meal "*ad libitum*" and failed to reduce the hunger, satiety, or food consumption
Hulmi *et al.* [2015]	78 untrained but physically active healthy men (34.4 ± 1.3 years, 1.80 ± 0.08 m, 83.6 ± 1.4 kg)	3 groups: WP (n=25), CHO (isocaloric (maltodextrin) (n=25) or WP + CHO (n=28)	randomized, controlled	37.5 g WPC (30 g PTN, 5 g lactose, <1 g LIP), 34.5 g (maltodextrin) CHO, or 37.5 g WPC (30 g PTN) and 34.5 g maltodextrin; after exercise; with a full meal 1 to 2 h after the workout	without controlled diet	12 weeks	strength training	WPC reduced BF and abdominal fat and increased LBM

Study	Participants	Groups	Type of study	Supplementation	Diet	Length	Type of exercise	Results
Gomes *et al.* [2017]	30 women who recovered at least 5% of the weight lost 24 months or longer after bariatric surgery (5 ± 11 years, 35.7 ± 5.2 kg/m² BMI, time elapsed since the surgery: 69 ± 23 months)	2 groups: WPC + hypocaloric diet (n=15) and hypocaloric diet (n=15)	randomized, double-blind, parallel	0.5 g/kg weight WPC + hypocaloric diet (1 g/kg weight day, 45% CHO, 20% LIP) or hypocaloric diet (1 g/kg weight day, 55% CHO, 20% LIP); WPC daily dose ingested in 3 portions: during breakfast and small meals (with fruit and milk, yogurt, or with water)	hypocaloric diet	16 weeks	usual physical activity	WPC showed increased weight and BF loss
Berryman *et al.* [2017]	63 men (25 ± 2.5 years, 178 ± 6, 83.8 ± 9.5 kg, 26.4 ± 2.1 kg/m² BMI)	3 groups: CON (n=21), MOD (n=24) or HIGH (n=18)	randomized, double-blind	CON (day: 64 g CHO, 1 g PTN; night: 113 g CHO, 6 g LIP, 5 g PTN, 0.9 g EAA, 0.4 g BCAA, 0.2 g leucine); MOD (day: 36 g CHO, 1 g LIP, 20 g WPI, 11.8 g EAA, 5.6 g BCAA, 3.1 g leucine; night: 68 g CHO, 8 g LIP, 44 g WPI, 18.9 g EAA, 8.7 g BCAA, 4.1 g leucine) and HIGH (day: 32 g CHO, 1 g LIP, 39 g WPI, 23.5 g EAA, 11.3 g BCAA, 6.2 g leucine; night: 56 g CHO, 9 g LIP, 55 g WPI, 23.6 g EAA, 10.9 g BCAA, 5.1 g leucine); in the form of a beverage; consumed immediately after exercise, between lunch and dinner, and before bed	without controlled diet	46 days	2 interventions: pre-supplementation (18 to 19 days with dietary restriction and aerobic exercise) and supplementation (unrestricted diet, aerobic training and supplementation)	no significant effects on weight, LBM, or % BF occurred in the CON, MOD, or HIGH groups
Jakubowicz *et al.* [2017]	56 participants (26 men and 30 women) (58.9 ± 4.5 years, 32.1 ± 0.9 kg/m² BMI)	3 groups: WBdiet (n=19), PBdiet (n=19) and CBdiet (n=18)	Parallel randomized clinical trial	High-energy and PTN breakfast (660 ± 25 kcal): WBdiet (25% LIP, 50% CHO and 25% PTN – 42 g, including 28 g WPC); PBdiet (25% LIP, 50% CHO and 25% PTN – 42 g, including 7 g eggs, 20 g tuna, 7 g soy; CBdiet (25% LIP, 64% CHO and 11% PTN, including 17 g soy).	With controlled diet: similar lunch (560 ± 20 kcal), and dinner (280 ± 15 kcal), but breakfast differed between groups	12 weeks	no data	WBdiet caused greater weight loss (7.6 ± 0.3 kg) and satiety than the PBdiet (6.1 ± 0.3 kg) and CBdiet (3.5 ± 0.3 kg)

CHO: carbohydrates; LIP: lipids; BF: body fat; PTN: proteins; TRP: high-intensity strength training; REE: rest energy expenditure; SPI: soy protein isolate; BMI: body mass index; WC: waist circumference; LBM: lean body mass; BLG: β-lactoglobulin; ALA: α-lactoalbumin; CON, MOD, and HIGH: names of the supplementation groups, wherein CON refers to the placebo and MOD and HIGH to moderate and high protein content. WBdiet: whey protein breakfast diet; PBdiet: protein breakfast diet; CBDdiet: carbohydrate breakfast diet (from Jakubczyk *et al.* [2017]).

and promoted weight and body fat loss among women [Gomes *et al*., 2017]. These results can be attributed to the WP content, which correlates with decreased energy consumption because it increases satiety, thereby decreasing energy consumption [Bendtsen *et al*., 2013] and/or increasing resting energy expenditure [Hackney *et al*., 2010]. Furthermore, these proteins participate in beta-oxidation processes [Acheson *et al*., 2011] and lipolysis [Hector *et al*., 2015] and can be recommended for body fat loss after strength training [Hulmi *et al*., 2015]. Conversely, Berryman *et al*. [2017] found no changes in weight, lean body mass, or body fat measurements in young men supplemented with WPI 3 times per day immediately after aerobic physical exercise in the morning, between lunch and dinner, and before bed.

Nutritional monitoring and a training program in line with an individual's goals are important for realizing beneficial effects from the consumption of different WP formulations because protein supplementation alone will most likely fail to cause the expected effects. As shown above, the RDA established by the DRI for the population is a key tool for nutritionists. Accordingly, the quantity of daily protein that should be ingested by a sedentary adult is 0.8 g/kg weight/day [IOM 2002/2005], whereas this need will vary with the individual's physical training intensity and objectives. According to the Brazilian Society of Exercise and Sports Medicine [Sociedade Brasileira de Medicina do Exercício e do Esporte, 2009], an intake of 1.2 to 1.6 g/kg protein/day is recommended for endurance athletes, whereas strength athletes may benefit from an intake of 1.6 to 1.7 g/kg protein/day, although an intake higher than 3.0 g/kg protein/day may benefit body composition [Jäger *et al*., 2017]. To optimize protein supplementation, nutritionists must evaluate the individual's diet, lifestyle, sports modality, goals, and training phase before choosing the supplement.

This study examined available evidence regarding the effects of consuming different varieties of WP on muscle hypertrophy, physical performance, response to muscle injury, weight loss, and body composition changes. However, this review had limitations due to difficulties in comparing studies that involve various methods and include small groups, heterogeneous samples, and differences in age, trained or untrained individuals, dosage, administration routes, supplement varieties, the degree of diet control, the study period length (ranging from hours to months), and the physical training modalities. These difficulties stress the need for further studies on WP supplementation with larger and more homogeneous samples and study periods longer than 12 weeks. Furthermore, more specific training protocols, which should focus on hypertrophy, physical performance, response to muscle injury, weight loss, and changes in body composition, are needed and should be adequately integrated with the supplementation protocol to consider dosage, administration route, and food intake to obtain more consistent results.

CONCLUSION

The papers analyzed in the present study showed that supplementation with either WPI or WPC was related to increased MPS and that WPH caused muscle hypertrophy and improved physical performance. Few studies showed results concerning muscle damage regarding the efficacy of WP varieties in reducing cell damage markers monitored by CK, LDH, and TNF-α. A higher reduction in weight, satiety, and improved body composition occurred with WPC supplementation, without compromising bodily lean mass loss.

WPH intake provided more advantages compared to WPC and WPI, and improved muscle torque and hypertrophy peak associated to strength exercise and reduced muscle damage, caused by reduced CK levels associated to aerobic exercises. With these findings, the present study contributes to assign the WP variety according to its use by athletes, sportspeople, and general population.

RESEARCH FUNDING

We would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarships and Universidade do Vale do Taquari – Univates for the financial support granted for this research paper.

CONFLICT OF INTERESTS

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

REFERENCES

1. Acheson, K.J., Blondel-Lubrano, A., Oguey-Araymon, S., Beaumont, M., Emady-Azar, S., Ammon-Zufferey, C., Monnard, I., Pinaud, S., Nielsen-Moennoz, C., Bovetto, L. (2011). Protein choices targeting thermogenesis and metabolism. *The American Journal of Clinical Nutrition*, *93*(3), 525–534.
2. Amaral, G.V., Silva, E.K., Cavalcanti, R., Martins, C.P.C., Andrade, L.G.Z.S., Moraes, J., Alvarenga V.O., Guimarães, J.T., Esmerino, E., Freitas, M.Q., Silva, M.C., Raices, R.S.L., Sant'Ana, A.S., Meireles, M.A.A., Cruz, A.G. (2018). Whey-grape juice drink processed by supercritical carbon dioxide technology: physicochemical characteristics, bioactive compounds and volatile profile. *Food Chemistry, 239*, 697–703.
3. Aoi, W.A., Takanami, Y., Kawai, Y., Morifuji, M., Koga, J., Kanegae, M., Mihara, K., Yanohara, T., Mukai, J., Naito, Y., Yoshikawa, T. (2011). Dietary whey hydrolysate with exercise alters the plasma protein profile: a comprehensive protein analysis. *Nutrition*, *27*(6), 687–692.
4. Areta, J.L., Burke, L.M., Ross, M.L., Camera, D.M., West, D.W.D., Broad, E.M., Jeacocke, N.A., Moore, D.R., Stellingwerff, T., Phillips, S.M., Hawley, J.A., Coffey, V.G. (2013). Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. *The Journal of Physiology*, *591*(9), 2319–2331.
5. Atherton, P.J., Etheridge, T., Watt, P. W., Wilkinson, D., Selby, A., Rankin, D., Smith, K., Rennie, M.J. (2010). Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *The American Journal of Clinical Nutrition* , *92*(5), 1080–1088.
6. Atherton, P.J., Kumar, V., Selby, A.L., Rankin, D., Hildebrandt, W., Phillips, B.E, Williams, J.P., Hiscock, N., Smith, K. (2017). Enriching a protein drink with leucine augments muscle protein

synthesis after resistance exercise in young and older men. *Clinical Nutrition*, *36*(3), 888–895.
7. Babault, N., Deley, G., Le, Ruyet P., Morgan, F., Allaert, F.A. (2014). Effects of soluble milk protein or casein supplementation on muscle fatigue following resistance training program: a randomized, double-blind, and placebo-controlled study. *Journal of the International Society of Sports Nutrition*, *11*, art.no. 36.
8. Baer, D.J., Stote, K.S., Paul, D.R., Harris, G.K., Rumpler, W.V., Clevidence, B.A. (2011). Whey protein but not soy protein supplementation alters body weight and composition in free-living overweight and obese adults. *Journal of Nutrition*, *141*(8), 1489–1494.
9. Bendtsen, L.Q., Lorenzen, J.K., Bendsen, N.T., Rasmussen, C., Astrup, A. (2013). Effect of dairy proteins on appetite, energy expenditure, body weight, and composition: a review of the evidence from controlled clinical trials. *Advances in Nutrition: An International Review Journal*, *4*(4), 418–438.
10. Berryman, C.E., Sepowitz, J.J., McClung, H.L., Lieberman, H.L., Farina, E.K., McClung, J.P. Ferrando, A.A., Pasiakos, S.M. (2017). Supplementing an energy adequate, higher protein diet with protein does not enhance fat-free mass restoration after short-term severe negative energy balance. *Journal of Applied Physiology*, *122*(6), 1485–1493.
11. Biocatalysts. The use of enzymes in the production of whey protein hydrolysates. (2014). Available from [http://www.biocatalysts.com/2015/10/technical-bulletin-the-use-of-enzymes-for-whey-protein-hydrolysis/] (accessed 01 December 2017).
12. Blacker, S.D., Neil, C.W., Joanne, L.F., James, L.J.B., Mark, E.T.W. (2010). Carbohydrate *vs* protein supplementation for recovery of neuromuscular function following prolonged load carriage. *Journal of the International Society of Sports Nutrition*, *7*(2).
13. Brancaccio, P., Maffulli N., Buonauro, R., Limongelli, F.M. (2008). Serum enzyme monitoring in sports medicine. *Clinics in Sports Medicine*, *27*(1), 1–18.
14. Brancaccio, P., Maffulli, N., Limongelli, F.M. (2007). Creatine kinase monitoring in sport medicine. *British Medical Bulletin*, *81–82*. 209–30.
15. Brown, M.A., Stevenson, E.J., Howatson, G. (2018). Whey protein hydrolysate supplementation accelerates recovery from exercise-induced muscle damage in females. *Applied Physiology, Nutrition, and Metabolism*, *43*(4), 324–330.
16. Buckley, J.D., Thomson, R.L., Coates, A.M., Howe, P.R.C., DeNichilo, M.O., Rowney, M.K. (2010). Supplementation with a whey protein hydrolysate enhances recovery of muscle force-generating capacity following eccentric exercise. *Journal of Science and Medicine in Sport*, *13*(1), 178–181.
17. Burd, N.A., Yang, Y., Moore, D.R., Tang, J.E., Tarnopolsky, M.A., Phillips, S.M. (2012). Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men. *The British Journal of Nutrition*, *108*(6), 958–962.
18. Burke, L.M., Winter, J.A., Cameron-Smith, D., Enslen, M., Farnfield, M., Decombaz, J. (2012). Effect of intake of different dietary protein sources on plasma amino acid profiles at rest and after exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, *22*(6), 452–462.
19. Burnley, E.C.D., Olson, A.N., Sharp, R.L., Baier, S.M., Aleke, D.L. (2010). Impact of protein supplements on muscle recovery after exercise-induced muscle soreness. *Journal of Exercise Science & Fitness*, *8*(2), 89–96.
20. Cannon, J.G. (2000). Inflammatory cytokines in non pathological states. *News in Physiological Sciences, 15*(6), 298–303.
21. Cappato, L.P., Ferreira, M., Moraes, J., Pires, R.P.S., Rocha, R.S., Silva, R.C., Neto, R.P.C., Tavares, M.I.B., Freitas, M.Q., Rodrigues, F.N., Calado, V., Raices, R.S.L., Silva, M.C., Cruz, A.G. (2018). Whey acerola-flavoured drink submitted Ohmic Heating: bioactive compounds, antioxidant capacity, thermal behavior, water mobility, fatty acid profile and volatile compounds. *Food Chemistry*, *263*, 81–88.
22. Chen, W.C., Huang, W.C., Chiu, C.C., Chang, Y.K., Huang, C.C. (2014). Whey protein improves exercise performance and biochemical profiles in trained mice. *Medicine and Science in Sports and Exercise*, *46*(8), 1517–1524.
23. Chungchunlam, S.M.S., Henare, S.J., Ganesh, S., Moughan, P.J. (2017). Effects of whey protein and its two major protein components on satiety and food intake in normal-weight women. *Physiology & Behavior*, *175,* 113–118.
24. Churchward-Venne, T.A., Breen, L., Di Donato, D.M., Hector, A.J., Mitchell, C.J., Moore, D.R., Stellingwerff, T., Breuille, D., Offord, E.A., Baker, S.K., Phillips, S.M. (2014). Leucine supplementation of a low protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double-blind, randomized trial. *The American Journal of Clinical Nutrition*, *99*(2), 276–286.
25. Cruzat, V.F., Krause, M., Newsholme, P. (2014). Amino acid supplementation and impact on immune function in the context of exercise. *Journal of the International Society of Sports Nutrition*, *11*, art.no. 61.
26. Cruzat, V.F., Rogero, M.M., Tirapegui, J. (2010). Effects of supplementation with free glutamine and the dipeptide alanyl-glutamine on parameters of muscle damage and inflammation in rats submitted to prolonged exercise. *Cell Biochemistry and Function*, *28*(1), 24–30.
27. Cruzat, V.F., Tirapegui, J. (2009). Effects of oral supplementation with glutamine and alanyl-glutamine on glutamine, glutamate, and glutathione status in trained rats and subjected to long-duration exercise. *Nutrition*, *25*(4), 428–435.
28. Davies, R.W., Carson, B.P., Jakeman, P.M. (2018). The effect of whey protein supplementation on the temporal recovery of muscle function following resistance training: A systematic review and meta-analysis. *Nutrients*, *10*(2).
29. Devries, M.C., Phillips, S.M. (2015). Supplemental protein in support of muscle mass and health: advantage whey. *Journal of Food Science*, *80,* Suppl. 1, A8-A15.
30. Duhamel, T.A., Green, H.J., Perco, J.G., Ouyang, J. (2005). Metabolic and sarcoplasmic reticulum Ca2+ cycling responses in human muscle 4 days following prolonged exercise. *Canadian Journal of Physiology Pharmacology*, *83*(7), 643–655.
31. Erskine, R.M., Fletcher, G., Hanson, B., Folland, J.P. (2012). Whey protein does not enhance the adaptations to elbow flexor resistance training. *Medicine and Science in Sports and Exercise*, *44*(9), 1791–1800.
32. Erskine, R.M., Jones, D.A., Williams, A.G., Stewart, C.E., Degens, H. (2010). Inter-individual variability in the adaptation of human muscle specific tension to progressive resistance training. *European Journal of Applied Physiology, 110*(6), 1117–1125.
33. FAO. Food And Agriculture Organization of the United Nations. (2013). Dietary protein quality evaluation in human nutrition: report of an FAO expert consultation. Available from [http://www.fao.

org/ag/humannutrition/35978-02317b979a686a57aa4593304ff-c17f06.pdf] (accessed 01 December 2017).

34. Farup, J., Rahbek, S.K., Riis, S., Vendelbo, M.H., Paoli, F., Vissing, K. (2014b). Influence of exercise contraction mode and protein supplementation on human skeletal muscle satellite cell content and muscle fiber growth. *Journal of Applied Physiology, 117*(8), 898–909.
35. Farup, J., Rahbek, S.K., Vendelbo, M.H., Matzon, A., Hindhede, J., Bejder A., Ringgard, S., Vissing, K. (2014a). Whey protein hydrolysate augments tendon and muscle hypertrophy independent of resistance exercise contraction mode. *Scandinavian Journal of Medicine & Science in Sports*, *24*(9), 788–798.
36. Gomes, D.L., Moehlecke, M., Silva, F.B.L., Dutra, E.S., D'Agord, S.B., Carvalho, K.M.B. (2017). Whey protein supplementation enhances body fat and weight loss in women long after bariatric surgery: a randomized controlled trial. *Obesity Surgery, 27*(2), 424–431.
37. Grala A.P., Teixeira M.A.R., Nishitania T.C., Melosi A.F., Jacinto J.L., Aguiar A.F. (2017). Effects of leucine supplementation associated with resistance training on muscle strength in young subjects. *Journal of Health Science*, *19*(2), 63–67 (in Portuguese; English abstract).
38. Guimarães, J.T., Silva, E.K., Freitas, M.Q., Meireles, M.A.A., Cruz, A.G. Non-thermal emerging technologies and their effects on the functional properties of dairy products. (2018). *Current Opinion in Food Science, 22*, 62–66.
39. Hackney, K.J., Bruenger, A.J., Lemmer, J.T. (2010). Timing protein intake increases energy expenditure 24 h after resistance training. *Medicine and Science in Sports and Exercise*, *42*(5), 998–1003.
40. Halford, J.C., Boyland, E.J., Lawton, C.L., Blundell, J.E., Harrold, J.A. (2011). Serotonergic anti-obesity agents: past experience and future prospects. *Drugs*, *71*(17), 2247–2255.
41. Hamarsland, H., Laahne, J.A.L, Paulsen, G., Cotter, M., Borsheim, E., Raastad, T. (2017). Native whey induces higher and faster leucinemia than other whey protein supplements and milk: a randomized controlled trial. *BMC Nutrition*, *3*(10).
42. Hansen, M., Bangsbo, J., Jensen, J., Bibby, B.M., Madsen, K. (2015). Effect of whey protein hydrolysate on performance and recovery of top-class orienteering runners. *International Journal of Sport Nutrition and Exercise Metabolism*, *25*(2), 97–109.
43. Haraguchi, F.K., Abreu, W.C., Paula, H. (2006). Whey protein: composition, nutritional properties, applications in sports and benefits for human health. *Revista de Nutrição*, *19*(4), 479–488 (in Portuguese; English abstract).
44. Hector, A.J., Marcotte, G.R., Churchward-Venne, T.A., Murphy, C.H., Breen, L., von Allmen, M., Baker, S.K., Phillips, S.M. (2015). Whey protein supplementation preserves postprandial myofibrillar protein synthesis during short-term energy restriction in overweight and obese adults. *The Journal of Nutrition, 145*(2), 246–252.
45. Hulmi, J.J., Laakso, M., Mero, A.A., Häkkinen, K., Ahtiainen, J.P., Peltonen, H. (2015). The effects of whey protein with or without carbohydrates on resistance training adaptations. *Journal of the International Society of Sports Nutrition*, *12,* art.no. 48.
46. IOM. Institute of Medicine. (2002/2005). Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients): panel on Macronutrients, Subcommittee on Upper Reference Levels of Nutrients, Subcommittee on Interpretation and Uses of Dietary Reference Intakes, and The Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board. Available from [https://www.ncbi.nlm.nih.gov/nlmcatalog/101237303] (accessed 01 December 2017).
47. Jäger, R., Kerksick, C.M., Campbell, B.I., Cribb, P.J., Wells, S.D., Skwiat, T.M., Purpura, M., Ziegenfuss, T.N., Ferrando, A.A., Arent, S.M., Smith-Ryan, A.E., Stout, J.R., Arciero, P.J., Ormsbee, M.J., Taylor, L.W., Wilborn, C.D., Kalman, D.S., Kreider, R.B., Willoughby, D.S., Hoffman, J.R., Krzykowski, J.L., Antonio, J. (2017). International Society of Sports Nutrition Position Stand: protein and exercise. *Journal of the International Society of Sports Nutrition*, *14,* art.no. 20.
48. Jakubowicz, D., Froy, O. (2013). Biochemical and metabolic mechanisms by which dietary whey protein may combat obesity and Type 2 diabetes. *The Journal of Nutritional Biochemistry*, *24*(1), 1–5.
49. Jakubowicz, D., Wainsteina, J., Landaua, Z., Ahrenc, B., Barnead, M., Bar-Dayana, Froy, O. (2017). High-energy breakfast based on whey protein reduces body weight, postprandial glycemia and HbA1C in Type 2 diabetes. *Journal of Nutritional Biochemistry*, *49,* 1–7.
50. Joy, J.M., Lowery, R.P., Wilson, J.M., Purpura, M., Souza, E.O., Wilson, S.M.C, Kalman, D.S., Dudeck, J. E., Jäger, R. (2013). The effects of 8 weeks of whey or rice protein supplementation on body composition and exercise performance. *Nutrition Journal*, *12,* art.no. 86.
51. Kankanamge, R., Jeewanthi, C., Lee, N-K., Lee, S-K., Yoon, Y.C., Paik, Hyun-D. (2015). Physicochemical characterization of hydrolysates of whey protein concentrates for their use in nutritional beverages. *Food Science and Biotechnology*, *24*(4), 1335–1340.
52. Kim, J., Chulhyn, L., Joohyung, L. (2017). Effect of timing of whey protein supplement on muscle damage markers after eccentric exercise. *Journal of Exercise Rehabilitation*, *13*(4), 436–440.
53. Koury, J.C., Donangelo, C.M. (2003). Zinc, oxidative stress and physical activity. *Revista de Nutrição*, *16*(4), 433–441 (in Portuguese; English abstract).
54. Kumar, V., Selby, A., Rankin, D., Patel, R., Atherton, P., Hildebrandt, W., Williams, J., Smith, K., Seynnes, O., Hiscock, N., Rennie, M.J. (2009). Age-related differences in the dose–response relationship of muscle protein synthesis to resistance exercise in young and old men. *The Journal of Physiology*, *587*(1), 211–217.
55. Lockwood, C.M., Roberts, M.D., Dalbo, V.J., Smith-Ryan, A.E., Kendall, K.L., Moon, J.R., Stout, J.R. (2017). Effects of hydrolyzed whey versus other whey protein supplements on the physiological response to 8 weeks of resistance exercise in college-aged males. *Journal of the American College of Nutrition*, *36*(1), 16–27.
56. Lollo, P.C.B, Amaya-Farfan, J., Faria, I.C., Salgado, J.V.V., Chacon-Mikahil, M.P.T., Cruz, A.G., Oliveira, C.A.F., Montagner, P.C., Arruda, M. (2014). Hydrolysed whey protein reduces muscle damage markers in Brazilian elite soccer players compared with whey protein and maltodextrin. A twelve-week in-championship intervention. *International Dairy Journal*, *34*(1), 19–24.
57. Macnaughton, L.S., Wardle, S.L., Witard, O.C., McGlory, C., Hamilton, D.L., Jeromson, S., Lawrence, C.E., Wallis, G.A., Tipton, K.D. (2016). The response of muscle protein synthesis after whole body resistance exercise is greater after 40 g than 20 g

of whey protein ingested. *Physiological Reports*, *4*, art.no. UNSP e 12893.
58. Madureira, A.R., Tavares, T., Gomes, A.M., Pintado, M.E., Malcata, F.X. (2010). Invited review: physiological properties of bioactive peptides obtained from whey proteins. *Journal of Dairy Science*, *93*(2), 437–455.
59. Mahan, L.K., Escott-Stump, S. (1998). Proteínas. In: Krause: *Alimentos, nutrição e dietoterapia*. 9. ed. Roca, São Paulo, pp. 63–76, ISBN: 8572412409 (in Portuguese).
60. Markus, C.R., Oliver, B., Haan, E.H.F. (2002). Whey Protein rich in alfa-lactoalbumin increases the ratio of plasma tryptophan to the sum of the other large neutral amino acids and improves cognitive performance in stress-vulnerable subjects. *The American Journal of Clinical Nutrition*, *75*(6), 1051–1056.
61. Marshall, K. (2004). Therapeutic applications of whey protein. *Alternative Medicine Review*, *9*(2), 136–156.
62. McArdle, W. D., Katch, F. I., Katch, V. L. (2014). *Nutrição para o esporte e o exercício*. 3th edition. Guanabara Koogan, Rio de Janeiro, RJ. pp. 33–41, ISBN: 9788527716659 (in Portuguese).
63. Mitchell, C.J., D'Souza, R.F., Fanning, A.C., Poppitt, D., Cameron-Smith, D. (2017). Short communication: Muscle protein synthetic response to microparticulated whey protein in middle-aged men. *Journal of Dairy Science*, *100*(6), 4230–4234.
64. Mitchell, W.K., Phillips, B.E., Williams, J.P., Rankin, D., Lund, J.N., Smith, K., Atherton, P.J. (2015). A dose- rather than delivery profile-dependent mechanism regulates the "muscle-full" effect in response to oral essential amino acid intake in young men. *Journal for Nutrition*, *145*(2), 207–214.
65. Moberg, M., Apro, W., Ohlsson, I., Ponten, M., Villanueva, A., Ekblom, B., Blomstrand, E. (2014). Absence of leucine in an essential amino acid supplement reduces activation of mTORC1 signalling following resistance exercise in young females. *Applied Physiology, Nutrition and Metabolism*, *39*(2), 183–194.
66. Monteiro, C.A., Cannon, G., Moubarac, J.C., Levy, R.B., Louzada, M.L.C., Jaime, P.C. (2018). The UN decade of nutrition, the NOVA food classification and the trouble with ultra-processing. *Public Health Nutrition, 21*(1), 5–17.
67. Monteyne, A., Martin, A., Jackson, L., Corrigan, N., Stringer, E., Newey, J., Rumbold, P.L.S, Stevenson, E., James, L.J. (2018). Whey protein consumption after resistance exercise reduces energy intake at a post-exercise meal. *European Journal of Nutrition*, 57(2), 585–592.
68. Moore, D.R., Robinson, M.J., Fry, J.L., Tang, J.E., Glover, E.I., Wilkinson, S.B., Prior, T., Tarnopolsky, M.A., Phillips, S.M. (2009). Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *The American Journal of Clinical Nutrition*, *89*(1), 161–168.
69. Morton, R.W., Murphy, K.T., McKellar, S.R., Schoenfeld, B.J., Henselmans, M., Helms, E., Aragon, A.A., Devries, M.C., Banfield, L., Krieger, J.W., Phillips, S.M. (2018). A systematic review, meta-analysis and meta-regression of the effect of protein supplementation on resistance training-induced gains in muscle mass and strength in healthy adults. *British Journal of Sports Medicine, 52*(6), 376–384.
70. Naclerio, F., Larumbe-Zabala, E. (2016). Effects of whey protein alone or as part of a multi ingredient formulation on strength, fat-free mass, or lean body mass in resistance-trained individuals: a meta-analysis. *Sports Medicine*, *46*(1). 125–137.
71. Niess, A.M., Simon, P. (2007). Response and adaptation of skeletal muscle to exercise–the role of reactive oxygen species. *Frontiers in Bioscience*, *12*, 4826–4838.
72. Nogiec, C.D., Kasif, S. (2013). To supplement or not to supplement: a metabolic network framework for human nutritional supplements. *PLoS One, 8*(8), art.no. e68751.
73. Oliveira, D.F., Bravo, C.E.C., Tonial, I.B. (2012). Whey of milk: a valuable product. *Revista do Instituto de Laticínios Cândido Tostes, 385*. 64–71 (in Portuguese).
74. Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., Pedersen, B.K. (1999). Pro-and anti-inflammatory cytokine balance in strenuous exercise in humans. *The Journal of Physiology*, *515*(1), 287–291.
75. Pal, S., Radavelli-Bagatini, S. (2013). The effects of whey protein on cardiometabolic risk factors. *Obesity Reviews*, *14*(4), 324–343.
76. Pasiakos, S.M., Austin, K.G., Lieberman, H.R., Askew, E.W. (2013). Efficacy and safety of protein supplements for U.S. Armed Forces personnel: consensus statement. *The Journal of Nutrition*, *143*(11), 1811S-1814S.
77. Petry, E.R., Cruzat, V.F., Heck, T.G., Leite, J.S., Homem de Bittencourt, P.I. Jr, Tirapegui, J. (2014). Alanyl-glutamine and glutamine plus alanine supplements improve skeletal redox status in trained rats: involvement of heat shock protein pathways. *Life Sciences*, *94*(2), 130–136.
78. Rahbek, S.K., Farup, J., Müller, A.B., Vendelbo, M.H., Holm, L., Jessen, N., Vissing, K. (2014). Effects of divergent resistance exercise contraction mode and dietary supplementation type on anabolic signalling, muscle protein synthesis and muscle hypertrophy. *Amino Acids*, *46*(10), 2377–2392.
79. Rigon, T.V., Rossi, R.G. (2012). Who and why use dietary supplements? *Revista Brasileira de Nutrição Esportiva*, *6*, 420–426 (in Portuguese).
80. Roberts, J., Zinchenko, A., Suckling, C., Smith, L., Johnstone, J., Henselmans, M. (2017). The short-term effect of high versus moderate protein intake on recovery after strength training in resistance-trained individuals. *Journal of the International Society of Sports Nutrition*, *14,* art.no. 44.
81. Rohde, T.M.D, MacLean, D.A., Richter, E.A., Kiens, B., Pedersen, B.K. (1997). Prolonged submaximal eccentric exercise is associated with increased levels of plasma IL-6. *American Journal of Physiology – Endocrinology and Metabolism*, *273*(1), E85–E91.
82. Soares, L.L., Pimenta, E.M., Barros, A.F.S., Lessa, L.B., Pussieldi, G.A. (2012). Analysis of serum creatine kinase in athletes in college football after an intermittent session. *Motricidade*, *8*(S2), 439–446 (in Portuguese).
83. Sociedade Brasileira de Medicina do Exercício e do Esporte. (2009). Dietary changes, water replacement, food supplements and drugs: evidence of ergogenic action and potential health risks. Revista Brasileira de Medicina do Esporte, *15*(3) (in Portuguese).
84. Stark, M., Lukaszuk, J., Prawitz, A., Salacinski, A. (2012). Protein timing and its effects on muscular hypertrophy and strength in individuals engaged in weight-training. *Journal of the International Society of Sports Nutrition*, *9,* art.no. 54.
85. Stefanetti, R.J., Lamon, S., Rahbek, S.K., Farup, J., Zacharewicz, E., Wallace, M.A., Vendelbo, M.H., Russell, A.P., Vissing, K. (2014). Influence of divergent exercise contraction mode and whey protein supplementation on atrogin-1, MuRF1, and FOXO1/3A in human skeletal muscle. *Journal of Applied Physiology*, *116*(11), 1491–1502.

86. Tahavorgar, A., Vafa, M., Shidfar, F., Gohari, M., Heydari, I. (2014). Whey protein preloads are more beneficial than soy protein preloads in regulating appetite, calorie intake, anthropometry, and body composition of overweight and obese men. *Nutrition Research*, *34*(10), 856–861.
87. Teixeira, F.J., Matias, C.N., Monteiro, C.P., Valamatos, M.J., Reis, J., Tavares, F., Batista, A., Domingos, C., Alves, F., Sardinha, L.B., Phillips, S.M. (2018). Leucine metabolites do not enhance training-induced performance or muscle thickness. *Medicine and Science in Sports and Exercise, 32*(4), 1020–1028.
88. Tsivitse, S.K., McLoughlin, T.J., Peterson, J.M., Mylona, E., McGregor, S.J., Pizza, F.X. (2003). Downhill running in rats: influence on neutrophils, macrophages, and MyoD+ cells in skeletal muscle. *European Journal of Applied Physiology*, *90*(5–6), 633–638.
89. Uchida, M.C., Nosaka, K., Ugrinowitsch, C., Nosaka K., Aoki M.S. (2009). Effect of bench press exercise intensity on muscle soreness and inflammatory mediators. *Journal of Sports Sciences*, *27*(5), 499–507.
90. Van De Vyver, M., Myburgh, K.H. (2012). Cytokine and satellite cell responses to muscle damage: interpretation and possible confounding factors in human studies. *Journal of Muscle Research and Cell Motility*, *33*(3–4), 177–185.
91. Volek, J.S., Volk, B.M., Gómez, A.L., Kunces, L.J., Kupchak, B.R., Freidenreich, D.J., Aristizabal, J.C., Saenz, C., Dunn-Lewis, C., Ballard, K.D., Quann, E.E., Kawiecki, D.L., Flanagan, S.D., Comstock, B.A., Fragala, M.S., Earp, J.E., Fernandez, M.L., Bruno, R.S., Ptolemy, A.S., Kellogg, M.D., Maresh, C.M., Kraemer, W.J. (2013). Whey protein supplementation during resistance training augments lean body mass. *Journal of the American College of Nutrition*, *32*(2), 122–135.
92. Waitzberg, D.L, Logullo P. (2006). Proteínas. In Waitzberg, D.L. (Eds.), *Nutrição oral, enteral e parenteral na prática clínica*, 3th edition. Atheneu, São Paulo, pp. 35–54, ISBN: 9788573792553 (in Portuguese).
93. Wallace, Douglas C. (2011). Genetic and molecular aspects of sport performance. In C. Bouchard, E.P. Hoffman (Eds.), *Mitochondrial Medicine in Health and Disease: Interface Between Athletic Performance and Therapeutics,* Blackwell Publishing Ltd, Oxford, Inglaterra, Reino Unido, pp. 14–32.
94. Welch, K.D., Davis, T.Z., Van Eden, M.E., Aust, S.D. (2002). Deleterious iron-mediated oxidation of biomolecules. *Free Radical Biology & Medicine*, *32*(7), 577–583.
95. West, D.W., Burd, N.A., Coffey, V.G., Baker, S.K., Burke, L.M., Hawley, J.A., Moore, D.L., Stellingwerff, T., Phillips, S.M. (2011). Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. *The American Journal Clinical of Nutrition*, *94*(3), 795–803.

Submitted: 2 July 2018. Revised: 7 October and 21 November 2018. Accepted: 3 December 2018. Published on-line: 22 January 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 101–108
DOI: 10.31883/pjfns-2019-0010
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Intermittent Microwave-Vacuum Drying Effects on Pears

Onur Taskin[1], Ahmet Polat[1], Nazmi Izli[1], Baris Bulent Asik[2]*

[1]Department of Biosystems Engineering, Faculty of Agriculture, Bursa Uludag University, Gorukle Campus, 16059, Bursa, Turkey
[2]Department of Soil Science and Plant Nutrition, Faculty of Agriculture, Bursa Uludag University, Gorukle Campus, 16059, Bursa, Turkey

Key words: microwave-vacuum drying, mineral content, protein, rehydration ratio, color, scanning electron microscopy

In the present study, the effects of intermittent microwave-vacuum drying on the "Deveci" pear in terms of the drying kinetics, mineral content, protein content, rehydration ratio, color, energy, specific energy and microstructure were investigated. According to the drying treatments, increased microwave power (100 and 200 W) and vacuum (200 and 400 mmHg) applications provided higher drying rates and protein content. Dried pear samples had a higher mineral content than fresh samples because of the increasing dry matter content. At the higher vacuum level experiments, the energy consumption reduced and the rehydration ratio increased. Moreover, the higher microwave power caused a decrease of the L^* (lightness) value. However, a regular pore size and pore distribution in all drying experiments were observed in the microstructures of microwave-vacuum-dried samples.

INTRODUCTION

The pear (*Pyrus communis* L.) belongs to the *Rosaceae* family [Antal *et al*., 2017]. It is a fruit native to temperate zones and is cultivated in Europe [Guiné *et al*., 2007]. According to the Food and Agriculture Organization data [FAO, 2018], the worldwide pear production in 2014 was estimated at approximately 25.8 million tons. Fully mature pears play an important role in the human diet and nutrition as sources of dietary fiber, vitamins C and B_6, and minerals such as magnesium and potassium [Lutovska *et al*., 2016]. They are eaten in both the natural and processed forms as purees, jams, and dried pears [Santos, et al., 2014]. In fact, dried pears are widely used in bakery products, gravies, and compotes [Doymaz & İsmail, 2012].

Drying is a complex biological and chemical reaction whose mechanism is not yet entirely understood [Ferreira *et al*., 2008]. However, it is widespread industrial preservation method used to decrease water content of agricultural products to minimize chemical, biochemical, and microbiological deterioration [Doymaz & İsmail, 2012], as well as to prolong the shelf life of the fruit, and avoid spoilage and contamination during storage. Using an appropriate drying procedure also minimizes storage and transportation costs [Doymaz, 2013]. Therefore, drying methods have been garnering increased attention, and detailed studies have been conducted on the effects of different [Lüle & Koyuncu, 2015] or hybrid drying [Huang *et al*., 2015] methods on food quality. In recent years, drying with a microwave vacuum has been used as a hybrid method for top-quality dried products [Cui *et al*., 2003]. The advantage of the microwave-vacuum process is drying acceleration under low temperature by an increased pressure gradient between layers [Therdthai & Zhou, 2009]. If the microwave being used is not applied properly, poor-quality products may be manufactured due to irregular drying depending on product characteristics, shape, and size [Han *et al*., 2010; Kuş, 2016]. Thus far, intermittent microwave application during drying has been an effective methodology to avoid improper heating and improve product quality and energy efficiency within the product during microwave off times [Zhang *et al*., 2017].

There are ample literature data on the drying of pears using various drying methods, such as osmotic pre-treated convective air drying [González-Martínez *et al*., 2006], solar drying [Guiné *et al*., 2007], sun drying [Ferreira *et al*., 2008], hot air drying [Doymaz & İsmail, 2012], ultrasound-assisted infrared drying [Dujmić *et al*., 2013], solar stove of greenhouse type drying [Guiné *et al*., 2013], convective drying [Lutovska *et al*., 2016; Santos *et al*., 2014], osmo-vacuum drying [Amiripour *et al*., 2015], forced air convection drying [Dotto *et al*., 2017], microwave drying [Kuş, 2016], intermittent convective drying [Silva *et al*., 2014], and infrared and freeze drying [Antal *et al*., 2017]. To the best of our knowledge, no study has been reported on the intermittent microwave-vacuum drying effect on pear slices. The pear cultivar "Deveci" is one of the best winter-type pear cultivars in Turkey, originating from Anatolia, that has recently gained popularity because

* Corresponding Author: Tel.: +90 224 294 16 04; Fax: +90 224 294 14 02; E-mail nazmiizli@gmail.com (N. Izli)

of the high fruit quality, production and storage capacity [Ozturk & Ozturk, 2014]. Therefore, this study was aimed to investigate the drying kinetics of "Deveci" pear slices and drying effects on the mineral content, protein content, rehydration ratio, color, energy specific energy and microstructure analyses when using intermittent microwave-vacuum drying conditions.

MATERIALS AND METHODS

Drying experiments

Wholly matured and healthy fruits of "Deveci" pears were selected for this study and were stored before drying at 4±0.5°C for a day. The initial moisture content of fresh samples was determined by oven drying (ED115; Binder, Tuttlingen, Germany) at 105°C [Celen & Kahveci, 2013] and was found to be 5.67 (g water/g dry matter). The samples were dried until the final moisture content of 0.1 (g water/g dry matter). The samples were horizontal sliced into 5±0.04-mm thickness using a food slicer (Nicer Dicer, China).

The drying experiments were carried out in a custom--modified intermittent microwave-vacuum dryer with a power output of 900 W and a microwave frequency of 2450 MHz. It consists of a microwave oven (NE1846; Panasonic, Japan), vacuum pump (VRT404; Bacca, Taiwan), voltage variac (DVT2.5; Devotrans, Turkey), pulse controller (MCB9; Entes, Turkey), and glass container (Figure 1). Before starting the drying experiments, the glass container with the dimensions of 200 (diameter) × 92 (height) mm with the pear to be dried was positioned inside the microwave cavity, and the pressure inside the container was managed by a pressure regulator valve connected to a 0.18-kW vacuum pump and was monitored using a vacuum gauge. Once the vacuum level was reached, the microwave oven was run, and various output power levels and the pulse ratio were adjusted using a voltage variac and pulse controller, respectively [Kumar & Shrivastava, 2017; Zaki *et al*., 2007]. The weight of the sample (80 g) was determined by the digital balance (Radwag, Radom, Poland) and was recorded at 10-min intervals after turning off the microwave-vacuum dryer [Zaki *et al*., 2007]. The experimental conditions of drying included: microwave power of 100 and 200 W, vacuum pressure of 200 and 400 mmHg, and pulse ratio of 3 and 4. The pulse ratio (PR) for each run was computed as PR = $(t_{on} + t_{off})/t_{on}$, where t_{on} is the magnetron power "on" time and t_{off} is the magnetron power "off" time [Gunasekaran & Yang, 2007]. During the study, 20 s of t_{on} and 40 s of t_{off} represented PR=3, and 15 s of t_{on} and 45 s of t_{off} represented PR=4.

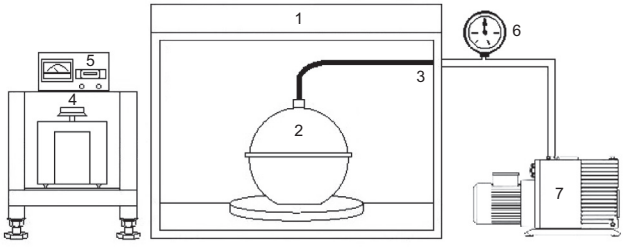


FIGURE 1. Schematic diagram of the laboratory apparatus: (1) dryer, (2) glass container, (3) teflon hose, (4) variac, (5) pulse controller, (6) vacuum gauge, and (7) vacuum pump.

Determining mineral contents

The total contents of some elements were determined by using dry samples after microwave treatment of the 0.20–0.50 g samples with 4 mL HNO_3 and 3 mL H_2O_2 in a microwave oven (MWS 2 DAP 60K, Berghof, Germany). Contents of Na, K, and Ca were determined by the flame emission method using a flame photometer (6361, Eppendorf Elex, Germany), whereas these of P, Mg, Fe, Cu, Zn, and Mn was determined in the extracts using inductively coupled plasma optical emission spectroscopy (ICP OES) and expressed in mg/kg (Optima 2100 DV, Perkin Elmer, United States).

Determining protein contents

The Kjeldahl method was used to determine the protein content in pear samples. The conventional factor 6.25 was used to obtain the protein content of fruits [AOAC, 1960]. Digestion/distillation unit (K-437/K-350, Buchi, Switzerland) was used for nitrogen determination.

Rehydration ratio

Dried pear samples were rehydrated by immersion in a distilled water bath at a controlled temperature of 25°C. The weight of dehydrated pear samples used in each experiment was 5±0.02 g. Rehydration of dried pears was stopped after three hours. Next, the samples were drained with a tissue paper to remove excess water from the surface. The data were quantified in terms of the rehydration ratio (RR) using the following formula [Zielinska *et al*., 2016]:

$$RR = \frac{\text{Mass of the rehydrated sample}}{\text{Mass of the dried sample}} \quad (1)$$

Color measurements

The color of the pear sample was measured before and after drying using a colorimeter (Hunter Lab, MSEZ-4500L, USA) at four different points on the pear's sample for all experiments. The color values were indicated as L^* (whiteness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness). Additionally, L_0^*, a_0^* and b_0^* represented the color parameters of the fresh samples. The following formulas were used to calculate Chroma C (Eq. 2), hue angle α (Eq. 3), and total color difference ΔE (Eq. 4), [İzli, 2017]:

$$C = \sqrt{(a^2 + b^2)} \quad (2)$$

$$\alpha = \tan^{-1}\left(\frac{b}{a}\right) \quad (3)$$

$$\Delta E = \sqrt{(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2} \quad (4)$$

Energy and specific energy consumption

The energy consumption values of the intermittent microwave-vacuum drying processes were measured using a power meter (EU TS-836A; Floureon, China). The drying devices

(*i.e*., the microwave oven and vacuum pump) were connected to the power meter that was on the electric grid. During the drying conditions, the energy consumption values were recorded by the power meter in kWh [Orikasa *et al*., 2018]. The energy required to evaporate a unit mass of water from the sample (specific energy consumption, SEC) was calculated for each drying experiment. It was defined in terms of the MJ/kg of water removed and was used as process parameters of optimization [Kumar & Shrivastava, 2017].

$$SEC\ (MJ/kg) = \frac{t_{on} M(1-m_f) \times 10^{-6}}{M_i\,(m_i - m_f)} \quad (5)$$

where: t_{on} is the total power on time (s), M is microwave input power (W), M_i is the initial mass of the sample (kg), m_i is the initial moisture content (g water/g dry matter), and m_f is the final moisture content (g water/g dry matter).

Microstructure analysis

The effect of different drying conditions on the microstructure of pear samples was observed using a scanning electron microscope (EVO 40, Carl Zeiss, Oberkochen, Germany). Particles extracted from the dried samples were vertically cut into 1-mm-thick slices and coated with gold-palladium. All samples were examined under high vacuum (20 kV) (SCD-005, Baltec, Wetzlar, Germany), and then their microphotographs were taken [Tian *et al*., 2015].

Statistical analysis

The study was performed using randomized plots factorial design of experimental type. All experiments were done in triplicate. The data were subjected to the analysis of variance (ANOVA) using JMP software (Version 7.0; SAS Institute Inc., Cary, NC, USA). The least significant difference (LSD) test was used to compare the means at the 5% significance level ($P<0.05$).

RESULTS AND DISCUSSION

Drying kinetics

The drying rates of the "Deveci" pear dried by the intermittent microwave-vacuum methodology are shown in Figure 2. A higher microwave power and vacuum application provided higher drying rates. The shortest drying time of pears (150 min) was noted at process conditions "200 W - 400 mmHg – PR=3", and the longest one (460 min) at "100 W – 200 mmHg – PR=4". These results indicate that it was possible to decrease the drying time by 310 min. A higher microwave power with more microwave energy made the pears have a higher temperature and increased the evaporation rate. With a rapid increase in the mass transfer rate of the pear, the drying time was reduced. The drying rates of the pear dried by constant microwave power and a pulse ratio under a vacuum of 400 mmHg were higher than those dried under 200 mmHg because a higher vacuum resulted in a lower boiling point of water and a higher evaporation rate. Several authors reported the total times for the drying of the pear to be 525, 480, 360 and 255 min in a convective dryer at 50, 57, 64 and 71°C, respectively [Doymaz, 2013]; as well as 20, 19, 16, 14 and 1260 min in an infrared dryer at 40, 50, 60, 70°C and in a freeze dryer (−47°C condenser temperature and 85–95 Pa absolute pressure), respectively [Antal *et al*., 2017]. On the other hand, the results were in good agreement with those reported by Cheenkachorn *et al.* [2012] who used a microwave-vacuum method to dry papaya cubes. Their drying times at a vacuum pressure of 400 mmHg, were 72.6, 115.5, 38.2, and 84 min at "400 W - PR=2", "400 W - PR=3", "800 W - PR=2", and "800 W - PR=3", respectively. Additionally, the drying time decreased with vacuum pressure increase.

Mineral content

The mineral contents of intermittent microwave-vacuum dried "Deveci" pears are shown in Table 1. The "Deveci" pears were found a significant source of K, whereas their Mn contents were low. The mineral contents of pears exposed to different drying treatments were higher than those of fresh fruits because of the increased dry matter ratio. The highest K levels determined at "100 W - 200 mmHg – PR=4", "100 W - 400 mmHg – PR=3", and "200 W - 400 mmHg – PR=4" did not differ significantly ($P>0.05$). On the other hand, the high-

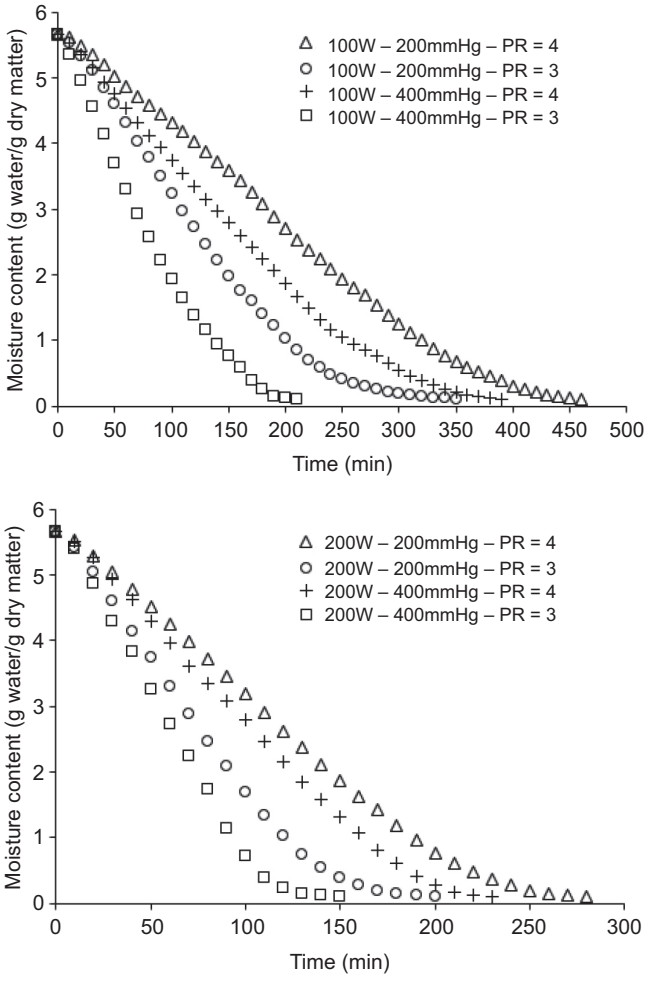


FIGURE 2. The moisture content of the "Deveci" pear *vs.* time under different drying conditions of pulses (PR=3 and 4), microwave powers (100 and 200 W), and vacuum levels (200 and 400 mmHg).

TABLE 1. Mineral contents of "Deveci" pears (mg/kg) fresh and dried under different conditions of pulses (PR=3 and 4), microwave powers (100 and 200 W), and vacuum levels (200 and 400 mmHg).

Minerals	Fresh	100 W 200 mmHg PR=4	100 W 200 mmHg PR=3	100 W 400 mmHg PR=4	100 W 400 mmHg PR=3	200 W 200 mmHg PR=4	200 W 200 mmHg PR=3	200 W 400 mmHg PR=4	200 W 400 mmHg PR=3
P	464±6^{d}	1446±27^{a}	1049±144^{bc}	1206±33^{abc}	1300±92^{ab}	1206±291^{abc}	1159±76^{bc}	979±26^{c}	1238±6^{abc}
K	353±136^{e}	6470±114^{ab}	3521±662^{d}	3424±167^{d}	7533±1107^{a}	3445±588^{d}	4383±472^{cd}	6987±551^{a}	5523±13^{bc}
Ca	64.8±0.8^{f}	291±25^{cd}	258±40de	364±25^{ab}	292±34^{cd}	279±14de	224±34^{e}	423±7^{a}	343±25^{bc}
Mg	47.1±1.5^{d}	284±11^{b}	228±4^{c}	290±16^{b}	378±20^{a}	223±16^{c}	235±6^{c}	376±48^{a}	338±4^{a}
Na	246±2^{e}	733±56^{a}	536±52^{d}	629±5^{bc}	640±30^{ab}	621±73^{bcd}	629±17^{bc}	579±49^{cd}	685±0.4^{ab}
Fe	3.97±1.00^{c}	8.84±0.88^{ab}	6.71±1.30^{bc}	8.19±0.54^{ab}	10.96±2.37^{a}	6.82±2.26^{bc}	6.58±034^{bc}	8.71±1.43^{ab}	8.41±0.75^{ab}
Cu	2.89±0.03^{d}	7.29±0.24^{b}	5.53±0.92^{c}	7.39±0.44^{b}	8.87±0.52^{a}	7.46±1.36^{ab}	6.98±0.37^{bc}	6.82±0.55^{bc}	7.69±0.41^{ab}
Zn	2.35±0.07^{c}	5.20±0.01^{ab}	3.93±0.51^{b}	4.82±0.22^{ab}	5.58±0.08^{a}	5.38±1.21^{a}	4.03±0.73^{b}	5.14±0.75^{ab}	5.12±0.43^{ab}
Mn	0.56±0.02^{f}	0.94±0.04^{ef}	1.34±0.01^{de}	3.47±0.01^{a}	2.06±0.12^{c}	1.83±0.13^{c}	1.66±0.01^{cd}	2.74±0.55^{b}	1.89±0.01^{c}

$^{a-g}$ means with different letters in the same row differ significantly (P<0.05).

est Mn level was found at "100 W - 400 mmHg – PR=4". There were significant differences between drying experiments in terms of contents of all minerals compared with the fresh samples (P<0.05). Al Juhaimi *et al*. [2016] reported the mineral contents of three pear varieties ("Santa Maria", "Deveci" and "Ankara"). The P, K, Ca, Mg, Fe, and Mn contents of fresh "Deveci" pears were found to be 164.3, 1255.4, 42, 101.5, 19.7, and 1.5 mg/kg, respectively. On the other hand, contents of Na, Cu, and Zn were closer to those of the presented study. The differences between the studies can be attributed to the growth conditions of "Deveci" pears which were picked in the province of Bursa and Konya. After drying 5-mm slices of "Deveci" pears for 15, 20, and 25 h at 70°C, the K content increased to 5298, 7245.6, and 7649.1 mg/kg, respectively, similar to that in our presented study. In another previous study performed by Clary *et al.* [2007], grapes dried at 71 °C using microwave-vacuum dehydration had higher contents of Ca, Na, and K than sundried and fresh grapes. However, lower values were reported for Fe in that study.

Protein analysis

Figure 3 demonstrates that the protein content of the dried "Deveci" pears was higher than that of the fresh fruit because of the decreased water content. Compared with the protein content, an increase in common or individual microwave power and vacuum level provided higher protein values. Between drying treatments, the values obtained at "200 W - 400 mmHg – PR=3" and "100 W - 400 mmHg – PR=3" did

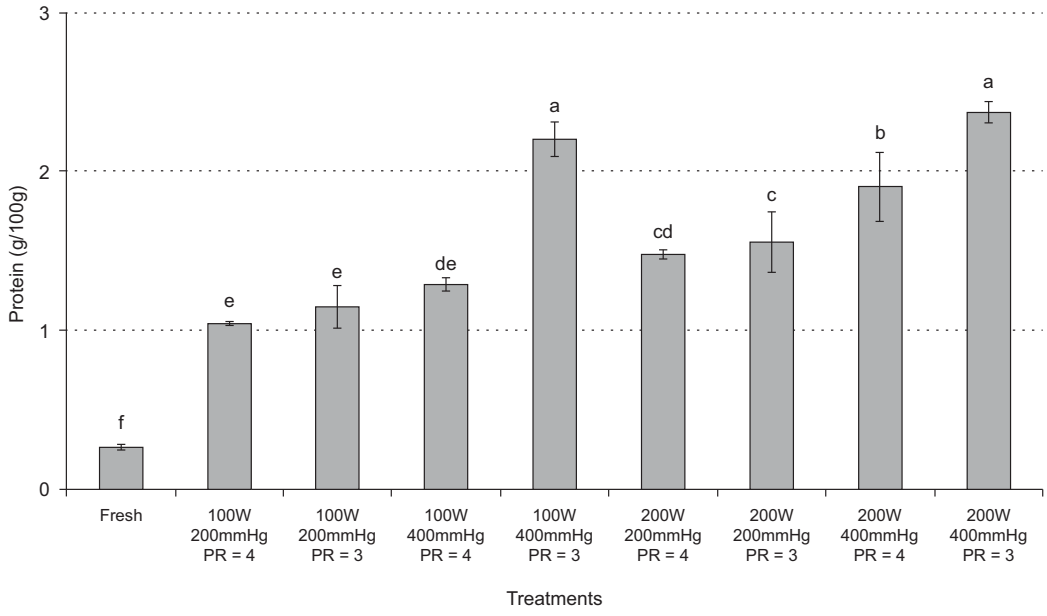


FIGURE 3. Protein content of "Deveci" pears fresh and dried under different conditions of pulses (PR=3 and 4), microwave powers (100 and 200 W), and vacuum levels (200 and 400 mmHg).

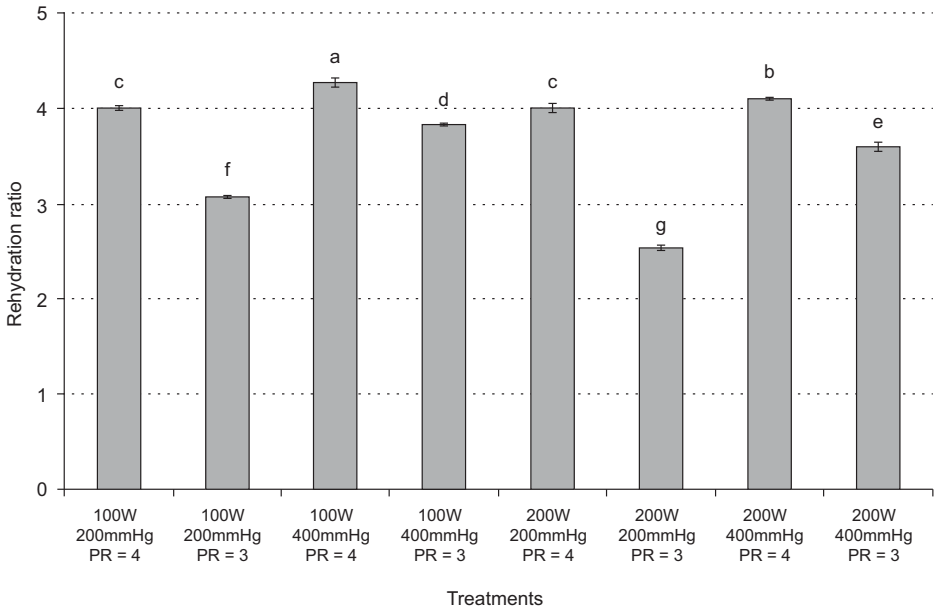


FIGURE 4. Rehydration ratio of "Deveci" pears dried under different conditions of pulses (PR=3 and 4), microwave powers (100 and 200 W), and vacuum levels (200 and 400 mmHg).

not differ significantly (P>0.05) and these process conditions resulted in the highest protein content of pears, whereas treatments at "100 W - 200 mmHg – PR=4" and "100 W - 200 mmHg – PR=3" caused the lowest protein content with comparable values (P>0.05). The possible reason for such a result might be the longer drying time. In an earlier study, protein content of fresh "Deveci" pear cultivar was found to be 3.8 g/L as reported by Öztürk *et al*. [2009], and an intermittent microwave-vacuum effect on the protein results was reported by Dermelj *et al*. [1995] and although impulse technique was gentler, a virtual increase was not observed in the protein content.

Rehydration ratio

The change in the rehydration ratio with change in the microwave power, pulse ratio, and vacuum level is shown in Figure 4. The rehydration ratio was found to be different between the drying treatments, and a higher rehydration ratio was observed at "100 W - 400 mmHg – PR=4". The higher pulse ratio (PR=4) increased the rehydration ratio values, but the microwave power modes did not show any meaningful influence. However, the increased vacuum level experiments presented a higher rehydration ratio. Similarly, Changrue *et al*. [2008] found for strawberry drying with a microwave--vacuum method that the generated power modes were inadequate to affect the rehydration property. According to Jiang *et al*. [2014], the pulse-spouted microwave vacuum-dried banana cubes had a lower final rehydration ratio (3.6) than freeze (3.9) and microwave freeze dried samples (3.7).

Color analysis

The color parameters of the fresh "Deveci" pears and these dried by intermittent microwave-vacuum are presented in Table 2. All drying experiments provided higher values of redness (a^*) and yellowness (b^*) but lower lightness (L^*). Drying conditions significantly (P<0.05) affected color values of "Deveci" pear samples. Using higher microwave power at a constant pulse ratio and vacuum level caused lower values of L^*. Furthermore, the maximum a^* value for the dried samples was $a^* = 17.3$ (P<0.05) in the drying experiment at "200 W - 200 mmHg – PR=3". Meanwhile, the significantly (P<0.05) higher b^* and C values were achieved at "100 W - 200 mmHg – PR=3". Compared with the fresh sample, a significant (P<0.05) decrease was determined in the α value in all experiments. In the present study, the flesh color of the fresh "Deveci" pears was found to be higher in L^* and a^* values but lower in b^*, C and α values than those previously reported by Ulubaş Serçe *et al*. [2010] using the same method. A similar effect of the microwave-vacuum drying method on the L^* value was found by Bai-Ngew *et al*. [2015].

Energy and specific energy analyses

Table 3 shows the effects of the microwave power, pulse ratio, and vacuum level on the energy and specific energy consumption values of the dried "Deveci" pear. The lowest energy consumption value was at "200 W - 400 mmHg – PR=3", likely due to the shortest drying time, despite the large power and pulse ratio input of the devices used in these processes. The energy consumption values of the "100 W - 400 mmHg – PR=3" and "200 W - 400 mmHg – PR=4" experiments were found to be approximately 1.4 and 1.5 times higher. The effect of the vacuum level clearly demonstrated that a strong relationship exists to reduce the energy consumption. For the same reason, the energy consumption values in the constant microwave power and pulse ratio experiments at a higher vacuum level were lower. Considering the specific energy consumption of "Deveci" pear drying, a higher vacuum level with a constant microwave power and pulse ratio resulted in a decreased consumption. The specific energy consumption, however, was increased by raising the microwave power from 100 W to 200 W. Because of the shorter drying times,

TABLE 3. Energy and specific energy consumption values of "Deveci" pears dried under different conditions of pulses (PR=3 and 4), microwave powers (100 and 200 W), and vacuum levels (200 and 400 mmHg).

Drying methods	Drying time (min)	Energy consumption (kWh)	Specific energy consumption (MJ/kg)
100 W 200 mmHg PR=4	460	2.6	1.6
100 W 200 mmHg PR=3	350	2.3	1.6
100 W 400 mmHg PR=4	390	2.2	1.4
100 W 400 mmHg PR=3	210	1.3	1.0
200 W 200 mmHg PR=4	280	1.6	1.9
200 W 200 mmHg PR=3	200	1.3	1.9
200 W 400 mmHg PR=4	230	1.3	1.6
200 W 400 mmHg PR=3	150	0.9	1.4

similar energy consumption results were reported by Liu *et al*. [2017] to dry asparagus cookies, and the lowest value was defined in pulse-spouted microwave vacuum drying. Additionally, microwave-vacuum drying time of the tomato was found to be approximately 4.8 times shorter than hot air drying as reported by Orikasa *et al*. [2018] because of the large power input of the devices used in that process.

Scanning electron microscopy (SEM) analysis

The effect of various drying conditions on the tissue structure of the dried "Deveci" pear slices was observed using scanning electron microscopy (Figure 5). The micrographs were examined by 1000× microscopy. The microwave-vacuum dried samples were demonstrated to be more regular in pore size and pore distribution in general. The cause may be rapid and extensive vaporization during microwave vacuum drying [Bai-Ngew *et al*., 2011]. Similarly, Jiang *et al*. [2014] provided better drying uniformity and production quality with pulsed microwave vacuum dried samples of banana. Again, the microwave-assisted vacuum-dried Litchis' structure was revealed to be clear and porous as shown by Song *et al*. [2015].

CONCLUSIONS

Quality analyses of "Deveci" pears regarding intermittent microwave-vacuum drying were performed. Overall, according to the experimental results, the "200 W - 400 mmHg – PR=3" drying condition showed the lowest drying time, the lowest energy consumption, and a higher protein content. When comparing the mineral content, the K, Mg, Fe, Cu and Zn levels were high at "100 W - 400 mmHg – PR=3". Moreover, higher microwave power usage did not have a meaningful influence on the rehydration ratio. Total color difference (ΔE) ranged between 35.7 ("200 W - 200 mmHg – PR=3") and 21.2 ("100 W - 400 mmHg – PR=3"). However, the structure of the dried samples was revealed to be clear and porous. Under these conditions, intermittent microwave-vacuum drying was proven to be an alternative for "Deveci" pear drying by some quality comparisons.

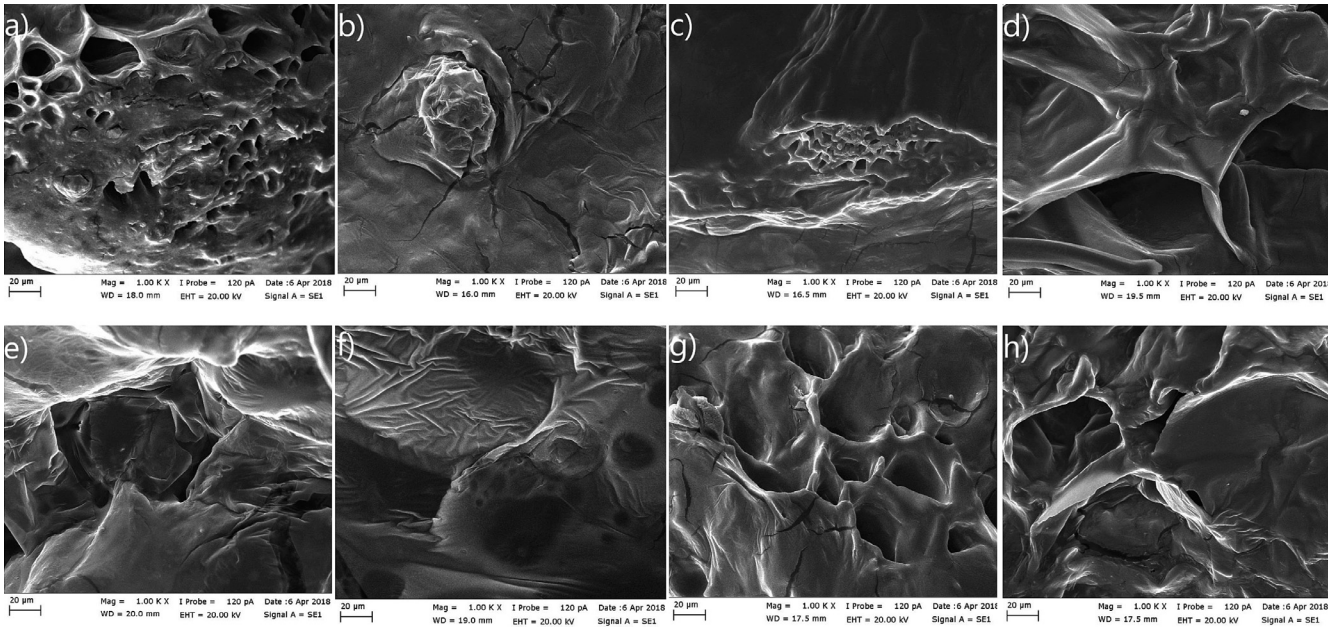


FIGURE 5. SEM images of dried pears: (a) 100 W - 200 mmHg – PR=4, (b) 100 W – 200 mmHg – PR=3, (c) 100 W - 400 mmHg – PR=4, (d) 100 W - 400 mmHg – PR=3, (e) 200 W - 200 mmHg – PR=4, (f) 200 W – 200 mmHg – PR=3, (g) 200 W- 400 mmHg – PR=4, and (h) 100 W – 400 mmHg – PR=3.

RESEARCH FUNDING

This research was financially supported by the Research Foundation of Uludag University (Project No. OUAP(Z)-2017/1).

CONFLICT OF INTERESTS

The authors report no declarations of interest.

REFERENCES

1. AlJuhaimi, F., Uslu, N., Özcan, M.M., ElBabiker, E.F., Ghafoor, K. (2016). Effect of drying on antioxidant activity, total phenol and mineral contents of pear fruits. *Journal of Food Safety and Food Quality*, *67*, 164–167.
2. Amiripour, M., Habibi-Najafi, M.B., Mohebbi, M., Emadi, B. (2015). Optimization of osmo-vacuum drying of pear (*Pyrus communis* L.) using response surface methodology. *Journal of Food Measurement and Characterization*, *9*(3), 269–280.
3. Antal, T., Tarek-Tilistyák, J., Cziáky, Z., Sinka, L. (2017). Comparison of drying and quality characteristics of pear (*Pyrus communis* L.) using mid-infrared-freeze drying and single stage of freeze drying. *International Journal of Food Engineering*, *13*(4), 125–146.
4. AOAC (1960). Association of Official Analytical Chemists. *Official Methods of Analysis* (9th ed.). Washington, DC
5. Bai-Ngew, S., Therdthai, N., Dhamvithee, P. (2011). Characterization of microwave vacuum-dried durian chips. *Journal of Food Engineering*, *104*(1), 114–122.
6. Bai-Ngew, S., Therdthai, N., Dhamvithee, P., Zhou, W. (2015). Effect of microwave vacuum drying and hot air drying on the physicochemical properties of durian flour. *International Journal of Food Science & Technology*, *50*(2), 305–312.
7. Celen, S., Kahveci, K. (2013). Microwave drying behaviour of tomato slices. *Czech Journal of Food Science*, *31*(2), 132–138.
8. Changrue, V., Orsat V., Raghavan, G.S.V. (2008). Osmotically dehydrated microwave-vacuum drying of strawberries. *Journal of Food Processing and Preservation*, *32*(5), 798–816.
9. Cheenkachorn, K., Jintanatham P., Rattanaprapa, S. (2012). Drying of papaya (*Carica papaya* L.) using a microwave-vacuum dryer. *World Academy of Science, Engineering and Technology*, *6*, 899–903.
10. Clary, C.D., Mejia-Meza, E., Wang, S., Petrucci, V.E. (2007). Improving grape quality using microwave vacuum drying associated with temperature control. *Journal of Food Science*, *72*(1), E23–E28.
11. Cui, Z.W., Xu, S.Y., Sun, D.W. (2003). Dehydration of garlic slices by combined microwave-vacuum and air drying. *Drying Technology*, *21*(7), 1173–1184.
12. Dermelj, M., Bogenrieder, C., Hídvégi, M., Lásztity, R. (1995). Effect of microwave vacuum drying on protein and chlorophyll contents of blind nettle (*Urtica urens* L.). *Periodica Polytechnica Chemical Engineering*, *39*, 77–84.
13. Dotto, G.L., Souza, T.B., Simões, M.R., Morejon, C.F., Moreira, M.F.P. (2017). Diffusive-convective model considering the shrinkage applied for drying of pears (*pyrus spp.*). *Journal of Food Process Engineering*, *40*(4), 1–8, art. no. UNSP e12503.
14. Doymaz, İ. (2013). Experimental study on drying of pear slices in a convective dryer. *International Journal of Food Science & Technology*, *48*(9), 1909–1915.
15. Doymaz, İ., İsmail, O. (2012). Experimental characterization and modelling of drying of pear slices. *Food Science and Biotechnology*, *21*(5), 1377–1381.
16. Dujmić, F., Brnčić, M., Karlović, S., Bosiljkov, T., Ježek, D., Tripalo, B., Mofardin, I. (2013). Ultrasound-assisted infrared drying of pear slices: textural issues. *Journal of Food Process Engineering*, *36*(3), 397–406.
17. FAO (2018). Food and Agricultural Organization statistical database. http://faostat3.fao.org/download/Q/QC/E
18. Ferreira, D., Da Silva, J.A.L., Pinto, G., Santos, C., Delgadillo, I., Coimbra, M.A. (2008). Effect of sun-drying on microstructure and texture of S. Bartolomeu pears (*Pyrus communis* L.). *European Food Research and Technology*, *226*(6), 1545–1552.
19. González-Martínez, C., Cháfer, M., Xue, K., Chiralt, A. (2006). Effect of the osmotic pre-treatment on the convective air drying kinetics of pear var. Blanquilla. *International Journal of Food Properties*, *9*(3), 541–549.
20. Guiné, R.P.F., Barroca, M.J., Silva, V. (2013). Mass transfer properties of pears for different drying methods. *International Journal of Food Properties*, *16*(2), 251–262. doi:
21. Guiné, R.P.F., Ferreira, D.M.S., Barroca, M.J., Gonçalves, F.M. (2007). Study of the drying kinetics of solar-dried pears. *Biosystems Engineering*, *98*(4), 422–429.
22. Gunasekaran, S., Yang, H.W. (2007). Effect of experimental parameters on temperature distribution during continuous and pulsed microwave heating. *Journal of Food Engineering*, *78*(4), 1452–1456. doi:
23. Han, Q.H., Yin, L.J., Li, S.J., Yang, B.N., Ma, J.W. (2010). Optimization of process parameters for microwave vacuum drying of apple slices using response surface method. *Drying Technology*, *28*(4), 523–532.
24. Huang, L.L., Qiao, F., Fang, C.F. (2015). Studies on the microstructure and quality of iron yam slices during combined freeze drying and microwave vacuum drying. *Journal of Food Processing and Preservation, 39*(6), 2152–2160.
25. İzli, G. (2017). Total phenolics, antioxidant capacity, colour and drying characteristics of date fruit dried with different methods. *Food Science and Technology (Campinas)*, *37*(1), 139–147.
26. Jiang, H., Zhang, M., Mujumdar, A.S., Lim, R.X. (2014). Comparison of drying characteristic and uniformity of banana cubes dried by pulse-spouted microwave vacuum drying, freeze drying and microwave freeze drying. *Journal of the Science of Food and Agriculture*, *94*(9), 1827–1834.
27. Kumar, V., Shrivastava, S.L. (2017). Optimization of vacuum-assisted microwave drying parameters of green bell pepper using response surface methodology. *Journal of Food Measurement and Characterization*, *11*(4), 1761–1772.
28. Kuş, S. (2016). Mikrodalga enerjisiyle ayva ve armut meyvesinin kuruma davranışı ve modellemesi. MSc Thesis, Namık Kemal University, Tekirdağ, pp. 1–70, [https://tez.yok.gov.tr/UlusalTezMerkezi/tezSorguSonucYeni.jsp], Available: 08.10. 2018.
29. Liu, Z., Zhang, M., Fang, Z., Bhandari, B., Yang, Z. (2017). Dehydration of asparagus cookies by combined vacuum infrared radiation and pulse-spouted microwave vacuum drying. *Drying Technology*, *35*(11), 1291–1301.
30. Lüle, F., Koyuncu, T. (2015). Convective and microwave drying characteristics of sorbus fruits (*Sorbus domestica* L.). *Procedia – Social and Behavioral Sciences*, *195*(3), 2634–2643.

31. Lutovska, M., Mitrevski, V., Pavkov, I., Mijakovski, V., Radojčin, M. (2016). Mathematical modelling of thin layer drying of pear. *Chemical Industry and Chemical Engineering*, *22*(2), 191–199.
32. Orikasa, T., Koide, S., Sugawara, H., Yoshida, M., Kato, K., Matsushima, U., Tagawa, A. (2018). Applicability of vacuum--microwave drying for tomato fruit based on evaluations of energy cost, color, functional components, and sensory qualities. *Journal of Food Processing and Preservation*, *42*(6), 1–12.
33. Ozturk, A., Ozturk, B. (2014). The rootstock influences growth and development of 'Deveci' pear. *Turkish Journal of Agricultural and Natural Sciences*, *6*, 1049–1053.
34. Ozturk, I., Ercisli, S., Kalkan, F., Demir, B. (2009). Some chemical and physico-mechanical properties of pear cultivars. *African Journal of Biotechnology*, *8*(4), 687–693.
35. Santos, S.C., Guiné, R.P., Barros, A. (2014). Effect of drying temperatures on the phenolic composition and antioxidant activity of pears of rocha variety (*Pyrus communis* L.). *Journal of Food Measurement and Characterization*, *8*(20), 105–112.
36. Silva, V., Figueiredo, A.R., Costa, J.J., Guiné, R.P.F. (2014). Experimental and mathematical study of the discontinuous drying kinetics of pears. *Journal of Food Engineering*, *134*, 30–36.
37. Song, C.F., Cui, Z., Jin, G.Y., Mujumdar, A.S., Yu, J.F. (2015). Effects of four different drying methods on the quality characteristics of peeled litchis (*Litchi chinensis Sonn.). Drying Technology*, *33*(5), 583–590.
38. Therdthai, N., Zhou, W. (2009). Characterization of microwave vacuum drying and hot air drying of mint leaves (*Mentha cordifolia Opiz ex Fresen*). *Journal of Food Engineering*, *91*(3), 482–489.
39. Tian, Y., Wu, S., Zhao, Y., Zhang, Q., Huang, J., Zheng, B. (2015). Drying characteristics and processing parameters for microwave-vacuum drying of kiwifruit (*Actinidia deliciosa*) slices. *Journal of Food Processing and Preservation*, *39*(6), 2620–2629.
40. Ulubaş Serçe, Ç., Gazel, M., Çağlayan, K., Özgen, M. (2010). Effect of *Candidatus phytoplasma pyri* infection on fruit quality, total phenolic content and antioxidant capacity of 'Deveci' pear. *Julius-Kühn-Archiv*, *427*, 407–411.
41. Zaki, N.A.M., Muhamad, I.I., Salleh, L.M. (2007). Drying characteristics of papaya (*Carica papaya* L.) during microwave-vacuum treatment. *International Journal of Engineering and Technology*, *4*(1), 15–21.
42. Zhang, Z., Wei, Q., Liu, C., Li, D., Liu, C., Jiang, N. (2017). Comparison of four pretreatments on the drying behavior and quality of taro (*Colocasia esculenta* L. Schott) slices during intermittent microwave vacuum-assisted drying. *Drying Technology*, *35*(11), SI, 1347–1357.
43. Zielinska, M., Sadowski, P., Błaszczak, W. (2016). Combined hot air convective drying and microwave-vacuum drying of blueberries (*Vaccinium corymbosum* L.): drying kinetics and quality characteristics. *Drying Technology*, *34*(6), 665–684.

Submitted: 23 July 2018. Revised: 9 October, 3 November, and 13 December 2018. Accepted: 18 December 2018. Published online: 07 February 2019.

http://journal.pan.olsztyn.pl Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 109–110

68 VOLUME'S REVIEWERS

The Editors gratefully thank the following reviewers for their valuable help in reviewing manuscripts published in this volume and other papers reviewed between 16th October 2017 and 31st December 2018.

Abaide E.R., Regional University of the Northwest of the State of Rio Grande do Sul, Ijui, Brazil
Abd El-Moniem G.M., National Research Centre, Dokki, Cairo, Egypt
Al-Kateb H., Birmingham City University, Birmingham, UK
Andlauer W., Institute of Life Technologies, University of Applied Sciences and Arts Western Switzerland, Sion, Switzerland

Barba de la Rosa A.P., IPICYT, Instituto Potosino de Investigacion Cientifica y Tecnologica, San Luis Potosi, Mexico
Battino M., Universita Politecnica delle Marche, Ancona, Italy

Bawa S.H., The University of the West Indies, St. Augustine, Trinidad and Tobago
Bodoira R.M., Universidad Nacional de Córdoba (UNC), Cordoba, Argentina
Bondoc I.D., University of Agricultural Sciences and Veterinary Medicine, Iasi, Romania
Bueano Costa R.G., Empresa de Pesquisa Agropecuária de Minas Gerais, Instituto de Laticínios Cândido Tostes, Brazil

Capriles V., Universidade Federal de São Paulo, Brazil
Casarotti S.N., Sao Paulo State University, Sao Paulo, Brazil
Chuck-Hernandez C., Tecnológico de Monterrey, Mexico
Culetu A., National Institute of Research and Development for Food Bioresources IBA Bucharest, Romania

Dardevet D., INRA, Clermont-Ferrand, France
Darewicz M., University of Warmia and Mazury in Olsztyn, Poland
Dey P., Rutgers The State University of New Jersey, New Brunswick, USA

Estevez E., Universidade do Algarve, Faro, Portugal

Falcao S., Instituto Politecnico de Braganca CIMO, Braganca, Portugal
Florek M., University of Life Sciences in Lublin, Lublin, Poland

Fotschki B., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland

Gomes da Cruz A., Federal Institute of Education, Science and Technology of Rio de Janeiro (IFRJ), Brazil
Gu L., University of Florida, Gainesville, USA
Guil-Guerrero J.L., Universidad de Almeria, Spain
Guillen-Bejarano R., Consejo Superior de Investigaciones Científicas (CSIC), Sevilla, Spain
Gurak P.D., Universidade Federal do Rio de Janeiro, Brazil

Iskra M., Medical University of Poznań, Poland

Jafari S., Department of Food Materials and Process Design Engineering, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran, Iran
Jarmoluk A., Wrocław University of Environmental and Life Sciences, Poland
Jurgoński A., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

Karwowska M., Lublin University of Life Sciences, Poland

Khaliq G., Lasbela University of Agriculture Water and Marine Sciences, Balochistan, Pakistan
Kołakowski P., DuPont, Poland
Kowalski S., University of Agriculture in Kraków, Poland
Kręgiel D., Technical University of Łódź, Poland

Malinowska-Pańczyk E., Gdańsk University of Technology, Poland
Mancini S., Universita di Pisa, Italy
Manganaris G., Cyprus University of Technology, Lemesos, Cyprus
Mao Ching-Feng, Southern Taiwan University of Science and Technology, Tainan City, Taiwan
Marciniak-Łukasiak K., Warsaw University of Life Sciences, Warsaw, Poland
Markiewicz L., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

Mascheroni R.H., Consejo Nacional de Investigaciones Cientificas y Tecnicas CIDCA, Buenos Aires, Argentina
Mateo J., Universidad de Leon, Spain
Matusevicius P., Lithuanian University of Health Sciences, Veterinary Academy, Lithuania
Michalska A., Wrocław University of Environmental and Life Sciences, Poland
Minkiewicz P., University of Warmia and Mazury in Olsztyn, Poland

Olszewska M., University of Warmia and Mazury in Olsztyn, Poland
Oroian M., Stefancel Mare University of Suceava, Suceava, Romania

Palazolo G.G., Universidad Nacional de Quilmes, Buenos Aires, Argentina
Pasqualone A., Universita di Bari, Italy
Pavelkova A., Slovak University of Agricultural in Nitra, Slovakia
Pegg R.B., The University of Georgia, Athens, GA, USA
Pourfarzad A., Iran

Roseiro L.B., Lab. Nacional de Energia e Geologi, Amadora, Portugal
Rutkowska J., Warsaw University of Life Sciences, Warsaw, Poland
Rybarczyk-Płońska A., Warsaw University of Life Sciences, Poland

Shimoyamada M., University of Shizuoka, Japan
Stajic S., University of Belgrade, Republic of Serbia
Stankowić M., University of Kragujevac, Republic of Serbia
Strączkowski M., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Białystok, Poland
Szymczak M., West Pomeranian University of Technology in Szczecin, Szczecin, Poland

Tamas A., University of Nyiregyhaza, Hungary
Tontul I., Necmettin Erbakan University, Konya, Turkey

Wanasundara J., Agriculture and Agri-Food Canada Science & Technology Saskatoon Research and Development Centre, Saskatoon, Canada
Wesołowski M., Gdańsk Medical University, Gdańsk, Poland
Wiczkowski W., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland
Wojdyło A., Wrocław University of Environmental and Life Sciences, Wrocław, Poland
Wronkowska M., Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland

Yang Z., Jilin University, Changchun, China

Zabot G., Universidade Federal de Santa Maria, Santa Maria, Brazil

Żary-Sikorska E., University of Science and Technology, Bydgoszcz, Poland

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 1, pp. 111–113
http://journal.pan.olsztyn.pl

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.

Polish Journal of Food and Nutrition Sciences uses CrossCheck's iThenticate software to detect instances of similarity in submitted manuscripts. In publishing only original research, PJFNS is committed to deterring plagiarism, including self-plagiarism. Your manuscript may be screened for similarity to published materials.

COPYRIGHT LICENSE AGREEMENT referring to Authorship Responsibility and Acknowledgement, Conflict of Interest and Financial Disclosure, Copyright Transfer, are required for all authors, i.e. *Authorship Responsibility and Acknowledgement*: Everyone who has made substantial intellectual contributions to the study on which the article is based (for example, to the research question, design, analysis, interpretation, and written description) should be an author. It is dishonest to omit mention of someone who has participated in writing the manuscript ("ghost authorship") and unfair to omit investigator who have had important engagement with other aspects of the work. All contributors who do not meet the criteria for authorship should be listed in an Acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chairperson who provided only general support. Any financial and material support should also be acknowledged. *Conflict of Interest and Financial Disclosure*: Authors are responsible for disclosing financial support from the industry or other conflicts of interest that might bias the interpretation of results. *Copyright License Agreement*: Authors agree that papers accepted become the copyright of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland, and may not be published elsewhere without the Editor's permission in writing.

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.

UNAUTHORIZED USE AND COPYRIGHT AGREEMENT. Published manuscripts become the property of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (IAR&FR PAS) and may not be published elsewhere without written permission. Unauthorized use of the PJFNS name, logo, or any content for commercial purposes or to promote commercial goods and services (in any format, including print, video, audio, and digital) is not permitted by IAR&FR PAS.

MANUSCRIPTS. A manuscript in English must be single-sided, preferably in Times New Roman (12) with 1.5-point spacing, without numbers of lines. The Editor reserves the right to make literary corrections and to make suggestions to improve brevity. English is the official language. The English version of the paper will be checked by Language Editor. Unclear and unintelligible version will be sent to the author(s) for correction.

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.

The reference list should only include peer-reviewed full-text works that have been published or accepted for publication. Citations of MSc/PhD theses and works unavailable to international Editors, Reviewers, and Readers should be limited as much as possible.

References in the text must be cited by name and year in square parentheses (e.g.: one author – [Tokarz, 1994]; two authors – [Słonimski & Campbell, 1987]; more than two authors – [Amarowicz *et al*., 1994]). If more than one paper is published in the same year by the same author or group of authors use [Tokarz, 1994a, b]. Unpublished work must only be cited where necessary and only in the text by giving the person's name.

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.

PAGE CHARGES. A standard publication fee has been established at the rate of 450 EUR + tax (if applicable) irrespective of the number of pages and tables/figures. Payment instructions will be sent to Authors via e-mail with acceptance letter.

Information on publishing and subscription is available from:

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;
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

Nutrition