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PAPER

# ANTIOXIDANT AND ANTI-INFLAMMATORY CAPACITIES OF PEPPER TISSUES

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## ABSTRACT

The objective of this study was to investigate the antioxidant and anti-inflammatory activities of five pepper varieties tissues. Green Bell peppers had the highest total antioxidant contents; while Red Chilli variety had the lowest antioxidant activities (ABTS was 3.89 μmol TE/g fw, DPPH was 2.82 μmol TE/g fw and FRAP was 16.95 μmol TE/g fw). The methanolic extracts of different peppers showed strong but different anti-inflammatory activity values (8.22 μg/ml - 9.52 μg). Yellow Bell, Red and Green Chilli had the highest anti-inflammatory activity followed by Green and Red Bell extracts, respectively. The results suggest that these varieties of pepper could contribute as sources of important antioxidant and anti-inflammatory related to the oxidative stress and inflammation prevention.

*Keywords*: pepper, antioxidant, anti-inflammatory, cell line

## 1. INTRODUCTION

The interest in natural food full of antioxidants and their therapeutic properties have recently increased dynamically. Pepper fruit belongs to the genus Capsicum, *Solanaceae* family with of more than 200 varieties (ZIMMER *et al.*, 2012; ALLEMAND *et al*., 2016). Pepper has spread of names reckoning on location and type; and the most common pepper names are chili, bell, green and red or just pepper (SUNG *et al.*, 2016). Pepper is considered an excellent source of bioactive nutrients (AMINIFARD *et al.*, 2012). The nutritive composition of peppers depends mainly on the several factors, including cultivar, agricultural practice (organic or conventional), maturity and storage conditions (NIMMAKAYALA *et al.*, 2016). Pepper fruits are popular due to their characteristic as flavor, texture, firmness and bright colors (SILVA *et al*., 2014a). In addition, peppers consumption is recommended due to the positive bioactive compounds impact on health, such as minerals, vitamins and antioxidant compounds (SILVA *et al*., 2013; SILVA *et al*., 2014b). The phytochemicals in pepper fruits have been reported to possess many pharmacological and biochemical properties, such as anti-allergic, anti-carcinogenic, antioxidant and anti-inflammatory activities (ROKAYYA *et al.*, 2019). They can be eaten fresh, pickled, smoked, dried, or in sauces (ALVAREZ-PARRILLA *et al.*, 2012). In fact, in Chinese medicine, pepper has been used for of stomach aches, arthritis, rheumatism, skin rashes, dog/snake bites and flesh wounds treatments (KIM *et al.*, 2016). The antioxidant properties were tested in several studies by using different approaches (LONKAR and DEDON, *et al.*, 2011). Free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), and trolox equivalent antioxidant capacity (ABTS) assays are the three most frequently used for measuring the antioxidant activities (MAGALHAES *et al.*, 2011).
Oxidative stress plays an essential role in cardiovascular diseases and pathogenesis cancer (MONTECUCCO *et al.*, 2011). The redox stress triggers the activation of immune cells which release pro-inflammatory cytokines, reactive nitrogen and oxygen species causing pathological pathways and physiological imbalances (LONKAR *et al*., 2011).
The present study, therefore aims to determine the total antioxidant, flavonoid and phenol, measure the bioactive activities such as (FRAP), (ABTS), (DPPH), NO production and cell viability (MTT).

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and cells

ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP, Quercetin, Folin-Ciocalteau, Trolox reagents and ascorbic acid, were from (Sigma, Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) and LPS were purchased from Sigma Inc. (St. Louis, MO, USA). The murine machrophage cell line, RAW 264.7 was purchased institute of biological sciences (Shanghai, China).

### 2.2. Sample preparation

Five different pepper varieties purchased from Shenyang city, China: Yellow Bell, Red Bell, Green Bell, Red Chilli and Green Chilli, respectively. Bell type (Yellow, Red and Green) and Chilli type (Red and Green). Yellow, Red and Green are Bell type from flowering plants genus in the nightshade family *(Solanaceae);* pepper with thick skin fruits

(approximately 112–217 g in weight); Red and Green Chilli are the fruits of genus *Capsicum* plants which are nightshade family *(Solanaceae)* members. Chilli peppers are varieties with cone shape and medium size (61-91 g in weight). Peppers were purchased in a local supermarket at commercial maturity. All the pepper varieties were cleaned and cut tissues into cubic of 10 . 10 . 10 $mm^3$ before processing. Freeze-dried (FD) treatment was operated 2h at -80°C then put in freeze drying machine (ALPHA 1-4 LSC, Germany) at -50°C and 0.04 atm for 48h. Tissues were grounded to powder, packed in $N_2$-vacuumed amber bottles and stored at -80°C until use.

**2.3. Antioxidant extraction**

Pepper powder (1.5 g) of each sample was extracted with 10 ml of 80% methanol, by stirring and sonicating for 20 min. The extracts were centrifuged at ~3000g for 20 min, the supernatant was stored at 4°C.

2.3.1. Total antioxidant, phenolic and flavonoid content determination

The total antioxidant capacity was determined by using ascorbic acid as a standard. The results were expressed as μg of ascorbic acid equivalent (AAE) per mg (PARASAD *et al*., 2013). The flavonoid content was determined on triplicate aliquots of the homogenous pepper extract (ILAHY *et al.*, 2011). Thirty-microliter aliquots of the extract were used for flavonoid determination. Samples were diluted with 90 μl methanol, 6 μl of 10% aluminum chloride, 6 μl of 1mol/l potassium acetate were added and finally 170 μL of methanol was added. The absorbance was done at 415 nm after 30 min. Quercetin was used as a standard for calculating the flavonoid content (mg Qe/g of fw).

2.3.2. Antioxidant activity determination (ABTS), (DPPH) and (FRAP) assay

Antioxidant activity was measured using the $ABTS^{\cdot}$ decoloration method using radical $ABTS^{\cdot}$ (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (KAUR *et al.*, 2013). DPPH assay was to evaluate ability of antioxidants toward the stable radical DPPH. A 0.2 ml aliquot of a 0.0062 mM of DPPH solution, in 20 ml methanol (95%) was added to 0.04 ml of each extract and shaken vigorously (SHUMAILA *et al.*, 2013). FRAP was operated according to the procedure (YOUNG *et al.*, 2013). The FRAP reagent included 300 (mM) acetate buffer, pH 3.6, 10 (mM) TPTZ in 40 (mM) HCl and 20 (mM) $FeCl_3$ in the ratio 10:1:1. Reduction of the ferric-tripyridyltriazine to the ferrous complex, which was measured at 593 nm. Results were expressed at μmol TE/g fw.

**2.4. Anti-inflammatory activity**

2.4.1. Anti-inflammatory extraction

Extracts were prepared by homogenization of 4 g of freeze-dried sample in 10 ml of 80% methanol, using an Ultra Turrax Digital Homogeniser T-25 (Ika Werke GMBH & Co., Staufen, Germany). Further, supernatants were combined and filtered using Whatman No. 1 paper. The concentrate was evaporated under vacuum to dryness. Finally, the extract residue was dissolved in (DMSO) dimethyl sulfoxide solution to give a final concentration of 20 mg/ml.

2.4.2. Cell viability assay

Mitochondrial respiration was determined by a mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, treated cells ($1x10^5$ cells/ml) were incubated with 5 mg/ml (MTT) in 96-well plates for 4h and solubilized in DMSO (150 µl/well). The extent of the reduction of MTT within the cells were measured at 490 nm (ALET *et al.*, 2015).

2.4.3. NO production measurement

For NO production determination, the amount of $NO_2$ in the supernatant of the media was measured by the Griess method (SURESH and PRIYA, *et al.*, 2015). Cells were incubated for 24h, after which the cell culture medium (0.2 ml) were added to aqueous extract of pepper varieties containing the Griess reagents (1%) sulfanilamide, (0.1%) naphthyl ethylene diamine dihydrochloride in (5%) $H_3PO_4$. The NO production was then determined based on the absorbance at 540 nm.

**2.5. Statistical analysis**

Data from replications of all varieties were subjected to a variance analysis (ANOVA) using SPSS (16.0.). Significant difference between the means was determined by Duncan's New Multiple Range Test ($p<0.05$). The correlation between the studied parameters was determined by (PCA) using XLSTAT software.

## 3. RESULTS AND DISCUSSION

### 3.1. Total antioxidant, phenol and flavonoid contents

In our study, all the extracts exhibited high total antioxidant activity values from 9.52 µg AAE/mg fw in Green Chilli to 12.70 µg AAE/mg fw in Green Bell (Table 1).

**Table 1.** Total antioxidant, phenol and flavonoid contents of selected pepper tissue varieties.

| | Total Antioxidant µg AAE/mg | Total Phenol mM (TEAC) | Total Flavonid mg Qe/g |
|---|---|---|---|
| Yellow Bell | 12.68±0.15$^a$ | 20.53±0.64$^a$ | 89.85±7.45$^d$ |
| Red Bell | 11.57±0.60$^b$ | 21.09±0.93$^a$ | 141.67±10.12$^b$ |
| Green Bell | 12.70±0.57$^a$ | 19.91±2.14$^a$ | 113.60±7.96$^c$ |
| Red Chilli | 10.21±0.18$^c$ | 20.45±1.71$^a$ | 75.85±4.20$^d$ |
| Green Chilli | 9.52±0.10$^c$ | 20.67±1.63$^a$ | 163.95±8.35$^a$ |

Values are the average of three individual samples each analyzed in triplicate ±standard deviation. Different uppercase superscript letters respectively indicate significant difference ($p < 0.05$) analyzed by Duncan's multiple-range test.

The antioxidant activity can be attributed to flavonoids and polyphenolic compounds found in it (KIM *et al.*, 2016). Total phenol contents ranged from 19.91 mM (TEAC) in Green Bell to 21.09 mM (TEAC) in Red Bell. Red and Green Chilli total phenol had similar values, 20.45 mM (TEAC) and 20.67 mM (TEAC), respectively. Flavonoid contents ranged from 75.85 mg QE/g fw to 163.95 mg QE/g fw, Green Chilli peppers had the highest flavonoid contents followed by Red Bell and Green Bell. Red Chilli and Yellow Bell varieties had lower flavonoid contents 75.85 mg QE/g fw and 89.85 mg QE/g fw, respectively than the other varieties (113.60 - 163.95 mg QE/g fw).

### 3.2. Antioxidant activity (ABTS, DPPH and FRAP)

Antioxidant activity results of pepper varieties were expressed as μmol TE/g fw in (Fig. 1). The antioxidant activity measured by ABTS· assay was between 3.89 μmol TE/g fw for Red Chilli and 5.18 μmol TE/g fw for Yellow Bell. The results obtained by DPPH assay were between 2.82 μmol TE/g fw for Red Chilli and 43.29 μmol TE/g fw for Green Chilli. The values of pepper varieties obtained by FRAP assay were between 16.15 μmol TE/ g fw for Red Chilli and 46.36 μmol TE/ g fw for Green Bell. Similar results of antioxidant activity using DPPH and FRAP assays have been reported (RAHIM and MAT, 2012). However, the antioxidant capacity also depends on many other factors including environmental conditions, genetics, post-harvest storage conditions, production techniques used, ext. (ANNA *et al.*, 2014).

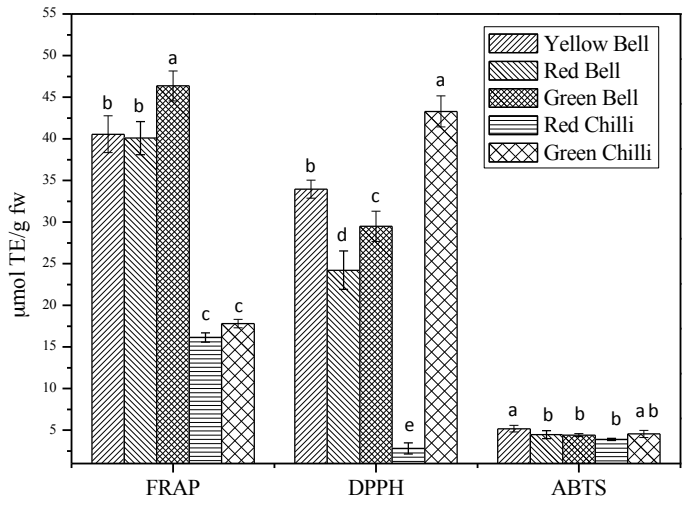


**Figure 1.** Antioxidant activities (ABTS, DPPH and FRAP) contents were expressed as (μmol TE/g fw).

### 3.3. Anti-inflammatory activity

The cytotoxicities of pepper extracts in LPS-induced macrophages were evaluated in a range of 0–200 μg extract/ml using MTT reduction assay after 24h (Fig. 2). Therefore, results indicated that the different concentration ranges of used in this study to treat the cells did not exert any cytotoxic effect. Analysis of NO production revealed that placing unstimulated RAW 264.7 cells in culture medium for 24h produced a basal amount of nitrite. When the cells were incubated with extracts from these varieties after treatment

with LPS for 24h, the medium concentration of nitrite increased markedly. Excessive production of NO in macrophages represents a probably noxious result, if not counteracted will cause the onset or progression of the many sickness pathologies (HAMIDREZA *et al.,* 2017). Significant concentration dependent inhibition was detected when cells were cotreated with LPS and various concentrations of the five variety extracts (Fig. 3).

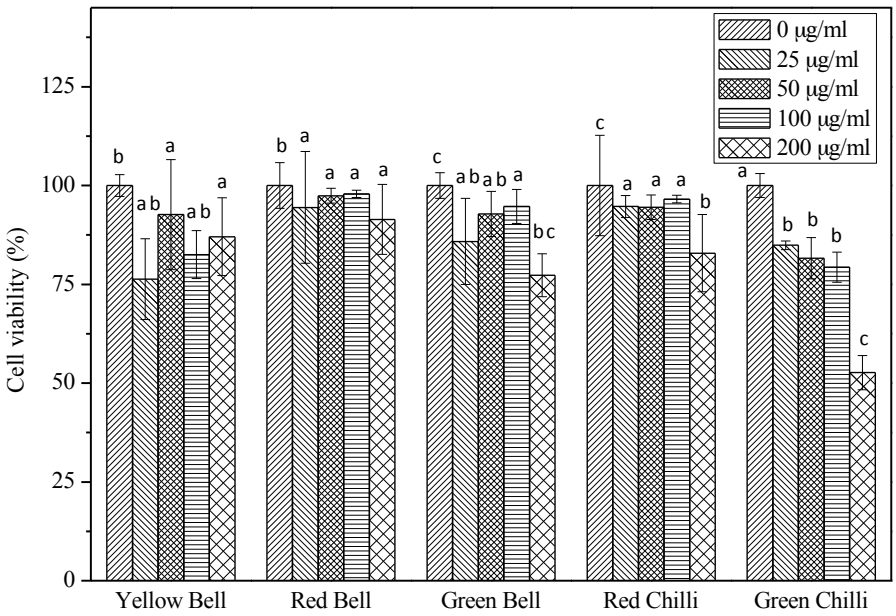


**Figure 2.** Cell viability.

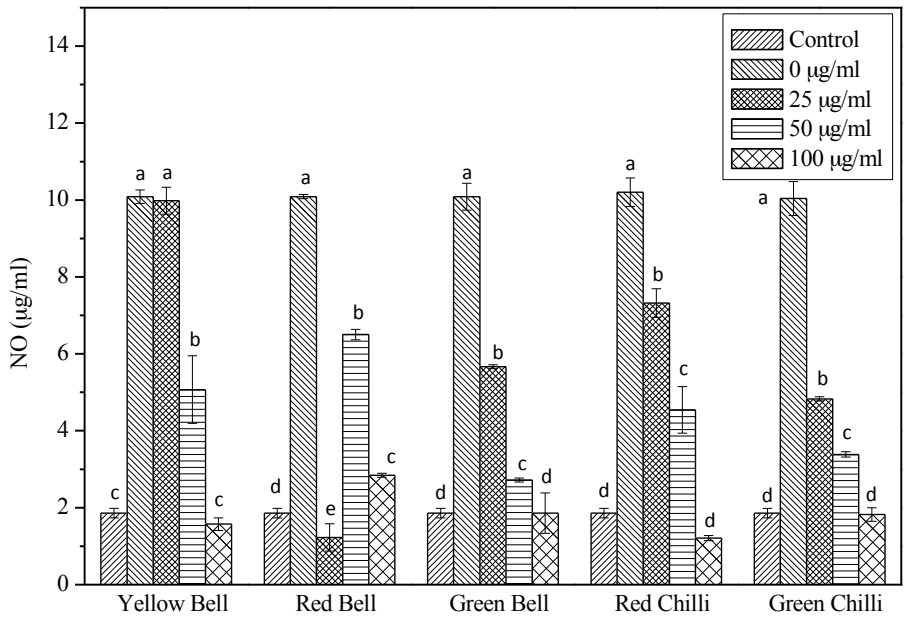


**Figure 3.** NO production by RAW 264.7 cells.

Pepper extracts induced a significant ($P<0.05$) dose-dependent suppression of NO production. All tested extracts showed high anti-inflammatory value in a range 0-100 μg concentration where Red, Green Chilli and Yellow Bell extracts were the best varieties followed by Green and Red Bell varieties. These results indicated that pepper had a noticeable effect on scavenging free radicals. NO production value grew equally in a dose dependant manner in all varieties except in Red Bell variety. A different reaction course was found for Red Bell. Red Bell showed an inverse relationship between the anti-inflammatory and the dose dependant manner at 25 μg/ml.

### 3.4. Correlation between antioxidant and anti-inflammatory activities

The analysis expressed that Green chili produced the lowest antioxidant level 9.52 μg AAE/mg fw with a little high anti-inflammatory activity 8.22 μg/ml (Fig. 4). Yellow Bell 12.68 μg AAE/mg fw and Green Bell 12.70 μg AAE/mg fw produced the highest antioxidant and high anti-inflammatory activity values 8.51 μg/ml and 8.23 μg/ml fw, respectively.

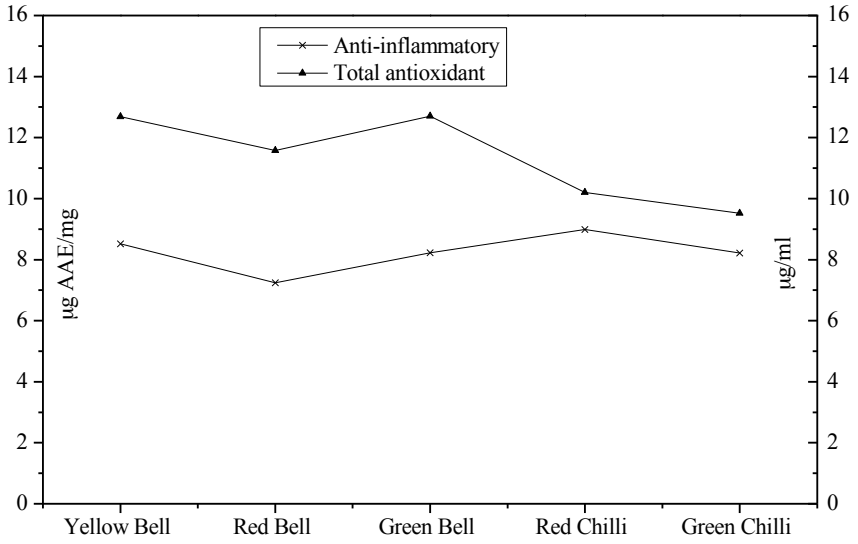


**Figure 4.** Correlation between antioxidant and anti-inflammatory activities.

The highest inflammatory activity level in Red Bell 7.25 μg/ml produced high antioxidant level 11.57 μg AAE/mg fw. Red Chilli produced a little high antioxidant and anti-inflammatory activity level 10.21 μg AAE/mg fw and 8.99 μg/ml, respectively.

### 3.5. Principal component analysis

Antioxidant and anti-inflammatory activities measurements had been submitted to (PCA) to presence of five subspecies of peppers, as seen in Table 2. The structuring accessions showed 70.38% of total variation. Axes were retained because they expressed 36.44% (axes 1), 33.94% (axes 2). Axes 2 was made positively by (DPPH), NO production, total phenol and flavonoid. The inertia was made negative by cell viability. Data projection on plans as

outlined by inertia axes of PCA from pepper samples showed vital significant variations between varieties. The (Figs. 5 and 6) present the plots of the scores/the correlation loadings respectively. In fact, once applying principal component analysis, it appeared that there was a discriminate structure. Yellow Bell and Green Bell were grouped together. As for Red Bell, Red and Green Chilli were individualized.

**Table 2.** Discriminate variables factors of principal components analysis.

| | F1 | F2 |
|---|---|---|
| Proper Value | 2.92 | 2.72 |
| Variability (%) | 36.44 | 33.94 |
| Cumulative (%) | 36.44 | 70.38 |
| Total Antioxidant | +29.57 | - |
| Total Phenol | - | +8.95 |
| Total Flavonoid | - | +33.62 |
| FRAP | +31.95 | - |
| DPPH | - | +23.61 |
| ABTS | +16.15 | - |
| Cell Viability | - | -13.22 |
| NO Production | - | +11.56 |

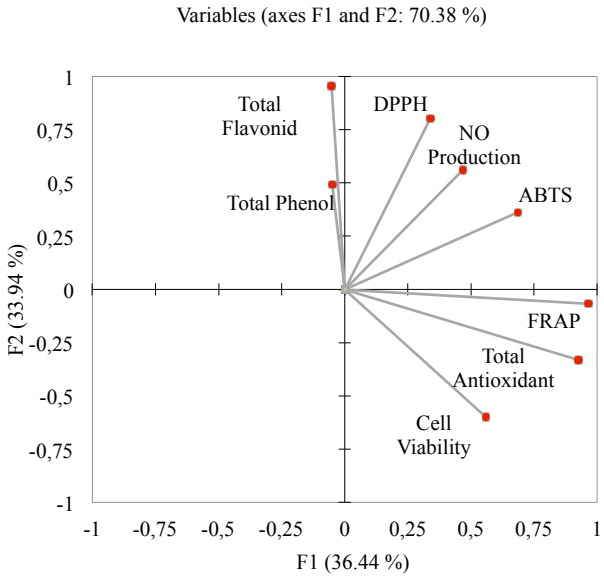


**Figure 5.** Plots of the scores for antioxidant and anti-inflammatory activities content.

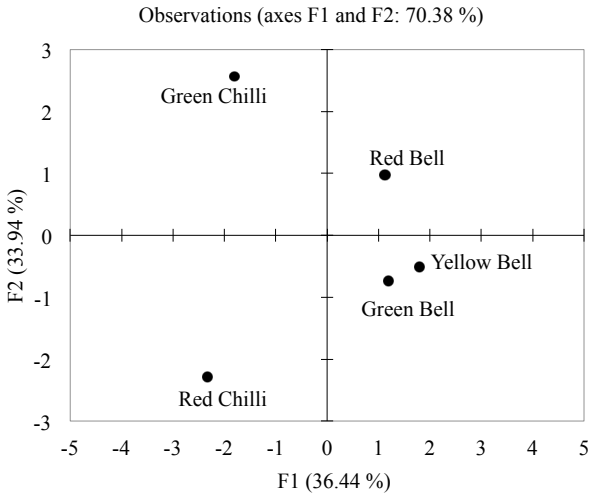


**Figure 6.** Plots of the x- loadings antioxidant and anti-inflammatory activities content.

## 4. CONCLUSION

The results of this study offer an experimental basis for the event of recent ways to provide knowledge of many pharmacological and biochemical properties. Although these results warrant further in-vivo studies, the presented in-vitro data suggest the potential of pepper to attenuate inflammation and oxidative stresses.

## ACKNOWLEDGMENTS

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PAPER

# ISOLATION OF BABY LIMA BEAN (*PHASEOLUS LUNATUS*) PROTEINS FRACTIONS AND EVALUATION OF THEIR ANTIOXIDANT ACTIVITY

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## ABSTRACT

The aim of this work was to obtain fractions of baby Lima Bean Protein Concentrate (LBPC) from (*Phaseolus lunatus*) and to evaluate their antioxidant activity. LBPC was prepared by alkaline extraction and isoelectric precipitation at pH 4.5. LBPC was subject to gastrointestinal digestion simulation. LBPC was fractionated using a DEAE Affi-Gel Blue Gel column. LBPC presented a protein content of 77.20% with a protein solubility capacity ranging between 37.34% to 99.98%. The antioxidant activity was evaluated using the FRAP, DPPH and ORAC methods. LBPC cationic fractions presented a high antioxidant activity when using the ORAC method with values ranging between 0.47 to 3.37 μmol TE/μmol of sample.

*Keywords*: baby lima bean, *Phaseolus lunatus*, simulated gastrointestinal digestion, protein concentrate, fractions, antioxidant activity

## 1. INTRODUCTION

Soybean Protein Isolate (SPI) are produced with defatted soybean flour. SPI is used in the food industry as a food ingredient for different purposes due to its functional properties such as a high solubility, a high foaming capacity and a high protein content. In Ecuador, soybeans and SPI imports for food purposes are high. The Government has implemented measures to promote the search for new native matrices that allow obtaining protein concentrates and isolates. Baby lima bean (*Phaseolus lunatus*) is a legume belonging to the Fabaceae family. This crop has been described for the exceptional potential in adaptation to lowland tropical conditions and potentially important as a food legume (DRAGO *et al.*, 2016; WU *et al*., 2016). Animal proteins, such as meat, milk, fish and eggs, are generally expensive and relatively difficult to acquire, which has led to a worldwide increase in research into vegetable protein sources. For their high protein content, legumes have formed an important part of this search being a cheaper, an alternative protein source and an important crop nitrogen fixative (SATHE and SALUNKHE 1981). Legumes are a source of quality protein in developing countries of South America such as Ecuador, Colombia, Peru and Venezuela. *Phaseolus lunatus* seeds have a high protein content with 26% of protein content (BETANCUR-ARCONA *et al., 2009).* The protein isolate obtained from *Phaseolus lunatus* presents a protein content of 71.13%-73.75%. This protein content in lima bean makes this plant to be an excellent protein source for food industry applications (CHEL-GUERRERO *et al.,* 2002). *Phaseolus lunatus* can be used to manufacture concentrate and isolate protein with excellent functionals properties.

The easiest way to obtain proteins from animal and plant sources is through alkaline extraction followed by isoelectric precipitation. For the characterization of the proteins present in protein isolates, different analytical techniques such as chromatography methods have been used. Many ion exchange columns (IEC) have been used to fraction different protein concentrates and isolate them to evaluate their biological activities and their possible use as functional ingredients in the food industry (CARRILLO *et al*., 2016a; 2016b; 2018; DE CASTRO and CASON 2017; GASPARD, 2017; TABTABAEI *et al*., 2017). *Phaseolus angularis* (red bean) globulins have been fractionated using an IEC of DEAE-Sepharose. Native and heated fractions were characterized using the electrophoresis technique. *Phaseolus vulgaris, Phaseolus lunatus, Canavalia ensiformis* and *Mucuna pruriens* legume plants belonging to the Fabaceae family have been hydrolyzed and fractionated with different methods such as ultrafiltration with membrane. Total hydrolysates and fractions have been described with different biological activities such as antioxidant, antibacterial, antihypertensive and antihyperglycemic activities using *in vivo* and *in vitro* models for their evaluation (CÁRDENAS *et al*., 2018; CUNSOLO *et al*., 2007; LUNA-VITAL *et al*., 2015; MAGAÑA *et al*., 2015; MAMILLA and MISHRA 2017; YOSHIDA 1989).

Different antioxidant activity methods are used to evaluate the potential of different compounds to determine the quality of foods of animal and vegetal sources. These antioxidant activity methods allow to enhance the nutritional and biological value of these components. These methods allow a biological characterization of bioactive compounds as polyphenols, proteins and peptides. Among the most used methods to evaluate antioxidant activity are the ORAC and FRAP methods. There is a high interest in finding new natural antioxidant compounds. Vegetal proteins can be a source of compounds with antioxidant abilities such as polyphenols, lipids, and proteins. Proteins can be hydrolyzed with different enzymes, being possible to simulate human condition of gastric digestion and duodenal digestion (HERNÁNDEZ-LEDESMA *et al*., 2004; ORSINI DELGADO *et al*., 2011; VILCACUNDO *et al*., 2018a;).

Protein concentrates and protein isolates with antioxidant activities have been reported by different authors (JE *et al*., 2005; OLAGUNJU *et al*., 2018; ORSINI-DELGADO *et al*., 2016; POWNALL *et al*., 2010). For example, quinoa protein concentrate (QPC) Chenopodium quinoa and amaranth protein concentrate (APC) Amaranthus caudatus have been described with antioxidant activity and the capacity to inhibit lipid peroxidation in the zebrafish model (VILCACUNDO *et al*., 2017; 2018b).
The aim of this study was to obtain baby lima bean (*Phaseolus lunatus*), protein concentrate, and determine its *in vitro* digestibility. Protein concentrate was fractionated using an exchange column, and the antioxidant activity was evaluated using the ORAC, FRAP and DPPH methods.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

Porcine gastric mucosa pepsin (4500 U/mg), porcine pancreas pancreatin (10000 U/mg), 2,2'-azobis (methylpropionamide)-dihidrochloride (AAPH), 2, 4, 6-tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium (FL) and trifluoracetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other reagents used in this study were of analytical grade.

### 2.2. Material vegetal

Baby lima bean (*Phaseolus lunatus*) samples were obtained from a native crop of the Recinto la Sequita of Manabí region (Manabí-Ecuador). The seeds of baby lima bean were collected in July,2016. The samples are registered in the ESPOL sample collection. The humidity of beans was determined with a value <10%. Then, the beans were ground using a Perten Laboratory mill 3100 and sifted in a sieve Advantech DuraTapTM DT168 with mesh # 70 (0.210mm). The obtained flour bean was vacuum packed and stored at laboratory temperature.

### 2.3. Proximate analysis

Proximal flour bean analysis and LBPC was performed, according to the official methods of the Official Association of Analytical Chemists (AOAC 1997). Moisture content was determined by the AOAC 925.10 method (AOAC 1997), protein by the AOAC 2001.11 Kjeldhal method (factor 6.25) (AOAC 2001), fat by the AOAC 922.06 method (AOAC 1997), and ash by the AOAC 923.03 method(AOAC 1997).

### 2.4. Preparation of LBPC

LBPC was prepared according to POVEDA *et al. (*2016). The bean flour was defatted with hexane (1:10 w/v) for 24 h. Then, 100 g of defatted flour was resuspended in Milli-Q water (1:10, *w:v*) at pH 9.5 for 15, 30, 45 and 60 min at 40°C. Then, the solution obtained was centrifuged at 5000 x g for 30 min at 25°C. The supernatant solution was adjusted to pH 4.5 using 1 N HCl and centrifuged for 20 min at 8000 x g at 25°C. The precipitated obtained was stored and then adjusted at pH 7.0 with 0.1 M NaOH. Then, the neutralized precipitated was lyophilized and frozen at -80°C.

## 2.5. Protein solubility capacity

LBPC were dissolved in distilled water at a concentration of 0.2% (*w:v*) and the pH of the suspension was adjusted to pH 2.0 – pH 10.0 using solutions of 0.001N HCl and 2N NaOH. The suspensions were shaken for 1 h and centrifuged at 10000 rpm for 10 min in a Sorvall Legend Micro 17 centrifuge (ThermoFhiser Scientific, Germany). The content of protein in the supernatant was analyzed with the BCA protein method. The content of soluble protein was expressed as the percentage of the content of protein present in the sample (PAZMIÑO *et al.*, 2018).

## 2.6. Fractionation of LBPC with an anion-exchange column

20 mg/mL of LBPC was centrifuged at 10000 x g for 20 min and 1 mL of supernatant was injected in the column of anionic exchange chromatography DEAE Affi-Gel® Blue Gel (Bio-Rad, Hercules, CA, USA). Then, 10 mL of buffer Tris-HCl 50 mM at pH 7.0 was loaded in the column to elute proteins not adhered in the column as proteins with charge positive. Then, 10 fractions of 1 mL each, were collected, then 10 mL of NaCl 500 mM were loaded in the column to elute the retained proteins (negative proteins) (QI *et al.*, 2001). 10 fractions of 1 mL each, were collected to be analyzed using the SDS-PAGE electrophoresis method. Protein contents of fractions were determined using the BCA method.

## 2.7. Gastric and duodenal digestion of LBPC

LBPC (10 mg/mL) was subject to simulated gastric digestion using pepsin enzyme (2000 U/mL) at pH 3.0 for 2 h at 37°C with agitation. The pepsin enzyme was inactivated by heating at 80°C for 10 min. One milliliter of gastric digestion was mixed with one milliliter of pancreatin enzymes (100 U/mL) at pH 7.0 for 2 h at 37°C. The enzymatic reaction was stopped by heating at 90°C for 10 min (PIÑUEL *et al.*, 2019).

## 2.8. % degree of hydrolysis by the Orthophthalaldehyde (OPA) assay

The hydrolysis degree (%DH) of gastric and duodenal digest from LBPC was determined using the OPA method described by PIÑUEL *et al.* (2019).
OPA solution: 25 mL of sodium tetraborate (100 mmoL/L) was mixed with 2.5 mL of 20% (w/v) sodium dodecyl sulfate, 40 mg of OPA was dissolved in 1.0 mL of methanol and 100 $\mu$L of 2-mercaptoethanol. The final volume was of 50 mL.
Derivatization OPA: 10 $\mu$L of the sample was mixed with 3.4 mL of the OPA solution and the mixture was stored at 25°C for 2 min. The absorbance was measured at 340 nm. %DH was determinate with the equation:

$$\%DH = Abs \times 1{,}934 \times d/c$$

where Abs is the sample absorbance, d is the factor of dilution and c is the concentration of protein in LBPC (mg/mL).

## 2.9. SDS-PAGE electrophoresis analysis of LBPC and their fractions

LBPC and LBPC fractions were analyzed using the SDS-PAGE electrophoresis method. The samples were analyzed using 15% polyacrylamide gels in a Mini-Protean

electrophoresis system (Bio-Rad, Hercules, CA, USA). The standard protein (Precision Plus ProteinTM Unstained standard, Bio-Rad) with a range of 10 kDa-250 kDa was used to determine the molecular weight of proteins present in the samples (QUINTEROS *et al.*, 2016; TOAPANTA *et al*., 2016).

### 2.10. RP-UHPLC analysis of LBPC fractions

LPBC and LBPC fractions were analyzed by RP-UHPLC technique using the Agilent 1200 infinity series UHPLC (Agilent Technologies, Waldbron, Germany). The signal of the chromatograms was registered at the wavelength of 280 nm. The separation of the samples was made with the help of a Zorbax EC C18 Agilent Poroshell 120 (4.6 mm x 50 mm x 2.7 $\mu$m) analytical column. The solvents used were solvent A [Milli-Q water + TFA 0.37%] and solvent B [acetonitrile + TFA, 0.270%]. Samples were eluted at 1.0 mL/min with a lineal gradient from 0% to 70% of solvent B for 15 min. Before analysis all samples were filtered with a membrane filter of 0.45 $\mu$m and then were centrifuged for 2 min at 12000 rpm at 4°C. Samples were injected for three times (LARA *et al.*, 2017).

### 2.11. Antioxidant activity of LBPC and their fractions

The colorimetric assays ferric ion reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was performed following the procedure described by BENZIE and STRAIN (1996) and PIÑUEL *et al*. (2019) respectively. The antioxidant capacity of LBPC and LBPC fractions was evaluated using an FRAP assay. The solutions used were 300 mM acetate buffer at pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ solution. A new working solution was prepared by a mixture of 25 mL acetate buffer with a 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ $6H_2O$ solution and then incubated at 37°C before use. LBPC and fractions (150 mL) were mixed with 2850 mL of the FRAP solution for 30 min in darkness. Readings of the colored product were measured at a wavelength at 593 nm. The standard Trolox linear curve was used as control (20 to 1200 mM). All results were expressed as mM of Trolox equivalents (TE) per g sample. All experiments were made in triplicate. LBPC and fractions were used to evaluate their antioxidant activity using the DPPH method. The ability to capture free radicals by antioxidants was analyzed using the radical species DPPH, measuring the decrease of the absorbance at 517 nm spectrophotometrically (spectrum SP-2100UV/SP spectrophotometer, China). Each assay was made five times, the value of antioxidant activity being expressed as mg of TE/100 g sample.

The oxygen radical absorbance capacity fluorescein (ORAC-FL) assay was made according to MOORE *et al* (2005). A synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) was used. Samples and Trolox standards were prepared with 50% acetone. All other reagents were prepared in a 75 mmol/L phosphate buffer (pH 7.4). Each well in a 96-well plate contained 30 µL of 20 µmoL/mg sample or 50% acetone for blank and 225 µL fluorescein (81.63 nmol/L). The plate with a cover was incubated for 20 min in 37°C, and then 25 µL AAPH (0.36 mol/L) were added to each well to start reaction, resulting in a final total volume of 280 µL. The fluorescence was recorded every minute for 2 h at 37°C, where excitation and emission of wavelengths were 485 and 528 nm. Trolox was used as standard (0-200 $\mu$moL) with a standard curve ($y=0.034x+0.6068$), $R^2=0.999$. Standards and samples were performed in triplicate. Results were expressed as $\mu$mol TE/$\mu$mol of sample.

### 2.12. Statistical analysis

Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The post hoc analysis was made by the Tukey and Dunnet test. All tests were considered significant at $P < 0.05$ using the software GraphPad Prism 4.

## 3. RESULTS AND DISCUSSION

### 3.1. Analysis proximal of flour bean and LBPC

The table 1 shows the proximate analysis of flour bean and LBPC. Flour bean present moisture with a value of 8.95% and LBPC present a value of 9.37%. Flour bean present a protein content of 20.48% and LBPC present a protein content of 77.20%. LBPC present an increase of protein content. The carbohydrates content of flour bean was 65.42%. LBPC present a decrease with a value of 10.63%. CHEL-GUERRERO *et al*. (2002); GALLEGOS-TINTORÉ, *et al*. (2004); TORRUCO-UCO *et al*. (2009); BETANCUR-ANCONA *et al*. (2009); GUZMÁN-MÉNDEZ *et al*. (2014) and DRAGO *et al*. (2016) report protein contents from lima bean with values of 71.13%, 71.13%, 71.80%, 69.90%, 71.88% y 74.06%. The protein content in this study is higher compared to the values reported. These differences are may be due to the varieties used in each study.

**Table 1.** Proximate analysis of flour bean and LBPC.

| Analysis | Flour bean (%) | LBPC (%) |
|---|---|---|
| Moisture | 8.95%±0.14 | 9.37%±0.26 |
| Protein | 20.48%±1.17 | 77.20%±0.02 |
| Fat | 1.57%±0.10 | 0.56%±0.03 |
| Ashes | 3.58%±1.23 | 2.24%±0.30 |
| Carbohydrates | 65.42±2.25 | 10.63±3.10 |

Results are expressed as mean±standard deviation (n=3)

### 3.2. Protein solubility capacity

Protein solubility is considered one of the most important functional properties in proteins. When a food protein has high solubility, this ingredient can be used for many industrial proposes, a food protein with low solubility decreases the industrial possibilities when used as an ingredient. Solubility capacity can affect other protein functional properties.

The flour bean solubility curve is shown in Fig. 1A. Proteins are more soluble in acidic regions (pH 2.0) and in alkaline regions (pH 12) with a value of 100%. Between pH 3.0 to pH 5.0 the solubility is reduced. Between pH 6.0 to pH 10.0 the solubility of proteins is relatively good. LBPC proteins solubility profile presents the typical U-shape of the legume extracts, with a minimum solubility at the isoelectric point and a greater solubility at low acidic pH and high alkaline pH (PAZMIÑO *et al.,* 2018). At pH 2.0 and pH 12.0,

LBPC present the highest percentage of solubility with values of 99.98±0.04% and 98.82±0.05%. At pH 6.0, these proteins present solubility values of 45.71±0.02% and at pH 8.0 these proteins present solubility values 57.95%. The lowest solubility was reached at pH 4.0 with a value of 37.34% (Fig. 1B). SEIDU *et al*. (2015) reported protein solubility of protein from skin lima bean with percentage between 25% to 90%. BETANCUR-ANCONA *et al*., 2009 reported protein solubility capacity of protein isolate obtained of lima bean with values of 15% to 70%. CHEL-GUERRERO *et al*., 2002 reported protein solubility of protein isolate from lima bean with values between 5% to 70%.

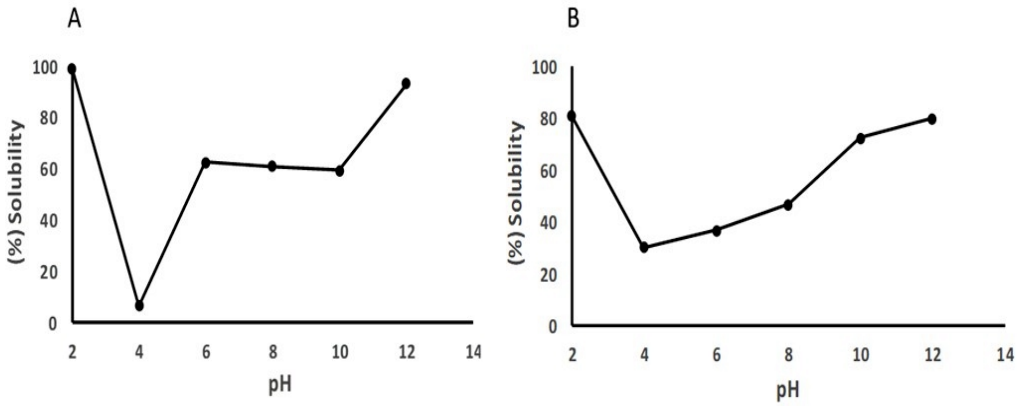


**Figure 1.** Percentage of solubility of flour and LBPC at different pHs.
A) flour bean B) LBPC.

### 3.3. LBPC protein profile and their digestibility

LBPC was obtained by alkaline extraction at pH 9.5 for 15-30-45 and 60 min of agitation at 40°C, followed by isoelectric precipitation at pH 4.5 and then analyzed by SDS-PAGE electrophoresis. Fig. 2A shows the LBPC proteins profile with bands between 10 kDa to 100 kDa. The bands with the higher intensity present molecular weights of 22 kDa, 25 kDa and 30 kDa. The bands with the lower intensity were the bands with low molecular weights with 10 kDa, 15 kDa, 20 kDa, 55 kDa and 60 kDa. The protein profile is composed as follows: one band of 100 kDa, one band of 70 kDa, doublet with 55 kDa and 60 kDa, triplet with 40 kDa, 35 kDa and 30 kDa, triplet of 20 kDa, 22 kDa and 25 kDa.
SPARVOLI *et al*. (1996) reported a similar protein profile identified as phaseolin from lima bean (*Phaseolus lunatus*) with molecular weights of one band (70 kDa), doublet (54 kDa - 58 kDa), triplet (32 kDa, 35 kDa and 38.5 kDa) and doublet (21 kDa - 25 kDa). These samples were analyzed for reaction of Western blot against phaseolin (*Phaseolus vulgaris*). The LBPC protein profile was the same at the different times alkaline extraction was assayed (15-30-45-60 min). Flour of *Phaseolus lunatus* presents a 20.48% of protein content. LBPC present a higher protein content with a value of 77.20% (Table 1).

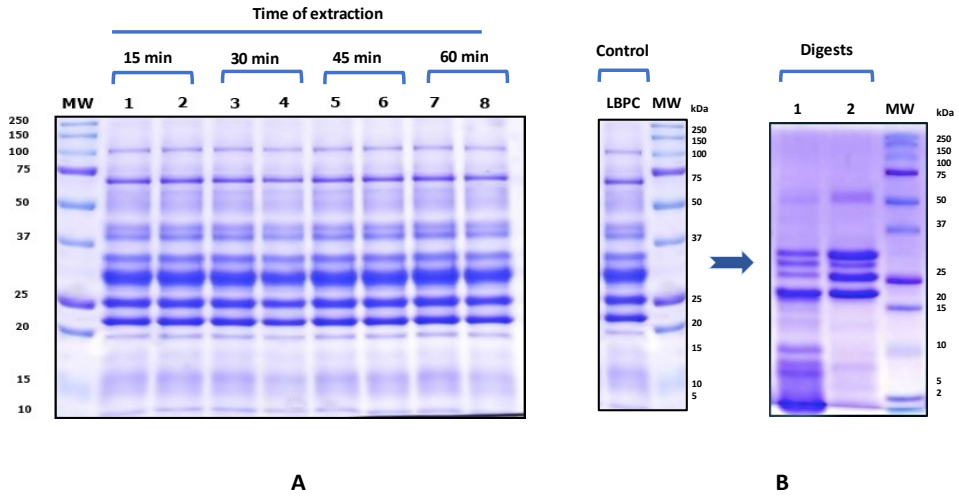


**Figure 2.** SDS-PAGE electrophoresis analysis of LBPC and gastric and duodenal digest of LBPC.
A) Profile protein of LBPC obtained at different times at 40°C and pH 4.5. MW (molecular weight).
B) LBPC control (without hydrolysis).
Lane 1: gastric digest of LBPC, lane 2: duodenal digest of LBPC.

In common bean (*Phaseolus vulgaris*) phaseolin protein content represents around 50% of the total seed protein (BOSCHIN *et al.*, 2014). It is made up of a small number of polypeptides and presents an extensive variation in the electrophoretic pattern, mostly observed among wild-growing beans. Comparison of electrophoretic patterns of total seed proteins of cultivated species belonging to the genus *Phaseolus* showed that the *Phaseolus lunatus* pattern is quite different from the others, lacking major polypeptides with molecular mass similar to those of phaseolin (LIOI, 1987).
Different authors have reported 7S globulin (vicilin) from *Phaseolus vulgaris*. with molecular weights between 40 kDa to 54 kDa. MONTOYA *et al.* (2008; 2010) reported the protein profile of a different variety of Phaseolus. They reported a high content of globulins and reported 2 to 6 bands between 40 kDa to 54 kDa. They identified these bands as Vicilin (7S globulin). CARRASCO-CASTILLA *et al.* (2012) reported *Phaseolus vulgaris* protein profiles. They identified ten protein bands with molecular weights (MW) ranging from 15 to 200 kDa in the protein isolate. The 41 kDa and 46 kDa bands, correspond to the phaseolin subunits and the most abundant proteins. The 15 kDa, 18 kDa, 25 kDa and 32 kDa, bands correspond to proteins belonging to the lectin-family.
GARCÍA-MORA *et al.* (2015) reported a protein profile from *Phaseolus vulgaris*. var. pinto, with bands between 10 kDa to 97 kDa. Bands with molecular weights of 25 kDa, 45 kDa and 50 kDa were identified as phaseolin. Phytohemagglutinins (32 kDa), $\alpha$-amylase inhibitor (18 kDa) and $\alpha$-amylase $\beta$ subunit (15 kDa) were identified in the pinto bean protein concentrate. Phaseolin band in *Phaseolus vulgaris* present high intensity and represent about 50% of total protein content. *Phaseolus lunatus* presents an absence of this high intensity band. Phaseolin protein of *Phaseolus lunatus* corresponds to another band with a different molecular weight.
LBPC and their fractions were subject to an *in vitro* gastrointestinal digestion simulation using pepsin enzyme for the gastric phase and a pepsin/pancreatin enzymes mix for the duodenal phase. In the gastric phase, bands with molecular weights of 20 kDa, 22 kDa and 25 kDa present resistance to hydrolysis with pepsin. We found bands with molecular

weights of 50 kDa and under 10 KDa. Phaseolin protein presents resistance to hydrolysis with pepsin/pancreatin (Fig. 2B).
In the duodenal phase, the protein profile of hydrolysate present bands with molecular weights between 20 kDa to 50 KDa. Bands with low molecular weight were partially hydrolyzed with pepsin/pancreatin. Bands of 20 kDa, 22 KDa and 25 KDa present resistance to duodenal hydrolysis. *Phaseolus lunatus* phaseolin protein presents resistance to hydrolysis with pepsin and pepsin/pancreatin. Phaseolin from different *Phaseolus* species has been reported to be resistant to enzymatic hydrolysis with pepsin and pepsin/pancreatin.
LBPC gastric digest present 9.3% DH and LBPC duodenal digest present 16.2% DH. These results are in accordance with the resistance at the hydrolysis observed in the electrophoresis analysis. BETANCUR-ANCONA *et al. (*2009) reported protein isolate (*Phaseolus lunatus*) hydrolysis using an enzymatic (trypsin, chymotrypsin and peptidase) mix, with a hydrolysis degree of 79.8% of HD. Different *in vitro* hydrolysis methods have been used to evaluate *in vitro* hydrolysis of common bean seeds. There are differences in the %HD reported in these studies. These differences must be due to the type and variety of seeds, geographic position of the cultivar and the differences in the method of hydrolysis and enzymes used, time of incubation, pHs of simulation, temperature, proportion of enzymes and combination of enzymes. For example, MONTOYA *et al.* (2008; 2010) reported hydrolysis of isolated phaseolin, treated and not treated thermally, of 43 varieties hydrolyzed with pepsin at pH 2.0 and hydrolyzed with pepsin and pancreatin dissolved at pH 7.5. In the gastric phase, at 120 min of incubation with pepsin the %HD was 5.2% in the unheated sample and 7.5% in the heated sample. In the duodenal phase, at 360 min of incubation with pepsin and pancreatin, the %HD was 11% to 27% for the unheated sample and 57% to 96% for the heated sample. The gastrointestinal digest presented a high % HD but the results were different depending on the variety of *Phaseolus vulgaris* used.
TORRUCO-UCO *et al. (2009) reported* hydrolysates of *Phaseolus vulgaris* from Mexico obtained with Alcalase and Flavourzyme for 30 min. They found a %DH of 49.48% and 26.05% respectively.

### 3.4. Characterization of fractions of LBPC by SDS-PAGE electrophoresis

LBPC fractions were obtained using a DEAE Affi-Gel Blue Gel chromatographic column. Ten cationic and anionic fractions were obtained to be analyzed with RP-UHPLC and SDS-PAGE electrophoresis. The anionic fractions numbered as fraction 1, F1, fraction 2, F2, fraction 3, F3 and fraction 4, F4 were chosen for their high protein content. The cationic fractions numbered as fraction 5, F5, fraction 6, F6, fraction 7, F7 and fraction 8, F8 were chosen for their high protein content. Fig. 3 shows the protein profile of cationic and anionic LBPC fractions. All fractions present an identical profile of proteins with molecular weights between 20 kDa to 50 kDa. Triplet with bands of 20 kDa, 22 kDa and 25 KDa were the bands with higher intensity. These bands correspond to the phaseolin protein. In the Cationic fractions, F5 and F6 present a higher protein content than F3 and F4. In the anionic fractions, F3 and F4 present a higher protein content than F5 and F6. The cationic fractions present a higher protein content than the anionic protein content. SPARVOLI *et al.* (1996) reported cationic fractions from *Phaseolus lunatus* obtained by an ion exchange chromatography with a Mono-Q HR5/5column coupled to the FPLC system. They reported nine cationic fractions with presence of triplet bands with molecular weights of 32 kDa, 35 kDa and 38.5 kDa in all fractions. These bands correspond to the

phaseolin protein. In this study, all fractions present triplet of bands but with lower molecular weights (20 kDa, 22 kDa and 25 kDa). The protein content fractions were determined using the BCA method. The anionic fractions, F1 (10.4%), F2 (11.5%), F3 (17.4%) and F4 (49.4%) have the percentage of protein content in brackets. These previous results show a correlation of the protein content with the intensity of the band in the gels of polyacrylamide. Cationic fractions F5 and F6 present a higher protein content with values of 77.9% and 77.2% of protein respectively.

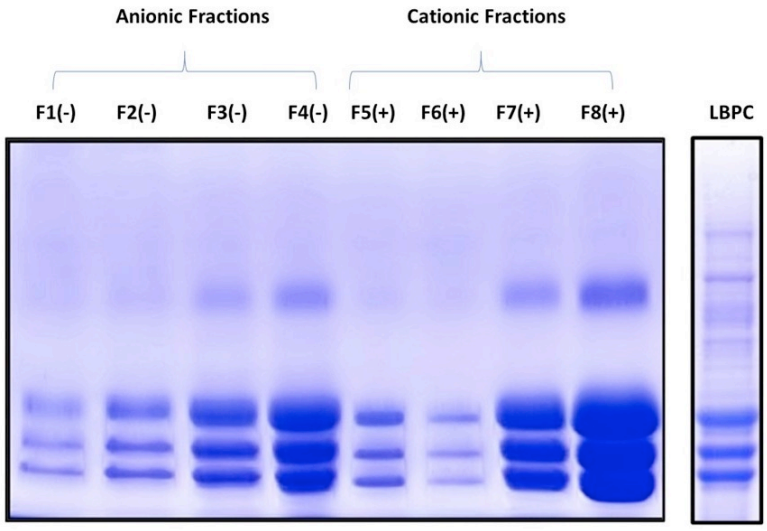


**Figure 3.** Protein profile of LBPC and cationic and anionic fractions using SDS-PAGE electrophoresis analysis.

### 3.5. Characterization of LBPC fractions by UHPLC analysis

Cationic and anionic fractions of LBPC were characterized using the UHPLC technique. The cationic fractions F5, F6, F7 and F8 have the same profile of proteins with four peaks in the chromatogram. F8 present higher intensity in the absorbance at 280 nm. Peak number one shows the highest intensity with a value of 20 AU. These results show a correlation with the intensity of the bands in the gel SDS-PAGE and the percentage of protein determined by the BCA method. These results suggest that this fraction present a higher protein content. These proteins are rich in tryptophan, this amino acid is absorbed at 280 nm (Fig 4).

LBPC anionic fractions show three peaks. F1 and F2 present minor absorbance at 280 nm. These results show a correlation with the SDS-PAGE electrophoresis results of these fractions. F3 and F4 present major absorbance at 280 nm and in the gel SDS-PAGE these fractions present a higher protein content because the staining is strong. Peak number one of fractions F3 and F4 shows a high content of tryptophan amino acid in these sequences, if we look at the polyacrylamide gel and the percentage of protein calculated by the BCA method (Fig. 5).

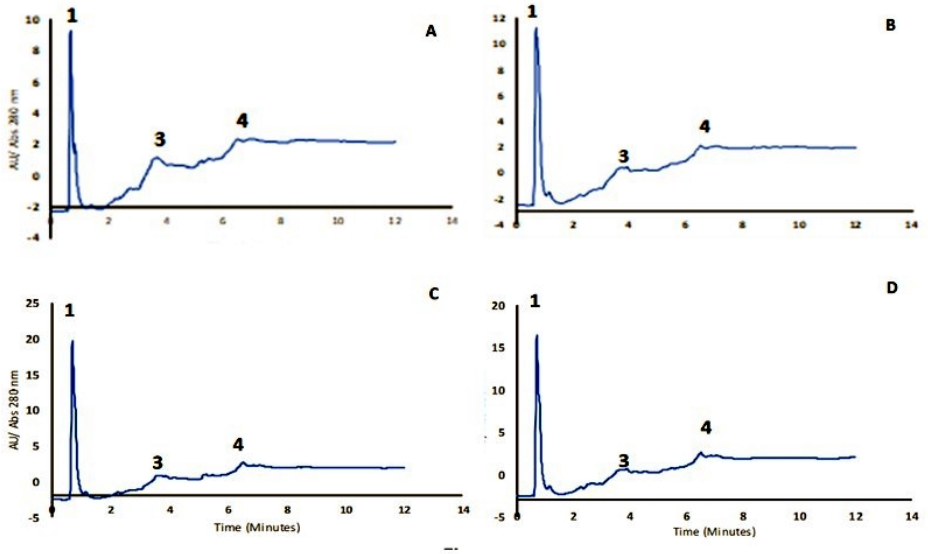


**Figure 4.** RP-UHPLC analysis of anionic fractions of LBPC. A) anionic fraction F1, B) anionic fraction F2, C) anionic fraction F3 (and D) anionic fraction F4.

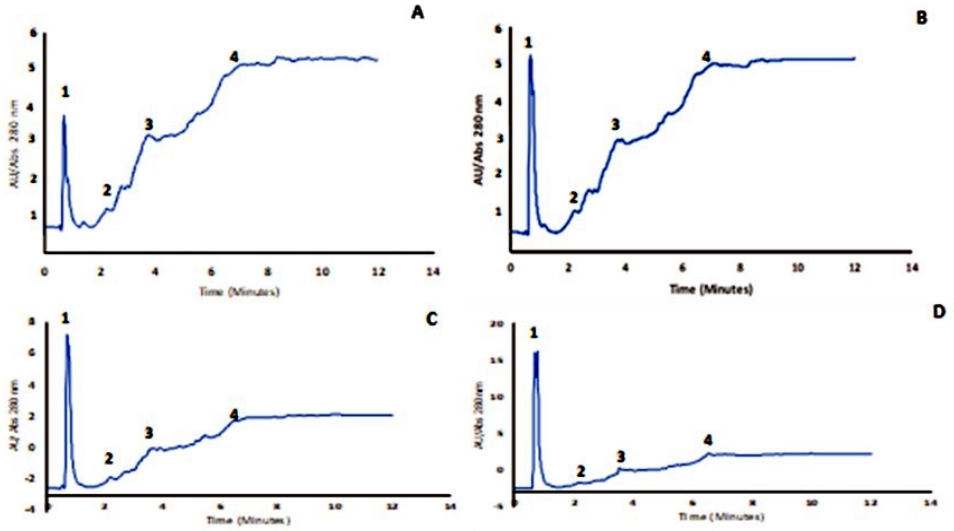


**Figure 5.** RP-UHPLC analysis of cationic fractions of LBPC. A) cationic fraction F5, B) cationic fraction F6, C) cationic fraction F7 and D) cationic fraction F8.

### 3.6. Antioxidant activity of LBPC and fractions of LBPC

Fig. 6 shows the results of LBPC and their fractions antioxidant activity, using the FRAP method. All samples assayed present antioxidant activity. LBPC present a value of 1.67±0.14 mg TE/g of sample. This is the highest value. The positive fractions present the

higher activities, F5 with value of 1.26±0.06 mg TE/g of sample, F6 present a value of 1.61±0.37 mg TE/g of sample, F7 present a value of 1.15±0.05 mg TE/g of sample, F8 present a value of 1.53±0.26 mg TE/g of sample. LBPC negative fractions were active with values between 1.15±0.05 to 1.23±0.18 mg TE/g of sample. F8 present the highest value with 1.23±0.18 mg TE/g of sample. Positive fractions were more active than negative fractions but the LBPC sample was more active than the negative fractions. This situation shows the correlation with the ORAC activity. In the ORAC activity, positive fractions were more active than negative fractions.

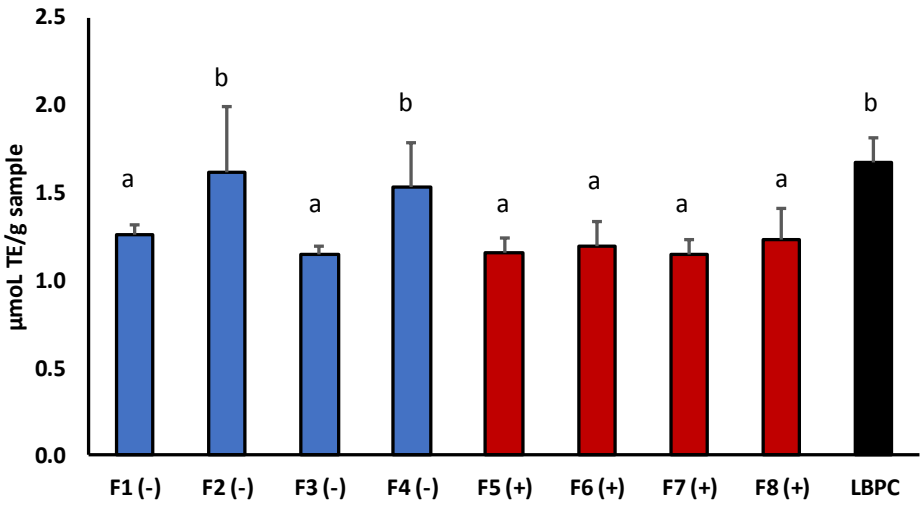


**Figure 6.** Antioxidant activity of LBPC and their fractions using the FRAP method. Different letters represent significant differences between LBPC vs sample as $P < 0.05$ $(n=3)$.

Antioxidant activity of LBPC fractions also was evaluated using the DPPH method. LBPC cationic fractions were more active than LBPC negative fractions. Fig. 7 shows the antioxidant activity of LBCP fractions. F5 present a value of DPPH of 189.38 µmoL TE/g of fraction, F8 present a value of DPPH of 191.33 µmoL TE/g of fraction, F7 show a value antioxidant of 196.17 µmoL TE/g of fraction and F8 show a value of 205.19 µmoL TE/g of fraction. This sample present higher antioxidant activity. LBPC negative fractions present value of DPPH between 36.79 to 48.96 µmoL TE/g of fraction. LBPC control present a value of 84.08 µmoL TE/g of LBPC. Different works have been reported fractions obtained of food proteins using different methods of isolation or separation with biological properties. For example, RODRIGUEZ SAINT-JEAN *et al.* (2013) have described fractions isolated of $\alpha S_2$ casein bovine with strong antiviral activity against the infectious hematopoietic necrosis virus of salmonid fish. The fractions were isolated using ion-exchange chromatography.
Fig. 8 shows the LBPC antioxidant activity results and their fractions using the ORAC method.

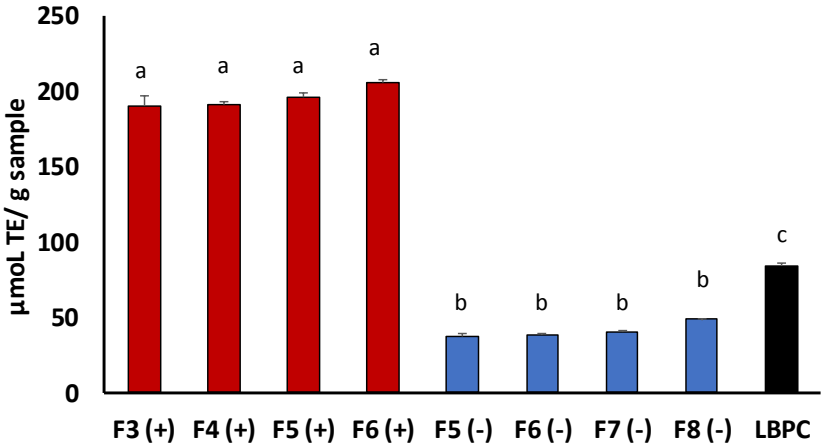


**Figure 7.** Antioxidant activity of LBPC and their fractions using the DPPH method. Different letters represent significant differences between LBPC vs sample as $P$ <0.05 ($n$=3).

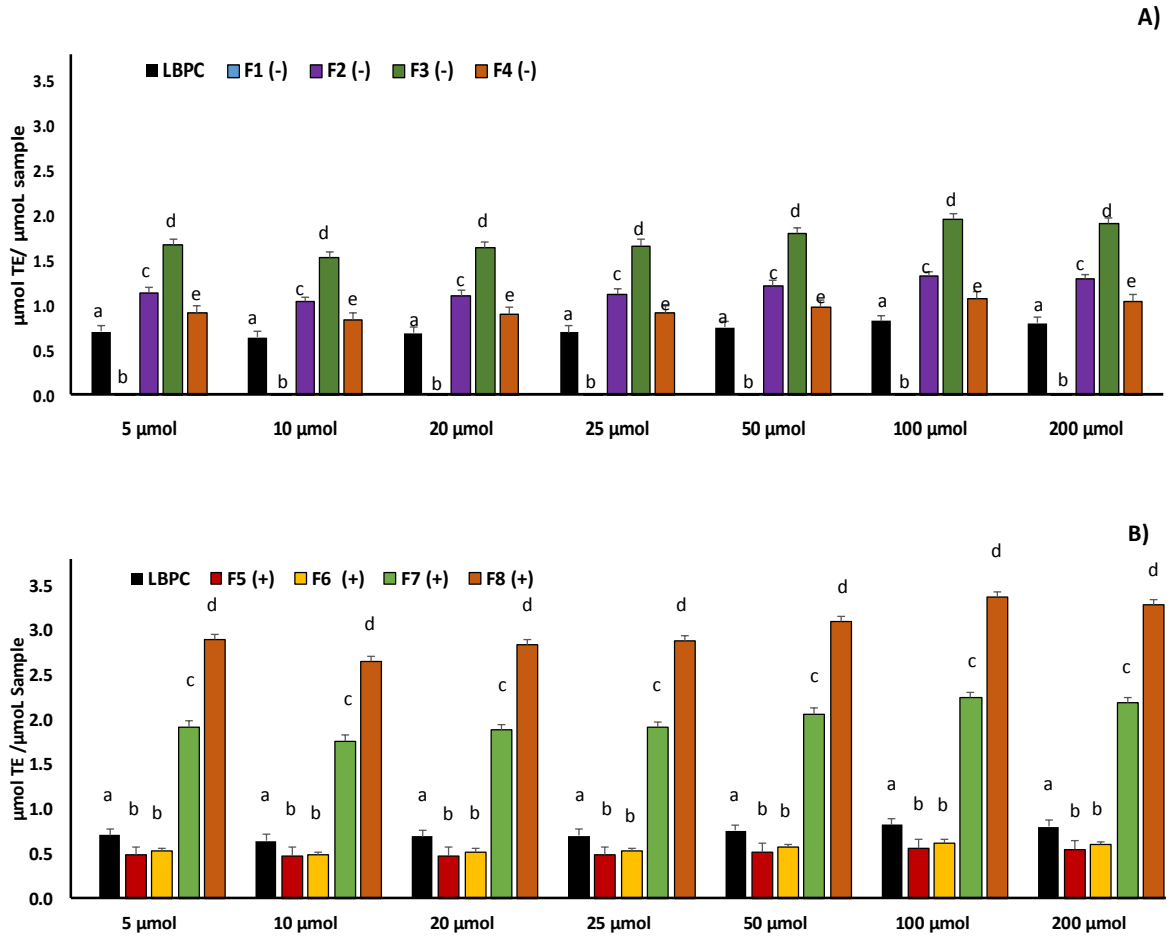


**Figure 8.** Antioxidant activity of LBPC and their fractions using the ORAC method. A) Anionic fractions, B) Cationic fractions. Different letters represent significant differences between LBPC vs sample as $P$ <0.05 ($n$=3).

Non-digested LBPC showed an ORAC value between 0.64±0.06 to 0.81±0.06 μmoL TE/ μmoL of sample. Vilcacundo *et al.* (2018) reported quinoa protein concentrates with values of 0.42±0.03 μmoL TE/μmoL of protein. NONGONIERMA *et al. (*2015) reported quinoa proteins with ORAC values of 0.26±0.07 μmoL TE/mg of sample. LBPC cationic fractions (F5 and F6) present lower antioxidant activity than LBPC with ORAC values between 0.47±0.09 to 0.56±0.09 μmoL TE/μmoL of sample and 0.48±0.03 to 0.61±0.03 μmoL TE/μmoL of sample respectively. The cationic fractions F7 and F8 present higher antioxidant activity than the F5 and F6 and anionic fractions (F1, F2, F3 and F4) with ORAC values between 1.75 to 2.24±0.03 μmoL TE/μmoL of sample and 2.64±0.05 to 3.37±0.05 μmol TE/μmoL of sample respectively. The anionic fraction F1 present an absence of antioxidant activity using the ORAC method, F3 and F4 present higher ORAC values (1.03±0.06 to 1.31±0.06 μmoL TE/μmoL of sample) and (1.52±0.06 to 1.94±0.06 μmoL TE/μmoL of sample). Fraction F4 present ORAC values between 0.83±0.08 to 1.06±0.08 μmoL TE/μmoL of sample.

CARRILLO *et al. (*2016a; 2016b) have described hydrolysates and fractions isolate of lysozyme protein with antioxidant activity and capacity to inhibit lipid peroxidation in zebrafish larvae, the fractions from lysozyme were isolated using the IEC technique. Peptides were identified by HPLC-ESI-MS-MS. Peptides presented antioxidant activity using the ORAC method. CARRILLO *et al*. (2018) have described fractions isolated of lysozyme protein with antibacterial activity against *Escherichia coli* and *Staphylococcus carnosus*. Fractions were separated using a membrane of cationic exchange chromatography technique. PIÑUEL *et al*. (2019) have reported antioxidant fractions obtained of digest of *Phaseolus vulgaris* separated using ultrafiltration membrane of 3 kDa and 10 kDa. Fractions with biological activities can be isolated of food proteins of animal or vegetal sources and can be used for different proposes. When the proteins are subject to enzymatic hydrolysis the peptides can be identified and synthetized to be studied.

## 4. CONCLUSIONS

Baby lima bean (*Phaseolus lunatus*) seeds from Ecuador used in this study present a high protein content. These seeds can be a new source of vegetable protein and used for different purposes in the food industry. In this study, LBPC was obtained using an alkaline extraction, followed by an isoelectric precipitation, LBPC present a complex proteins profile. Phaseolin is the most abundant protein in the *Phaseolus lunatus* protein concentrate. Phaseolin protein presents resistance to hydrolysis with pepsin in the gastric phase and to pepsin/pancreatin in the duodenal phase. LBPC present a high solubility and their fractions present a high antioxidant activity. For the previous reasons, *Phaseolus lunatus* proteins can have a potential use in the formulation and development of new functional ingredients food.

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PAPER

# CHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF HOMOGENISED RICOTTA CHEESE PRODUCED FROM BUFFALO WHEY

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## ABSTRACT

To extend the shelf life of buffalo Ricotta cheese, a process was assessed that included a second heat treatment followed by homogenisation and hot packaging. The microbiological and chemical characteristics as well as the oxidation degree of the product were determined over storage for 21 days using total antioxidant activity, redox potential, malondialdehyde content, and protein-bound carbonyl content. Homogenised buffalo Ricotta cheese has a longer shelf life than traditional Ricotta cheese, although the process could be optimised to reduce the total bacterial load during storage. No significant oxidative damage occurred during storage. This innovative process could promote the market expansion of Ricotta cheese.

*Keywords*: antioxidant activity, carbonyl groups, malondialdehyde, oxidation level, Ricotta cheese, shelf life

## 1. INTRODUCTION

Ricotta cheese is an Italian dairy by-product obtained from whey via the denaturation of whey proteins at 80-85°C. In Italy, almost all families purchase Ricotta cheese at least once a year (ASSOLATTE, 2018). The consumption of Ricotta cheese is favoured by its low price. Despite its lower commercial value with respect to that of other cheeses, the whey proteins in Ricotta cheese have a high biological value owing to their significant content of sulphur-containing amino acids (SMITHERS, 2008).
Ricotta cheese has a high moisture content and an initial pH above 6.0. Its shelf life varies according to the treatment applied after curd floating and the type of packaging (MUCCHETTI and NEVIANI, 2006). Fresh buffalo Ricotta cheese is marketed locally and mainly consumed within 1-2 days of production. Its shelf-life is about 4 days (ZOTTOLA, personal communication).
The fact that the guaranteed shelf life of this product is only a few days hampers its distribution to distant markets. Current markets absorb only a small amount of fresh buffalo Ricotta cheese in comparison to the actual production potential, indicating that a large proportion of the buffalo whey deriving from Mozzarella cheese manufacture is not destined for Ricotta cheese but disposed of or destined for non-dairy use. An increase in the shelf life of buffalo Ricotta cheese could lead to an increase in the market share; this would in turn lead to a reduction in the quantity of whey to be disposed of.
In addition, the recent recognition of the Protected Designation of Origin (PDO) to "Ricotta di Bufala Campana" could increase interest from domestic and foreign markets in a product with a longer shelf life.
Shelf life can be increased by reducing microbial contamination, improving the hygiene conditions of the cheese plant equipment and environment, and reducing the cooling time (OTTOGALLI *et al.*, 1981; MUCCHETTI and NEVIANI, 2006). Furthermore, packaging systems, such as vacuum packaging, can extend the shelf life of Ricotta cheese (PINTADO and MALCATA, 2000). Modified atmosphere (MA) packaging has been used to extend the shelf life of fresh Ricotta cheese by up to 14 days (MANCUSO *et al.*, 2014).
A system that ensures a longer shelf life for Ricotta cheese is heat packaging. After the whey is drained, Ricotta cheese is heat-packaged into suitable containers (MUCCHETTI *et al.*, 2002). Additionally, after the whey is drained, Ricotta cheese can be subjected to a second thermal treatment and a homogenisation treatment. The homogenisation process occurs at a low pressure and inhibits product syneresis. Then, the homogenised Ricotta cheese is heat-packaged in sealed plastic containers (MUCCHETTI *et al.*, 2002). The heat packaging is applied to cow Ricotta cheese at an industrial level and is less often applied to Ricotta cheese from other species, or in small- and medium-sized cheese plants.
Other product innovations in the food chain have been introduced to meet new consumer needs. These include changes in the manufacturing process, product composition, packaging, and product size and shape, and the introduction of a new method of using the product (LIPAN *et al.*, 2017). Some examples of innovation in Ricotta cheese have been described by SCARANO *et al*. (2019).
To fulfil the needs of the buffalo dairy industry, the present study was designed to improve the technology of small plants to extend the shelf life of buffalo Ricotta cheese. A homogenisation of traditional Ricotta cheese preceded by heat treatment was suggested. Homogenised buffalo Ricotta cheese, obtained as described above, differs in sensory properties from traditional buffalo Ricotta cheese. Homogenisation produces a fine and uniform consistency with consequently greater creaminess (WILBEY *et al.*, 2012).

There have been many studies regarding the chemical and microbiological characteristics of Ricotta cheese (MUCCHETTI and NEVIANI, 2006; MUCCHETTI *et al*., 2017), but few have focused on its oxidative characteristics (RAIA *et al*., 1996). The double heating treatments and homogenisation applied to buffalo Ricotta cheese induced us to study the oxidation degree of this type of product at different times of storage.
In dairy products, lipids are susceptible to oxidation, a biochemical process that contributes considerably to the degradation of the nutritional and sensory qualities of a product during manufacture and storage; this causes a significant reduction in shelf life (Bergamo *et al*., 1998). This alteration in lipids is determined not only by the absorption of oxygen by both free and esterified unsaturated fatty acids but also by other environmental factors, including exposure to light, high temperatures, and contact with metals (Fe, Cu, Co, Ni, and Mn) (MORTENSEN *et al*., 2004).
Protein oxidation, another type of oxidative damage in dairy products that affects the protein matrix during heat treatment, generally induces changes in amino acid residues and three-dimensional protein structures and may result in the loss of biological functionality (AUGUSTYNIAK *et al*., 2015; FENG *et al*., 2015).
The aim of this study was, therefore, to evaluate the microbiological and chemical characteristics of homogenised buffalo Ricotta cheese over storage for 21 days. Moreover, the degree of oxidation of the product was investigated by adapting analytical methods to specifically study the Ricotta cheese matrix.

## 2. MATERIALS AND METHODS

### 2.1. Process of Ricotta cheese

The trials were performed in a medium-sized cheese plant located in Italy in the Lazio region where buffalo Mozzarella and traditional Ricotta cheese are produced using an artisanal system. The raw material used to produce traditional Ricotta cheese utilises the sweet whey drained from Mozzarella curd. Other important phases of the process are reported in Fig. 1.
Approximately 1.5% (w/v) fresh cream from buffalo whey was added to the sweet whey. Then, approximately 0.3% (w/v) NaCl was added, and the whey mixture was heated continuously in a large open kettle by direct heating. When the whey grains began to float, *sieroinnesto* (natural whey cultures obtained from previous cheese making) was added at various amounts according to the extent of the *sieroinnesto* titratable acidity (2-3% v/v). The heating of the mixed whey was stopped at 85°C, and when the firm curd floated to the surface, it was collected into perforated hoops where the whey was drained. The Ricotta cheese was held for one hour at room temperature and then transferred to a cold room at 4°C.
While the traditional Ricotta cheese was cooling, the temperature, pH, and weight were measured.
To produce fresh Ricotta cheese with increased shelf life, the following process (Fig. 2) was applied.

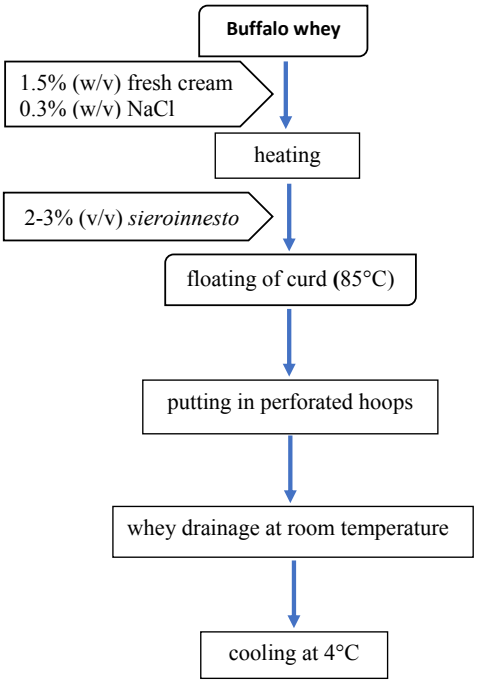


**Figure 1.** Flow-chart for the manufacture of traditional buffalo Ricotta cheese.

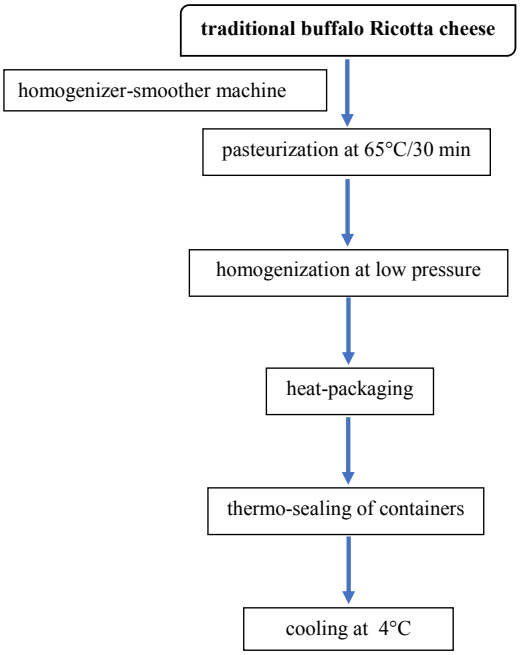


**Figure 2.** Flow-chart for the manufacture of homogenised buffalo Ricotta cheese

After draining, the traditional Ricotta cheese was transferred to a homogeniser-smoother machine. In this machine, which was equipped with a heating system, the Ricotta cheese was pasteurised at 65°C for 30 min and then homogenised at 15 bar.
Preliminary trials were carried out to determine the optimal initial moisture content of the product, pasteurisation parameters, and packaging conditions. The homogenisation process occurred at a low pressure. Finally, the product, namely homogenised buffalo Ricotta cheese, was immediately heat-packaged in sealed 250 g plastic containers to reduce spoilage risk, and then cooled in a room at 4°C.

**2.2. Analysed samples**

Sixteen samples were collected per day on three different days during production. Hermetically sealed 250 g Ricotta cheese packages arrived at the laboratory in refrigerated conditions and were stored at refrigeration temperature (4°C) for a period of 1, 7, 14, and 21 days.
Microbiological analyses were carried out on the refrigerated samples at the Animal Prophylaxis Research Institute for Lazio and Toscana Regions (IZSLT), Latina, Italy.
Chemical and oxidative analyses were carried out on frozen samples at the Council for Agricultural Research and Economics, Research Centre for Animal Production and Aquaculture of Monterotondo, Rome, Italy. For each sample, two Ricotta cheese packages were allowed to defrost overnight at 4°C. The sample was adequately homogenised, and the subsamples were taken for analyses. For each analysis, at least two replicates for each sample were performed.

**2.3. Microbiological analyses**

Bacteria in the fresh cheese samples were detected and enumerated in accordance with international standard methods. An initial suspension was prepared to achieve a uniform distribution of the sample microorganism content. The initial suspension was made by adding 225 ml of diluent to 25 g of sample for bacterial detection, and 90 ml of diluent to 10 g of sample for bacterial enumeration.
The samples were subjected to detection methods for *Salmonella* spp. (UNI EN ISO 6579-1:2017), *Listeria monocytogenes*, and *Listeria* spp. (UNI EN ISO 11290-1:2017).
*Salmonella* spp. were detected via pre-enrichment in buffered peptone water (BPW) broth incubated at 36±2°C for 18 h, enrichment in Rappaport-Vassiliadis soya peptone (RVS) broth and Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth at 37°C for 24 h, and plating on xylose lysine deoxycholate (XLD) agar and Salmonella-Shigella (SS) agar plates incubated at 37°C and examined after 24 h.
*Listeria monocytogenes* and *Listeria* spp. were detected via incubation in Half-Fraser broth at 30°C for 25±1 h, followed by a second enrichment with Fraser broth at 30°C for 25±1 h, which was then plated on Listeria agar, according to OTTAVIANI and AGOSTI (ALOA), and Oxford agar (LSM), and incubated at 37°C for 48 h.
*β-glucuronidase-positive Escherichia coli* was detected by incubating samples in tryptone-bile-glucuronic medium (TBX) at 44±1°C for 24 h (ISO 16649-2: 2001).
*Coagulase-positive staphylococci* (*Staphylococcus aureus* and other species) were detected using rabbit plasma fibrinogen agar medium incubated at 37±1°C for 24 h (UNI EN ISO 6888-2: 2004).
*Pseudomonas* spp. were detected by culturing the samples on penicillin and pimaricin agar (PPA) at 25°C for 48 h (ISO/TS 11059:2009- IDF/RM 225:2009).

*Enterobacteriaceae* was detected by culturing the samples on violet red bile glucose agar (VRBG) at 37°C for 24 h (UNI EN ISO 21528-2: 2017).
Yeasts and moulds with greater than 0.95 water activity were detected by incubating the samples on dicloran-rose bengal chloramphenicol agar (DRBC) at 25±1°C for five days (ISO 21527-1: 2008).
Total mesophilic counts (TMCs) were enumerated by incubating the 3M™ Petrifilm™ Aerobic Count Plate at 30±1°C for 72±3 h (AFNOR 3M 01/1-09/89).
Total psychrophilic counts (TPCs) were enumerated by incubating the 3M™ Petrifilm™ Aerobic Count Plate at 6.5±1°C for ten days (AFNOR 3M 01/1-09/89; ISO 6730: 2005 IDF 101).
Presumed mesophilic and thermophilic cocci were enumerated on M17 agar after aerobic incubation for 48 h at 30°C and at 44°C, respectively. Presumed mesophilic lactobacilli were enumerated on DeMan, Rogosa, and Sharpe (MRS) agar adjusted to pH 5.4 and incubated anaerobically at 37°C for 72 h. The result of bacteria enumeration was expressed as the number of microorganisms per gram of product.
Activity water (Aw) was determined with an Aqualab Lite (Decagon) principle dew-point measurement (ISO18787:2017).

### 2.4. Physical and chemical analyses

Homogenised Ricotta cheese samples were submitted to the following analyses: pH, moisture (IDF, 1986), total nitrogen (TN) (FIL-IDF, 1993), pH 4.6 soluble nitrogen (pH 4.6 SN) (FIL-IDF, 1991), fat (FIL-IDF, 2001), ashes (AOAC, 2000), and salt (IDF, 1988).

### 2.5. Antioxidant activity - the DPPH method

The antioxidant activity of the samples was evaluated using the DPPH (1,1-diphenyl-2-picryl-hydrazyl radical) method, as reported by Unal (2012), for milk and cheese, with some modifications. The purple stable free radical DPPH changes to a yellow colour following reduction by antioxidant molecules. This method is commonly used for the evaluation of the antioxidant capacity of plant extracts and food in various contexts (KATALINIC *et al.*, 2006; NIKOLOVA *et al.*, 2011).
Ricotta cheese samples (2 g) were mixed with 8 ml of 0.11 mM DPPH ethanolic solution. The control was prepared by adding 2 ml of ethanol to 8 ml of 0.11 mM DPPH ethanolic solution.
The mixtures were shaken vigorously and then left standing at room temperature for 20 min in the dark. Then, the mixtures were centrifuged for 10 min at 9000 g at 22°C.
The absorbance of the supernatant at 517 nm was measured using a double-beam UV-VIS spectrophotometer (Lambda 25, PerkinElmer). Absolute ethanol was used as a blank, and analyses were performed in duplicate.
The antioxidant activity was expressed as the percentage of inhibition of the DPPH radical according to the following equation:

$$\% \text{ antioxidant activity} = (A0 - As) / A0 \times 100$$

where A0 is the absorbance of the control (containing all reagents except Ricotta cheese sample),
and As is the absorbance of the tested sample.

Trolox was used as a standard to compare the antioxidant activity of the sample with a reference antioxidant. The antioxidant activity of the samples was expressed in Trolox equivalents (mmol Trolox eq. /100 g).

### 2.6. Redox potential determination

For each sample, the redox potential was determined using a potentiometric method that uses a pH meter (Metrohm 827 pH Lab) equipped with a platinum electrode (combined Pt-ring 6.0451.100) and the potential was expressed in millivolts (mV).

### 2.7. Malondialdehyde analysis

The method used for MDA determination was based on the detection of MDA - thiobarbituric acid (TBA) fluorescent complexes, in which the MDA present in a sample reacts with TBA, and the MDA-TBA complex results in an absorption peak at 532-535 nm (RAHARJO and SOFOS, 1993).
Ricotta cheese samples were prepared according to the methods of RAIA *et al.* (1996), with some modifications. A total of 0.4 g of the sample was homogenised in a solution of 3.4 ml of 10% w/v trichloroacetic acid (TCA) to precipitate proteins, and 0.20 ml of BHT (butylhydroxytoluene) 2.8% w/v ethanolic solution was added as an antioxidant to prevent further lipid peroxidation.
The mixture was heated at 90°C for 30 min, quickly cooled in an ice bath for 20 min, and centrifuged at 10,000 g for 10 min to separate the pellet containing the precipitated protein fraction.
A supernatant aliquot (300 µl) was added to 700 µl of 0.28% w/v TBA and incubated at 90°C for 30 min to induce the formation of the TBA-MDA complex, and the aliquot was then quickly cooled on ice for 20 min. After centrifugation at 10,000 g for 5 min, the supernatant was collected for the following HPLC analysis.
HPLC analysis was performed using a Shimadzu-SPD-M10A HPLC with a fluorimeter (RF-10A) and ZORBAX Eclipse Plus C18 column (4.6 x 250 mm x 5 $\mu$m). The isocratic mobile phase consisting of 5 mM sodium phosphate buffer (pH 7.0) and acetonitrile (80:20 v:v) was filtered on Whatman 0.2 $\mu$m filter paper. Aliquots (20 µl) were injected and analysed using a flow rate of 1 ml/min at room temperature. The fluorescent detector was set at an excitation wavelength of 515 nm and emission at 543 nm.
The concentration of the MDA-TBA complex was then calculated based on the calibration curve using a standard solution, and the values obtained were expressed as nmol/g.

### 2.8. Carbonyl determination

To evaluate the oxidative damage of proteins in the Ricotta cheese samples, the method of REZNICK and PACKER (1994) modified by FEDELE and BERGAMO (2001) for milk and cheese was used. The method is based on the derivatization of the carbonyl group with di-nitrophenol hydrazine (DNPH), and the chromophore produced can be detected at 360 nm.
In detail, Ricotta cheese samples (2 g) were weighed, and 5 ml of 0.2 M sodium citrate-NaOH (pH 8) at room temperature was added. After vigorous mixing for 5 min, 100 $\mu$l aliquots were incubated in the presence of 400 $\mu$l of 10 mM DNPH in 2.5 M HCl (in duplicate). In total, 400 $\mu$l of 2.5 M HCl (in duplicate) was added to the other 100 $\mu$l aliquots as a control (blank).

After incubation for 30 min in the dark at room temperature, 500 μl of cold 20% w/v TCA was added to each sample, and the samples were vigorously shaken. The mixtures were incubated for 20 min on ice and centrifuged for 10 min at 10,000 *g* at 4°C to cause protein precipitation.
The supernatant was removed, and 400 μl of 10% TCA was added to the pellet. After incubation for 10 min in the dark, the samples were centrifuged at 10,000 *g* for 10 min, after which the supernatant was removed.
To remove the free DNPH, the pellet was washed twice with 1 ml of ethanol/ethyl acetate (1:1 v/v) solution by centrifugation at 10,000 g for 10 min, and the supernatant was removed without disturbing the precipitate.
Pellets containing protein precipitates were then dissolved in 1 ml of 6 M guanidine HCl in 10 mM phosphate buffer (pH 2.3). The samples were incubated for 30 min in a 60°C water bath, and subsequently the absorbance was measured at 370 nm using a double-beam UV-VIS spectrophotometer (Lambda 25, PerkinElmer).
For the samples treated with 2 M HCl (control without DNPH), the protein concentration was determined by measuring the absorbance at 280 nm. The amount of protein was calculated from a bovine serum albumin (BSA) standard curve (0.5-2.5 mg/ml). The net absorbance was calculated as the difference between the absorbance of the sample with DNPH and the blank (without DNPH).
The concentration of protein-bound carbonyls (C Carb) was calculated by the molar extinction coefficient of 22,000/M/cm, according to the following formula:

$$\text{C Carb (nmol/mg prot)} = (\Delta \text{ Abs } 360 \ / \ 22 \text{ mM}) * 1000/\text{C prot}$$

where Δ Abs 360 = Abs 360 (DNPH) - Abs 360 (blank) is the net measured absorbance, and C prot is the protein content (g/L). The carbonyl concentration, expressed as nmol/mg of protein, was used to provide a global protein oxidation index.

### 2.9. Statistical analysis

All microbiological data were log-transformed. The GLM procedure of SAS software (SAS Institute Inc., 2007) was used for the statistical analysis of the chemical and oxidative analyses of Ricotta cheese. A factorial model, including the fixed effect of the storage time of Ricotta cheese, was used. The CORR procedure of SAS software (SAS Institute Inc., 2007) was also used.

## 3. RESULTS AND DISCUSSION

### 3.1. Process of making traditional buffalo Ricotta cheese

The initial titratable acidity of the whey transferred to the Ricotta cheese kettle ranged from 0.16 to 0.18 g of lactic acid/100 ml. ADDEO and COPPOLA (1983) found similar values of titratable acidity (0.17 g of lactic acid/100 ml) of starting whey from buffalo Mozzarella used for the manufacture of Ricotta cheese.
The process specifications of PDO "Ricotta di Bufala Campana" require a sweet whey with titratable acidity <0.16 g of lactic acid/100 ml. According to True (1973), in general, the initial titratable acidity of sweet whey destined to produce Ricotta cheese, should be less

than 0.16 g of lactic acid/100 ml. Optimal values are considered 0.13-0.14 g of lactic acid/100 ml.
In the traditional process of making buffalo Ricotta cheese, approximately 1.5% (w/v) cream was added to the whey.
According to "Ricotta di Bufala Campana" specifications, the addition of up to 6% (w/v) milk and 5% (w/v) fresh cream is allowed. The addition of milk or cream enriches the fat content of the whey and improves the sensorial characteristics of the Ricotta cheese (SHAHANI, 1979). Ricotta cheese manufactured with the addition of milk or cream is softer and creamier and has a delicate texture (PINTADO *et al.*, 2001). Fresh cream made from Mozzarella whey transfers a particular flavour to Ricotta cheese.
To enhance the coagulation and rise of the curd, *sieroinnesto* was added to hot whey. According to MUCCHETTI *et al.* (2017), in ovine Ricotta cheese production, it is not necessary to reduce the whey pH to favour protein aggregation, while cow and buffalo whey needs to be slightly acidified for better protein aggregation. Even the *sieroinnesto* addition can concur with a particular Ricotta flavour.
The amount of salt added to the whey (0.3%, w/v) was minimal with respect to the maximum amount allowed by "Ricotta di Bufala Campana" specifications (1%, w/v). NaCl dehydrates the whey proteins and has a destabilizing effect on BSA (FARKYE, 2004).
The industrial process of Ricotta cheese production (FARKYE, 2004) involves the stages described below. The whey is first neutralised to pH>6.5 (6.9-7.1) with a NaOH solution. Manipulation of the pH minimizes protein aggregation and produces a more cohesive coagulum (MODLER and EMMONS, 1989). The recommended temperature for milk addition (5-25%) is 65-70°C, while cream is added at 75-80°C. After the addition of cream, NaCl (0.5, v/v) is added. Then, an acetic or citric acid solution is added for coagulation and curd formation. Optimal coagulation and maximum yield occur at pH 5.6-5.8 (WEATHERUP, 1986).
In our trials, after three hours of cooling, the temperature of the Ricotta cheese decreased on average from 65°C to 17°C. The weight stabilised after three hours starting from the transfer to the basket. Weight loss for the traditional Ricotta cheese was approximately 15%. The final weight of the shapes was 400-450 g. After three hours, the pH increased from 6.22 to 6.76, while in Ricotta cheese from sheep's milk, the final pH ranged from 6.35 to 6.85 (CHERCHI *et al.*, 1999; SALVATORE *et al.*, 2014).

### 3.2. Process of homogenising buffalo Ricotta cheese

Regarding the optimal initial moisture of the product, according to preliminary trials, better results were achieved when the moisture content ranged from 67-70%.
The homogeniser-smoother machine used to produce homogenised Ricotta cheese was equipped with scraping blades, which stirred the product during heating. If the curd was too dry, the stirring was more difficult, and the curd adhered to the heating surface, reducing the effectiveness of the heat exchange. Moreover, the increase in temperature for low-temperature or batch pasteurisation (65°C/30 min) applied to heat the product was very slow.
Homogenised Ricotta cheese was heat-packaged, and to minimise microbiological pollution, the containers were thermo-sealed immediately after being filled. In the preliminary trials, we observed that the application of these conditions contributed to extending the shelf life of the homogenised Ricotta cheese. In fact, not sealing the containers immediately after filling them resulted in substantial contamination, particularly with mould and yeasts, after a few days (data not shown).

### 3.3. Microbiological characteristics

*Salmonella* spp. and *Listeria monocytogenes,* pathogens considered as food safety criteria (Reg CE 2073/2005), were not detected (below the detection limit), in accordance with the above regulations. In addition, *Listeria* spp. were not detected.
The counts of ß-*glucuronidase*-positive *Escherichia coli* and *coagulase*-positive staphylococci (*Staphylococcus aureus* and other species) are considered hygiene markers (Reg CE 2073/2005) and were lower than the detection limit of the method <10 colony forming units (cfu)/g (Table 1).

**Table 1.** Microbiological characteristics of homogenised buffalo Ricotta cheese during storage.
TMC: Total mesophilic count; TPC: Total psychrophilic count. Bacteria values are means±sd of three batches samples, expressed as log 10 cfu/g.

| | Storage time | | | |
|---|---|---|---|---|
| | **Day 1** | **Day 7** | **Day 14** | **Day 21** |
| ***ß-glucuronidase-positive Escherichia coli* (log 10 cfu/g)** | < 1 | < 1 | < 1 | < 1 |
| ***Coagulase-positive staphylococci* (log 10 cfu/g)** | < 1 | < 1 | < 1 | < 1 |
| ***Enterobacteriaceae* (log 10 cfu/g)** | < 1 | < 1 | < 1 | < 1 |
| ***Pseudomonas* spp. (log 10 cfu/g)** | < 1 | < 1 | < 1 | < 1 |
| **Yeasts (log 10 cfu/g)** | < 1 | < 1 | < 1 | < 1 |
| **Moulds (log 10 cfu/g)** | < 1 | 1.30 ± 0.43 | 2.00 ± 1.41 | < 1 |
| **TMC (log 10 cfu/g)** | 4.57 ± 0.41 | 6.42 ± 0.87 | 7.37 ± 0.47 | 8.27 ± 0.20 |
| **TPC (log 10 cfu/g)** | 2.52 ± 2.15 | 6.88 ± 1.45 | 7.35 ± 0.44 | 7.44 ± 0.90 |
| **Mesophilic lactococci (log 10 cfu/g)** | 4.34 ± 0.32 | 4.94 ± 0.30 | 7.24 ± 0.29 | 8.56 ± 0.63 |
| **Thermophilic lactococci (log 10 cfu/g)** | 4.31 ± 0.01 | 3.02 ± 2.86 | 7.20 ± 0.23 | 8.50 ± 0.71 |
| **Mesophilic lactobacilli (log 10 cfu/g)** | 2.72 ± 0.18 | < 1 | < 1 | < 1 |
| **Aw** | 0.995 ± 0.001 | 0.995 ± 0.001 | 0.994 ± 0.0014 | 0.993 ± 0.002 |

Additionally, the counts of *Enterobacteriaceae* and *Pseudomonas* spp., which are often associated with changes in the texture and colour of the cheese, were lower than the detection limit (<10 cfu/g).
Yeasts were <10 cfu/g, while moulds were present only in one sample at day seven and in another at day 14. Moulds were within the limits according to ISO 21527-1: 2008. *Penicillium* spp. was identified through macroscopic and microscopic traits.
These results show that in the homogenised buffalo Ricotta cheese made by the process described above, pathogens are undetected, and all microorganisms considered hygiene markers were lower than the detection limit.
Our results agree with those reported by PALMAS *et al*. (1994) during the storage of sheep Ricotta cheese subjected to direct hot packaging.

The average TMC of our samples increased from 4.57 on day 1 to 8.27 log10 cfu/g on day 21. The same trend was observed for TPC, changing from 2.52 on day one to 7.44 log10 cfu/g on day 21. Mesophilic and thermophilic lactococci increased from 4.34 and 4.31 log10 cfu/g on day one to 8.56 and 8.50 log10 cfu/g on day 21, respectively. Mesophilic lactobacilli were present only in day one samples, and thermophilic lactobacilli were not detected (Table 1).
According to MUCCHETTI *et al.* (2002), industrially produced fresh Ricotta cheese, and particularly Ricotta cheese subjected to a second heat treatment, had a lower total bacterial count compared to that found in our samples.
Lactic acid bacteria are the microorganisms most commonly represented in Ricotta cheese, and they are an important part of the total bacterial count (MUCCHETTI *et al*., 2017). The increase during storage in TMC and LAB could be due to heat resistant or post-contamination microorganisms, since the high pH and elevated $A_w$ do not limit microbial growth.
We assume that the microbiological characteristics of the product can be improved by the stricter control of environmental contaminants through the implementation of hygienic conditions during the process.
Furthermore, to reduce the number of heat-resistant microorganisms, higher temperatures could be used during the second heat treatment. Total bacterial count was lower during the storage of the product in industrial Ricotta cheese subjected to second heat treatment by 85-95°C (MUCCHETTI *et al*., 2002).
Moreover, an essential strategy for the control of mesophilic bacteria, such as spore-forming, is the reduction of cooling times and the strict maintenance of the cold chain during product storage.

### 3.4. Chemical characteristics

The chemical characteristics of the homogenised Ricotta cheese are reported in Table 2.

**Table 2.** Chemical characteristics of homogenised Ricotta cheese from buffalo whey.

| Sample number | Moisture (%) | Dry matter (%) | Protein (%) | Soluble protein (%) | Fat (%) | Ash (%) |
|---|---|---|---|---|---|---|
| **1** | 67.05 | 32.95 | 6.39 | 0.07 | 19.42 | 2.22 |
| **2** | 64.29 | 35.71 | 5.41 | 0.06 | 24.50 | 2.07 |
| **3** | 65.18 | 34.82 | 5.08 | 0.09 | 23.59 | 1.75 |
| **Mean** | 65.51 | 34.49 | 5.63 | 0.07 | 22.50 | 2.01 |
| **s.d.** | 1.41 | 1.41 | 0.68 | 0.01 | 2.71 | 0.24 |

The mean moisture content of the samples analysed was 65.51%, which was similar to that of the "Ricotta di Bufala Campana" cheese (64.58%) reported previously by several Authors (MUCCHETTI and NEVIANI, 2006). The moisture content of industrial Ricotta cheese from bovine and sheep whey is higher, 75.72% and 70.03%, respectively (MUCCHETTI *et al.*, 2002), both of which are made in large cheese plants where the

industrial process favours better whey recovery in the product than the traditional process (Salvadori DEL PRATO, 2001; MUCCHETTI *et al.*, 2002).
The mean protein and fat contents of homogenised buffalo Ricotta cheese were 5.63% and 22.50%, respectively, and the fat: protein ratio was 4.0. The samples analysed had lower protein and higher fat contents than the values found in "Ricotta di Bufala Campana" PDO, which were 9.43% and 19.09%, respectively (MUCCHETTI and NEVIANI, 2006). However, owing to the artisanal processing conditions, the data cited above are very variable: the protein content ranged from 7.10% to 13.46%, and the fat content ranged from 15.87% to 20.78%. The average composition of industrial cow milk Ricotta cheese is very different with 9.73% fat content, 9.12% protein content and a fat: protein ratio of 1.07 (MUCCHETTI and NEVIANI, 2006).
The mean value of pH 4 soluble protein was 0.07%, similar to that observed by MUCCHETTI *et al.* (2002) in Ricotta cheese from bovine whey at 0.10%. These data indicate the content of undenatured whey protein in Ricotta cheese. The use of other techniques such as ultrafiltration can increase the recovery of whey protein in Ricotta cheese (MAUBOIS and KOSIKOWSKI, 1978).
The mean ash content value was 2.01%, which was higher than that of "Ricotta di Bufala Campana" and cow Ricotta cheese (MUCCHETTI and NEVIANI, 2006); their values were 1.23% and 1.16%, respectively.

### 3.5. Physicochemical and oxidative characteristics

The physicochemical and oxidative characteristics of the homogenised Ricotta cheese from day 1 to 21 are reported in Figures 3, 4, 5, and 6. During product storage, the values of all characteristics were not significantly different.
The pH values (Fig. 3) decreased during storage from 6.90 to 6.55.

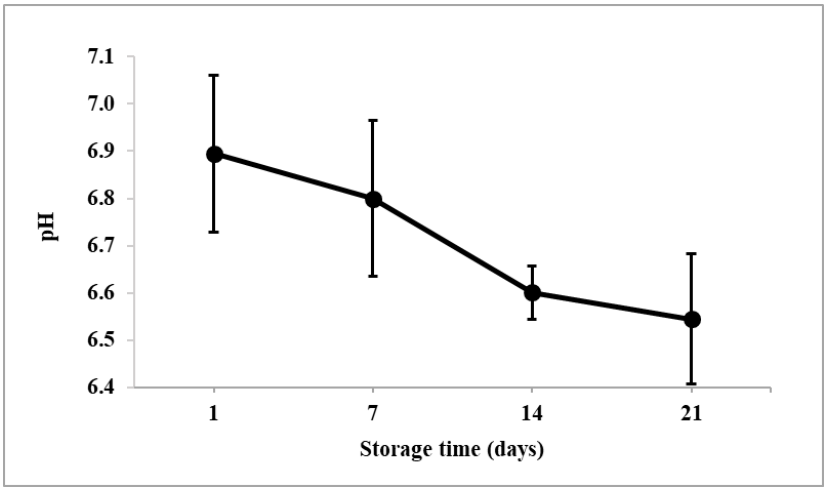


**Figure 3.** pH of homogenised buffalo Ricotta cheese during storage.

The increase in LAB during product storage could explain the pH decrease (MUCCHETTI *et al.*, 2002). According to HOUGH *et al.* (1999), the pH of Ricotta cheese packaged at temperatures between 65-68°C and stored at 6°C for 20 days decreased to approximately 6.0, and a significant correlation between pH and microbial growth was found. The pH of

fresh traditional sheep Ricotta cheese packaged in a modified atmosphere decreased from 6.54 on day 1 to 5.97 on day 21 of storage (Mancuso *et al.*, 2014).
The redox potential (Fig. 4) ranged from a minimum of 121 mV on day 1 to a maximum of 134 mV on day 14.

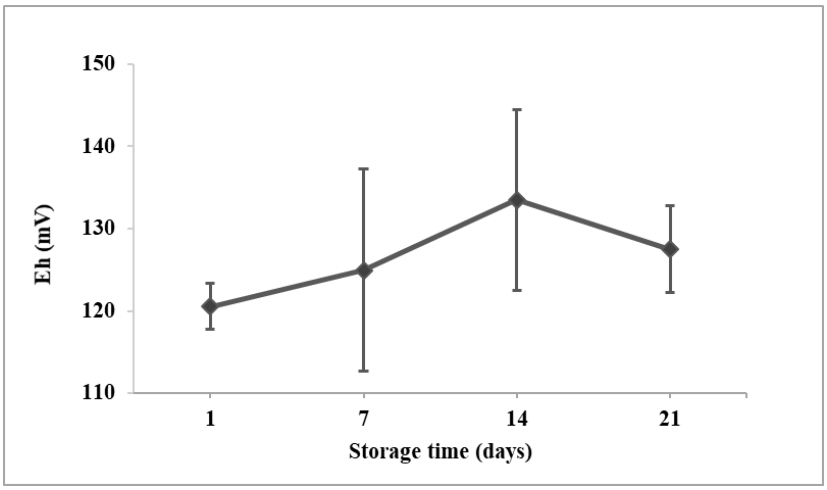


**Figure 4.** Redox potential of homogenised buffalo Ricotta cheese during storage.

The values found in industrial cow Ricotta cheese were positive (183 mV) (MUCCHETTI *et al.*, 2002). In the samples of homogenised buffalo Ricotta cheese not subjected to freezing before analysis, the redox potential was 82.3 mV (data unpublished).
Redox potential is an important parameter related to food stability. It is affected by many chemical and biological reactions (TANGO and GHALY, 1999), but it is not routinely measured (CALDEO, 2015).
The total antioxidant activity (Fig. 5) measured as Trolox eq. increased slightly from day one (13.69 mmol/100 g or 64.60%) to day 21 (14.05 mmol/100 g or 66.30%).

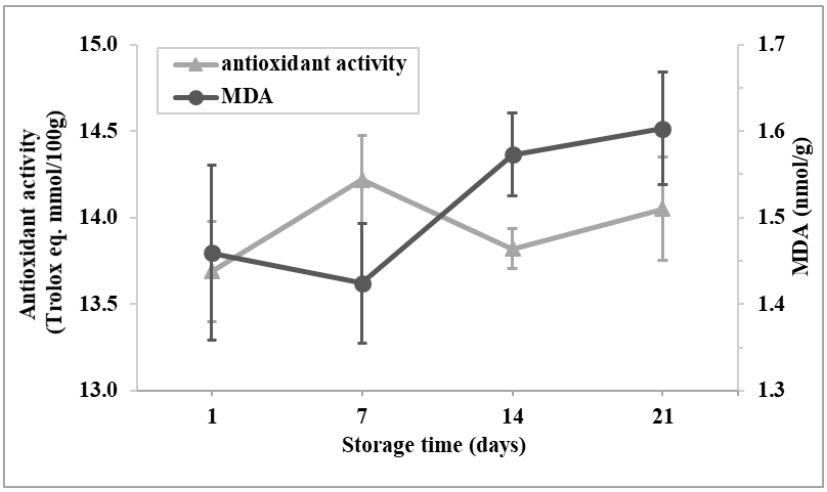


**Figure 5.** Antioxidant activity and MDA content in homogenised buffalo Ricotta cheese during storage.

In Cheddar cheese, antioxidant activity decreases with an increasing storage period, and in one study was 48% at the beginning of storage and 32% after three weeks of storage (LEE *et al.*, 2016). In another study, the antioxidant activity of Gouda cheese increased throughout the ripening period from 4.61% on day 0 to 16.38% on day 90 (KHAN *et al.*, 2018). The authors of the latter study attributed this increase to water-soluble peptides that have antioxidant properties.

The total antioxidant capacity measures the activity of many antioxidants that are active (KORPELA *et al.*, 1995; LINDMARK-MANSSON and AKESSON, 2000), and variations in activity depend on processing conditions. In milk, the changes upon heating were attributed not only to the thermal degradation of naturally occurring antioxidants, such as vitamins and enzymes, but also to the formation of novel oxidative species (CALLIGARIS *et al.*, 2004; ANDREI *et al.*, 2015).

In Figure 5, the MDA values are shown to slightly increase during storage, with a lower content at day seven (1.42 nmol/g or 0.103%) and a higher content at day 21 (1.60 nmol/g or 0.116%).

In contrast, one study found that the MDA content of buffalo Mozzarella cheese significantly increased after four days of storage (TATICCHI *et al.*, 2017). An increase in MDA content was also detected during the storage of high moisture Mozzarella from cow's milk (SEGAT *et al.*, 2013).

The average MDA content of homogenised Ricotta cheese (1.51 nmol/g or 0.11 mg MDA/kg) was similar to that of Grana Padano cheese (1.28 nmol/g) (Fedele and Bergamo, 2001) and semi-cooked Pecorino cheese (0.13 mg/kg) (BRANCIARI *et al.*, 2014).

In homogenised buffalo Ricotta cheese, a negative correlation (-0.76892; $P \leq 0.015$) between total antioxidant capacity and MDA content was found. Other Authors have confirmed the active role of antioxidants in preventing or limiting fat oxidation. FEDELE and BERGAMO (2001) reported a positive correlation between MDA and consumption of $\alpha$-tocopherol.

The MDA values of homogenised Ricotta cheese are far below those of other animal and vegetable foods (PAPASTERGIADIS *et al.*, 2012), despite the high fat content of this product. In fact, one of the factors that predisposes to lipid oxidation is the high fat content of food (DALSGAARD *et al.*, 2010; CITTA *et al.*, 2017).

The carbonyl content is shown in Fig. 6 and decreased from 3.93 nmol/mg protein at day one to 3.01 nmol/mg protein at day 21; the average content was 3.52 nmol/mg.

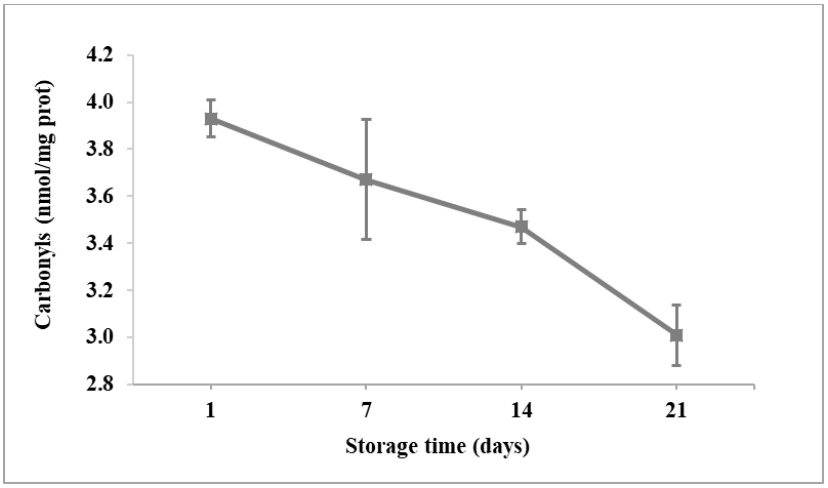


**Figure 6.** Protein-bound carbonyl content in homogenised buffalo Ricotta cheese during storage.

In comparison with that of other cheeses (FEDELE and BERGAMO, 2001; BALESTRIERI *et al.*, 2002), the carbonyl content of homogenised Ricotta cheese is similar to that of cooked cheeses, such as Grana Padano, Caciocavallo, Provolone and Pecorino Romano, and higher than that of fresh cheeses, including buffalo Mozzarella cheese.
Protein-bound carbonyl groups formed during heat treatment are currently used to evaluate the extent of protein oxidation (FEDYELE and BERGAMO, 2001; FEYNAILLE *et al.*, 2006). Moreover, carbonyl content was positively correlated with the temperature of heat treatment (FEDEYLE and BEYRGAMO, 2001; SCALOYNI *et al.*, 2002).

## 4. CONCLUSIONS

In conclusion, the process adopted to produce homogenised buffalo Ricotta cheese ensured that during storage, microorganisms considered hygiene markers were below the detection limit. However, the increase in total mesophilic and lactic bacteria during storage suggests stricter control of environmental contaminants. Higher temperatures are also recommended during the second heat treatment to reduce the number of heat-resistant microorganisms and to contain the acidification of the product.
Owing to its oxidative characteristics, homogenised buffalo Ricotta cheese is comparable to semi-cooked and cooked cheeses. The low content of malondialdehyde, which remains almost unchanged at the end of storage, confirms the active role of antioxidants present in dairy products in preventing or limiting fat oxidation.
The suggested process meets the needs of buffalo dairy producers to obtain a product with a longer shelf life and to increase the distribution to national and international markets away from the production area of origin.

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PAPER

# GRAPEVINE CANES WASTE FROM VENETO REGION AS A NEW SOURCE OF STILBENOIDS CONTENT

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## ABSTRACT

In the present paper we analyzed the stilbene accumulation in grape canes of seven autochthonous grape varieties from Veneto region, Italy, in comparison to two international cvs. In addition, we investigated the effect of pruning time on the stilbenes accumulation rate during the storage. Taking into account the effect of both pruning time (October, November and December) and storage time (from zero to twelve weeks at room temperature), cultivar Verdiso and Incrocio Manzoni 13.0.25 showed the highest accumulation of *trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin, in particular when the canes were harvested in October, highlighting the importance of the cultivar but also the effect of the pruning time on the accumulation of stilbenes in grape canes.

*Keywords*: autochthonous varieties, grape canes, piceatannol, resveratrol, viniferin

## 1. INTRODUCTION

The winemaking industry is responsible for large part of grape waste as pomace, grape canes, seeds and stems. Some waste as pomace and seeds are valued from food industry being popular as source of antioxidant polyphenols or for grape seed oil production. At the same time, there is still a need for alternative sources of resveratrol, as can be seen by the recent permission of resveratrol as a novel food ingredient in the European Union (Commission Implementing Decision (EU) 2016/1190). Stilbenoids are a small family of plant secondary metabolites derived from the phenylpropanoid pathway. They act as plant phytoalexins displaying different bioactivities and thus making them compounds of high current interest (FERNANDEZ-MAR *et al*., 2012). In the Vitaceae, stilbenoids accumulate in response to various biotic and abiotic stresses such as the attack of pathogen *Erysiphe necator Plasmopara viticola, Botrytis cinerea* and UV-C irradiation (SCHNEE *et al*., 2008; SCHNEE *et al*., 2013; PEZET *et al*., 2004; ALONSO-VILLAVERDE *et al*., 2011; ADRIAN and JEANDET, 2012; GRUAU *et al*., 2015; YIN *et al*., 2016). They can also be induced in response to plant hormones, such as ethylene and jasmonate (D'ONOFRIO *et al*. 2009; JIANG *et al*., 2015). In grapevine, the stilbene *trans*-resveratrol has attracted particular attention, not only because of its antimicrobial activity, but also due to its health benefits to humans, as antioxidant, anticarcinogenic, anti-inflammatory, cardioprotective and neuroprotective, among others (FLAMINI *et al*., 2013; BAVARESCO *et al*., 2012; SHEN *et al*., 2009).

Stilbenoids accumulate in different parts of grapevine, however, WANG *et al.* (2010) found the highest concentration of *trans*-resveratrol in grape canes. Grape canes waste is generated during winter annual pruning and represents a large source of waste derived from the viticulture industry, with an estimated volume of 1 to 3 t/ha year depending upon plantation density, climate, and vigor of the grape variety (DEVESA-REY *et al*., 2011; EWALD *et al*., 2017). Currently, emission protection regulations mostly prohibit the burning of grape canes, which was the traditional way of disposal of these woody residues (EWALD *et al*., 2017).

Grape canes can be considered as an unexploited source of stilbenoids, as proposed by several authors (VERGARA *et al*., 2012; LAMBERT *et al*., 2013; GORENA *et al*., 2014; HOUILLÉ *et al*., 2015; GUERRERO *et al*., 2016; EWALD *et al*., 2017). Different content of stilbenoids have been found in grape canes of *Vitis vinifera* stored at 40 to 45°C or at room temperature (20±3°C). VERGARA *et al.* (2012) compared the stilbenoid content in canes of several grape varieties cultivated in different regions and in two different years in Chile, finding *trans*-resveratrol in the range 446 to 6533 mg/kg DW, with the highest content found in Gewurztraminer variety. Lower values of *trans*-resveratrol were reported by LAMBERT *et al.* (2013) comparing grape canes harvested from 16 different varieties in France. These authors found the lowest content of *trans*-resveratrol in Chardonnay (190 mg/kg DW) and the highest content in Pinot noir cultivar (1526 mg/kg DW) while the *trans*-piceatannol and *trans*-ε-viniferin content were significantly higher in all cultivars when compared with VERGARA *et al.* (2012). GUERRERO *et al.* (2016) described that most abundant stilbenoid was *trans*-viniferin in all cultivars, which reached the highest concentration in Gewürztraminer cultivar. While EWALD *et al.* (2017) found the higher levels of *trans*-resveratrol and *trans*-viniferin in Pinot blanc and Sauvignon blanc harvested in Germany (3199-3329 mg/kg DW, respectively). ZHANG *et al.* (2011) studied the content of *trans*-resveratrol in grape canes of many different grape varieties, including local varieties cultivated in the seven major Chinese grape producing regions finding high variability.

Beside the genetic determinants, several other factors could explain these different results, such as the climate, the solvent used for the extraction of stilbenoids (RAYNE *et al.*, 2008), the temperature and time of grape canes storage (HOUILLÉ *et al.*, 2015). In addition, other factors, such as the pruning time, could affect the stilbene accumulation rate in canes. This factor has never taken in account before, in fact in many articles this data is not reported at all, and, when present, show to be highly variable, with pruning times varying from 1 to 4 months after the grape harvest.
Up to date there are no data available concerning stilbenoid content in grape cane waste of Italian grape varieties. In the present paper the stilbene accumulation in grape canes of seven autochthonous grape varieties from Veneto region, one of the most important wine producing regions in Italy, has been studied. In addition, the effect of pruning time on the stilbenes accumulation rate during the storage was taken in account.

## 2. MATERIALS AND METHODS

### 2.1. Plant materials

Grape canes of *Vitis vinifera* L. from Veneto region white varieties, such as Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso; and red varieties Incrocio Manzoni 13.0.25, Marzemino Biotipo 13, Raboso, were collected randomly from plants from a conventional vineyard at Oenological School of Conegliano, Province of Treviso, Italy (I.S.I.S.S – Istituto Statale di Istruzione Secondaria Superiore "G.B. Cerletti") (latitude 45° 87′ 69″ N and longitude 12° 28′ 53″ E). As reference, international varieties Sauvignon blanc INRA 316 and Pinot noir, grown in the same vineyard, were chosen because according to LAMBERT *et al*. (2013) these were the cultivars with the highest content of stilbenes. The canes were collected monthly in October (11$^{th}$), November (15$^{th}$) and December (13$^{th}$) (autumn-winter 2016-2017) from 30 selected plants for each variety. About 1.5 kg for each sampling and for each variety were obtained. The canes were cut into 10-20 cm long pieces and stored for three, six, nine and twelve weeks in well-aerated conditions in the dark, at room temperature. For control, a sample was immediately extracted after each pruning sampling point.

### 2.2. Stilbenoid extraction

The stilbenoid extraction was performed according to the procedure described by RAYNE *et al.* (2008) with some modifications. Briefly, the grape canes were ground with a coffee grinder (Imetec, Azzano San Paolo, BG, Italy). Three-stage extraction (in the dark to avoid stilbene isomerization) was performed by continuous stirring at room temperature using an 8:1 (v/w) 80% ethanol:sample ratio over a 60-min period for each extraction. During the first extraction, 250 $\mu$L of t-OH-stilbene 200 $\mu$g/mL in ethanol were added as internal standard. The extracts were vacuum filtered at 1.6 μm on glass microfibre filter (GF/A, Whatman) and combined and the solvent removed by rotary evaporation (Büchi model R-114, Flawil, Switzerland), then stored at -20°C. All the extractions were performed in triplicate. Before quantification, the extracts were defrosted at room temperature and homogenized. An aliquot of the extract (500 $\mu$L) was transferred to Eppendorf tubes and 500 $\mu$L of methanol were added. After centrifugation at 4000 × g for 1 min, part of the supernatant (500 $\mu$L) was transferred to HPLC vials.

## 2.3. HPLC analysis

The analysis of stilbenoids was performed according to the procedure described by VINCENZI *et al.* (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm x 250 mm, 5 μm, Agilent Technologies, Milano, Italy) at 40°C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient was 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B).
The gradient program was 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in 2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate was 1.0 mL/min and injection volume 20 μL. Detection was performed at 306 nm for *trans*-isomers for *trans*-resveratrol, *trans*-piceatannol and *trans*-ε-viniferin and at 285 nm for the corresponding *cis*-isomers. All the stilbene standards were obtained in *trans* form from Extrasynthese (Genay Cedex, France). The *cis*-isomers were obtained by UV-exposition of the corresponding *trans*-isomers, and were loaded in HPLC for the identification of the retention times. The concentration of individual stilbenes (both *trans*- and *cis*-forms) was calculated on the basis of peak areas using calibration curves of commercially available standards of *trans*-resveratrol, *trans*-piceatannol and *trans*-ε-viniferin, and correcting the value for the internal standard recovery. Data were analysed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were performed according to the procedure described by (SHRIVASTAVA and GUPTA, 2011).

## 2.4. Statistical analysis

Within each factor the results were evaluated by one-way analysis of variance (ANOVA), and values were analyzed by Tukey's test using the software STATISTICA 12.0 (StatSoft Inc, Tolson, USA). Results were expressed as mean values ± standard deviation (SD) and the value of $p < 0.05$ was considered statistically significant.
For the global analysis of alla data in order to take in account the interactions among different variables, a MANOVA test was applied and values were analyzed by Wilks's test using the software XLSTAT (Addinsoft).

# 3. RESULTS AND DISCUSSION

Considering that stilbenoid accumulation in cut canes depends on activation of related genes followed by active synthesis of resveratrol and its derivatives, as already reported by HOUILLÉ *et al.* (2015) and BILLET *et al.* (2018), it is expected that different grape varieties respond in different way after the injury for both total amount of stilbenoid produced and rate of their accumulation. Also, the climate and other environmental factors can affect the way the canes respond during the storage period, for this reason the canes of the seven varieties taken in consideration in this study were collected in the same year from plants grown in the same vineyard.
The evolutions of stilbenoids during the storage time for samples of different varieties harvested at different times is reported in Figs. 1, 2 and 3.

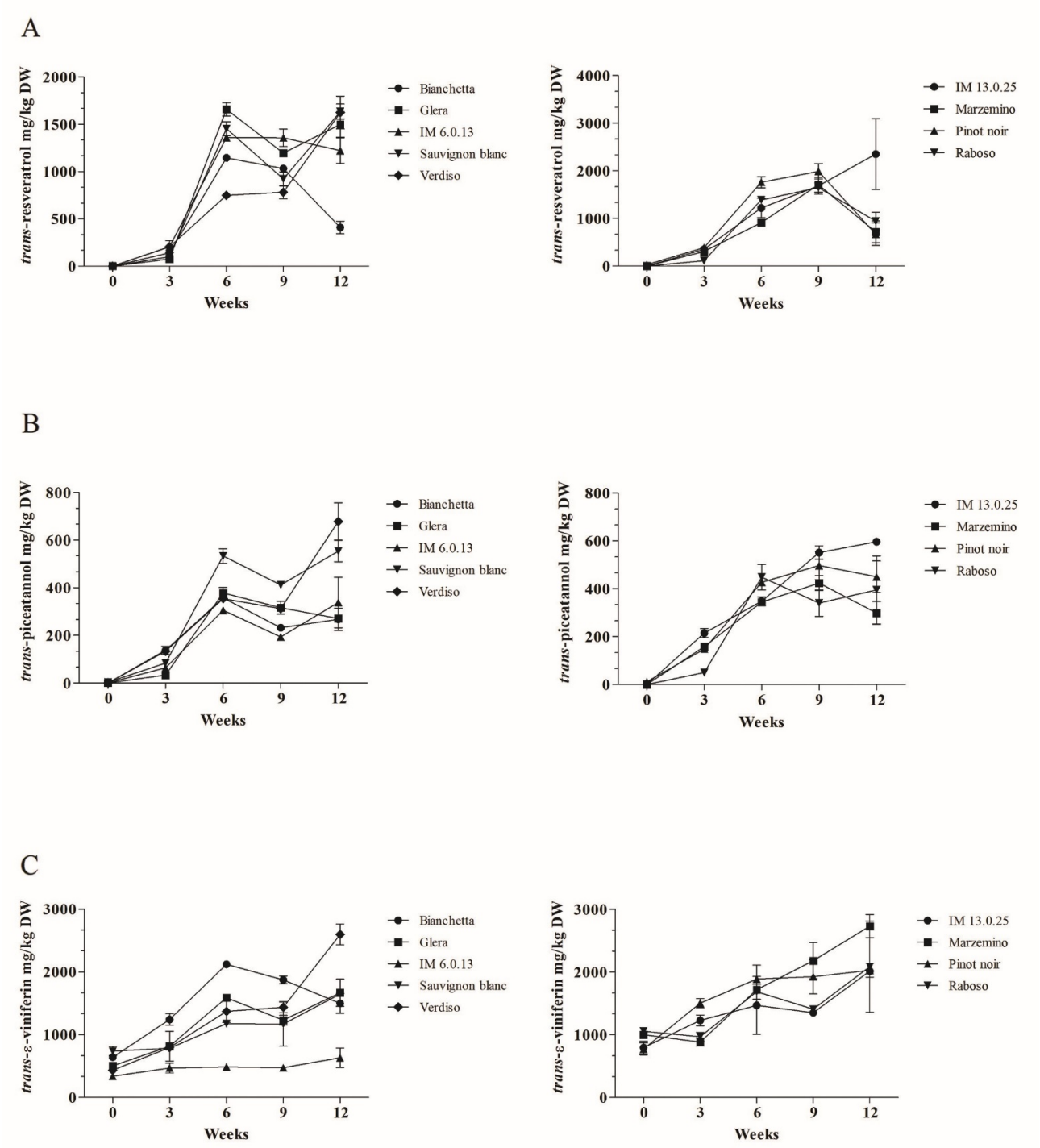


**Figure 1.** Content of *trans*-resveratrol (A), *trans*-piceatannol (B) and *trans*-ε-viniferin (C) on grape canes harvested in October (autumn–winter 2016–2017) from white varieties, Bianchetta, Glera, Incrocio Manzoni 6.0.1.3, Sauvignon blanc, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino, Pinot noir, Raboso, and stored at room temperature for three, six, nine, and twelve weeks. Results represent the mean ± SD of triplicate assays.

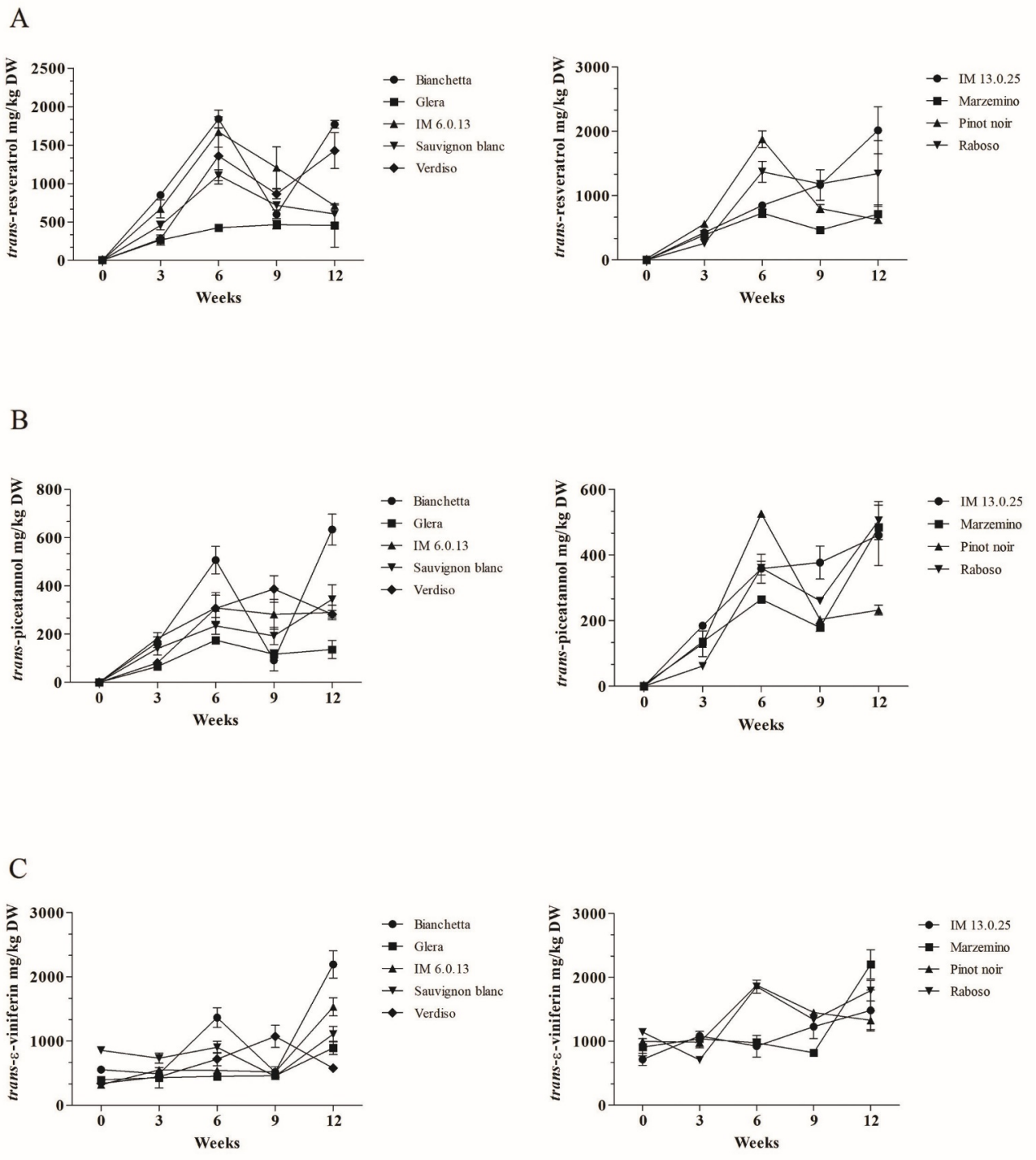


**Figure 2.** Content of *trans*-resveratrol (A), *trans*-piceatannol (B) and *trans*-ε-viniferin (C) on grape canes harvested at pruning time in November (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera, Incrocio Manzoni 6.0.1.3, Sauvignon blanc, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino, Pinot noir, Raboso, and stored at room temperature for three, six, nine, and twelve weeks. Results represent the mean ± SD of triplicate assays.

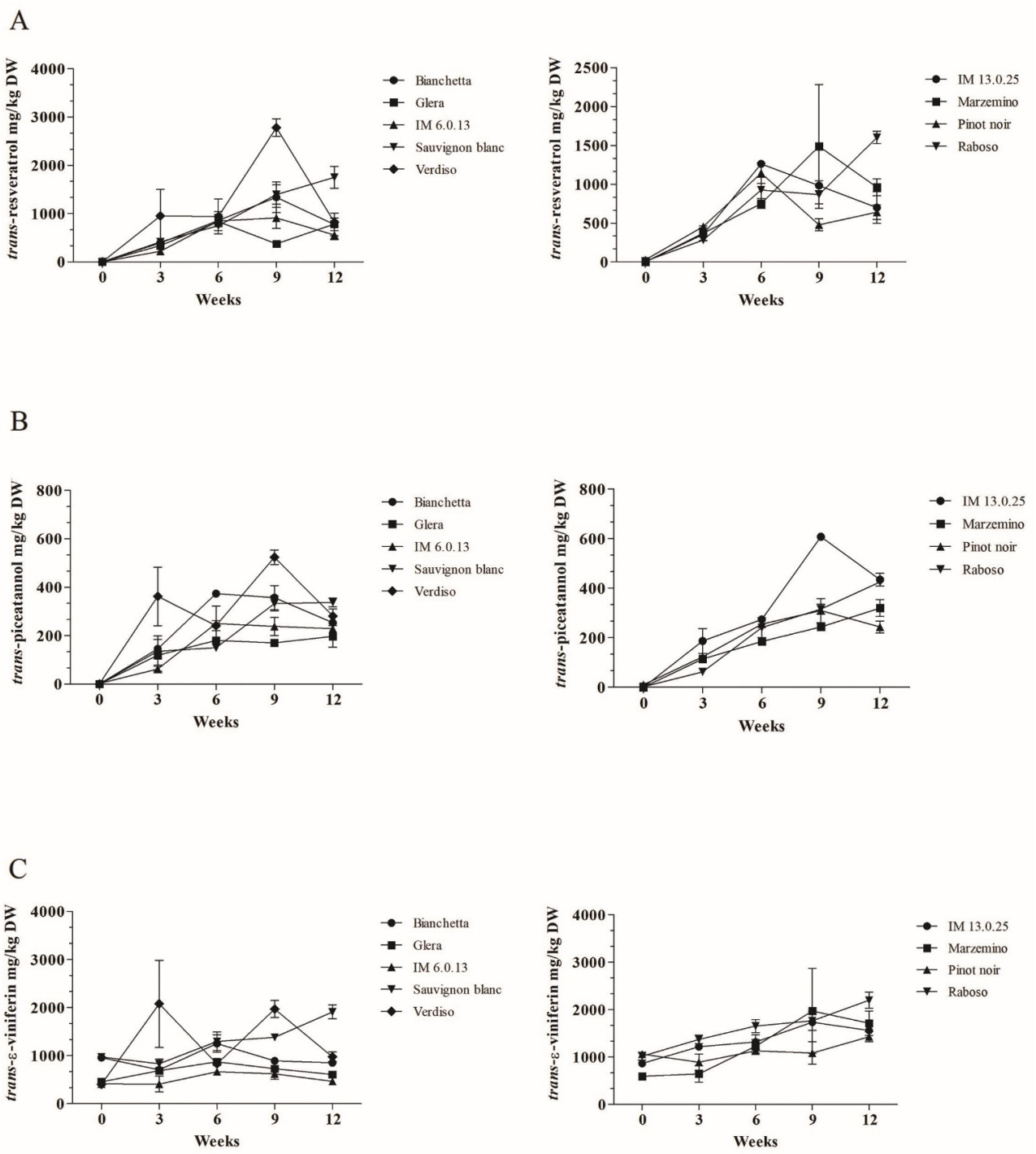


**Figure 3.** Content of *trans*-resveratrol (A), *trans*-piceatannol (B) and *trans*-ε-viniferin (C) on grape canes harvested at pruning time in December (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera, Incrocio Manzoni 6.0.1.3, Sauvignon blanc, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino, Pinot noir, Raboso, and stored at room temperature for three, six, nine, and twelve weeks. Results represent the mean ± SD of triplicate assays.

A MANOVA test was also applied to study the effect of different variables on the stilbenoids accumulation (Table 1).

**Table 1.** Results of MANOVA analysis on the total dataset.

| | Storage | Harvest | Variety | Storage* Harvest | Storage* Variety | Harvest* Variety | Storage* Harvest* Variety |
|---|---|---|---|---|---|---|---|
| F-value | 49,240 | 5,227 | 4,579 | 6,615 | 4,387 | 2,624 | 2,313 |
| GDL1 | 12 | 6 | 24 | 21 | 96 | 48 | 150 |
| GDL2 | 421 | 318 | 462 | 457 | 477 | 474 | 478 |
| p-value | < 0,0001 | < 0,0001 | < 0,0001 | < 0,0001 | < 0,0001 | < 0,0001 | < 0,0001 |

As a first observation, the basal level of *trans*-piceatannol and *trans*-resveratrol in fresh canes of Pinot noir was quite low (less than 40 mg/kg DW) and below the detection level in the other varieties, whereas the dimer *trans*-ε-viniferin was already present at concentrations 10 to 30 times higher in all the samples (Supplementary table 1). These results confirm the literature data (GORENA *et al.*, 2014; HOUILLÉ *et al.*, 2015; BILLET *et al.*, 2018), only VERGARA and colleagues (2012) found very high content of *trans*-resveratrol (between 2500 and 3500 mg/kg) already at time zero.
During the storage a large increase of stilbenoids was observed in all samples, confirming that the main variable driving their accumulation in grape canes is the storage time (highest F value in Table 1). However, a significant effect of the harvest time was also found (Table 1). Collectively, the canes pruned in October showed a gradual increase of stilbenes during all the storage period, whereas the canes collected in November showed a notable peak of accumulation of stilbene content after 6 weeks of storage for almost all the varieties (Figs. 1 and 2). HOUILLÉ *et al.* (2015) found the same results, i.e. a peak of accumulation of *trans*-resveratrol and *trans*-piceatannol after 6 weeks of storage, on eight cultivars (collected in December) and stored for two, four, six, eight and ten weeks. On the other hand, the pruning carried out in December demonstrated a different behavior in the accumulation of stilbenes (Fig. 3). For many varieties, in particular for Verdiso and Incrocio Manzoni 13.0.25 (IM 13.0.25) the peak of stilbene accumulation was retarded to 9 weeks of storage. This behavior of canes harvested at different times seems to show a slower stilbenoid response in canes harvested in October, probably due to the high quantity of stilbene synthase enzymes still present in the woody tissue, which under regulate the induction of the related genes after the injury. The synthesis of stilbenoids is instead more rapid with the evolution of canes toward winter dormancy until November, then the accumulation rate slowdown in canes harvested in December. This could explain the significant effect of the interaction storage*harvest time (Table 1).
The different cultivars showed different behaviors, and different responses to harvest time and storage conditions, as confirmed by the significant effect of variety, storage*variety and harvest*variety in the MANOVA test (Table 1).
Among the samples harvested in October, cultivar Verdiso, IM 13.0.25 and Marzemino showed the highest increase of *trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin after twelve weeks at RT (Fig. 1). Among the white varieties, Verdiso is one of the last to be harvested. Similarly, IM 13.0.25 and Marzemino are, among red varieties, those with the later harvest. It is not clear if this common behavior could be related to the similar accumulation rate of stilbenes in pruned canes. Regarding resveratrol, even though after 9

weeks Pinot noir was the cultivar with the highest content, confirming the high stilbene metabolism of this variety, after 12 weeks the content in IM 13.0.25 canes increased again reaching the highest value among all the varieties taken in consideration. Even for piceatannol, IM 13.0.25 presented concentration usually higher than Pinot noir.
The canes harvested in November demonstrated an increase for all stilbene compounds when maintained for twelve weeks at RT. Again, the cultivar IM 13.0.25 presented a constant increase of *trans*-resveratrol content reaching, after 12 weeks of storage, a value up to 2016±365 mg/kg DW, comparable with those found in October. In this group, a high increase of the content of *trans*-piceatannol (633±64 mg/kg DW) and *trans*-ε-viniferin (2193±213 mg/kg DW) was detected even on the cultivar Bianchetta (Fig. 2). Compared to Pinot noir, which reached after 6 weeks the maximum content of piceatannol and resveratrol among all varieties, Bianchetta and IM 13.0.25 were able to reach the same or higher quantities for both compounds after a storage of 12 weeks.
In grape canes harvested in December Pinot noir showed a very small accumulation of resveratrol and piceatannol, while again Verdiso and IM 13.0.25 were able to reach a high content of both compounds (Fig. 3) (Supplementary tables 2-5).
Our findings confirm the results previously found by GUERRERO *et al.* (2016), which highlight the importance of the cultivar in the accumulation of stilbenes in grape canes after pruning. In addition, for the first time, we demonstrated how the pruning time affects both the quantity and the accumulation rate of stilbenes in pruned canes.
The information on the maximum stilbene content recoverable from canes of different grapevine cultivars could be interesting for grape producers in order to obtain cane extracts with high stilbenes concentration from their own grape canes waste. These extracts can be the base for the purification of stilbenes to be used in the food or cosmetic industries, with a big economic income considering the value of food-grade resveratrol is about 2000-3000 US$/kg (ZHANG *et al.*, 2011). However, the crude cane extract could be also reused by grape growers in the same vineyard in an idea of circular economy. In fact, stilbenes have shown antifungal activity against different fungi. Until now, the antifungal activity *in vitro* of the crude cane extracts from Pinot noir, Gamaret and Divico cultivars against *Plasmopara viticola, Erysiphe necator* and *Botrytis cinerea* was reported by SCHNEE *et al.* (2013). Recently, the direct antifungal activity of crude cane extract from Pinot noir against *B. cinerea* was studied by monitoring the mycelium growth on nutrient agar medium, and also in grapevine plants *in vitro* and *in vivo* by DE BONA *et al*. (2019).

## 4. CONCLUSION

The information on the maximum stilbene content recoverable from canes of different grapevine cultivars could be interesting for grape producers, but many factors have to be taken in to account to obtain the highest yield of these compounds. First, the storage time is the main factor driving the increase of stilbenoids in grape canes, confirming the literature data, but we demonstrated for the first time that the total amount of stilbenoids and their rate of accumulation depends significantly on the pruning time. In addition the data reported in the present study confirm the importance of the cultivar in the accumulation of stilbenes in grape canes after pruning. Finally, this study showed that the cultivars Verdiso and Incrocio Manzoni 13.0.25 possess a high potential of stilbene accumulation, mainly when the canes were harvested in October.

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PAPER

# EFFECT OF MIXED STARTER CULTURES ON BIOGENIC AMINE FORMATION DURING THE RIPENING OF TUNISIAN DRY FERMENTED CAMEL MEAT SAUSAGE

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## ABSTRACT

The effect of mixed starter cultures on biogenic amine production was examined during the ripening process of dry camel meat sausage. Changes in pH, moisture content, proteolysis, microbial counts and lipid oxidation were also studied. The combination of three amine-negative bacteria, resulted in a drastic reduction of biogenic amine production. The highest total free amino acid concentration was observed in batches manufactured with mixed starter cultures. The bactericidal properties of *L. sakei* improved the hygienic quality of sausages by decreasing the number of Enterobacteriaceae. Inoculation of sausages with a mixture of strains, significantly delayed lipid oxidation and enhanced sensory characteristics.

*Keywords*: biogenic amine, fermented sausages, starter cultures, quality, ripening

## 1. INTRODUCTION

The formation of biogenic amines (BAs) in fermented sausages could affect the quality of the final products. BAs are mainly formed by the decarboxylation of amino acids. Biogenic amines could be harmful on health when they are consumed in large quantities; these molecules are responsible for food poisoning (LATORRE-MORATALLA *et al*., 2010; LORENZO *et al*., 2017). In fact, "tyramine and phenylethylamine have been associated with food histaminic intoxications and severe hypertensive crisis (MARINE-FONT *et al*., 1995)". Moreover, BOVER-CID *et al*. (1999) have reported that putrescine and cadaverine could contribute to the formation of heterocyclic carcinogenic nitrosamines.
Many factors contribute to the formation of BAs in dry fermented sausages such as ingredients, technological ripening conditions, acidification, and proteolysis during the ripening of dry fermented sausage. Many studies have reported that the production of biogenic amines in fermented meat products was related to the growth of Pseudomonas, enterobacteria, enterococci, and lactobacilli (DURLU-ÖZKAYA *et al*., 2001; SUZZI *et al*., 2003; LATORRE-MORATALLA *et al*., 2012).
Strains of lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) are usually used in the manufacture of dry fermented sausages. These strains should have the most important technological properties such as the adaptation to meat environment, development of red color, texture, and flavor of fermented meats (TALON *et al*., 2011). Moreover, the use of amine-negative strains with bacteriostatic activity can stopped the formation of BAs in the initial fermentation stage (BOVER-CID *et al*., 2000).
In the present study, the influence of mixed starter cultures of *L. sakei*, *S. carnosus* and *S. xylosus* on biogenic amine formation during the ripening of dry fermented camel meat sausage was studied. Besides the biogenic amine contents, evolution of microbial population, amino acids content, total lipid and total protein contents, pH, thiobarbituric acid (TBA), moisture content, textural and sensory characteristics were determined during the ripening of a dry fermented camel meat sausage.

## 2. MATERIALS AND METHODS

### 2.1. Sausage preparation

"The sausage formulation included 3.750 kg of camel meat (75%), 1.250 kg of hump fat (25%), 260 g of salt, 15 g of black pepper, 15 g of paprika, 60 g of glucose and 0.8 g of potassium nitrate. After chopping and mixing the ingredients, the mixture was divided into two batches (2.5 kg for each batch): batch 1, inoculated with a commercial starter culture starter A (20 g/200 kg): *L. sakei* + *S. carnosus* + *S. xylosus* (TEXEL SA-201, DANISCO, Paris, France) and batch 2, control without inoculation. Starter A was added to sausages according to manufacturer's recommendations. The mixture of each batch was stuffed into artificial casings, giving approximately 500 g as the final mass of each sausage and then placed in a fermentation chamber (BCR, CF 1 B, Antony, France). The sausages were fermented for 5 days at 24 °C and 80% relative humidity (RH). After 5 days of processing, the temperature was decreased to 14 °C for 23 days and the RH value was 80%. For sampling, three sausages of each batch at 0 day (mix before stuffing) and after 7, 14, 21 and 28 days of ripening were taken for microbiological, physicochemical and textural analysis. All reported values represent the mean of three random measurements of the sausage sample."

## 2.2. Microbiological analysis

"Sausage samples (10 g) of each batch were homogenized with 90 mL of sterile peptone water (Biolife, Milan, Italy) and decimal dilutions were prepared. Mesophilic LAB were enumerated on MRS (de Man, Rogosa and Sharpe) agar (Biolife) after 48 h of incubation at 30 °C. The number of staphylococci was determined on mannitol salt agar (Biolife) after incubation at 37 °C for 48 h. Yeasts and molds were enumerated on Sabouraud Dextrose Agar (Biokar, Beauvais, France) at 28 °C for 4 days. Total viable counts were determined on standard plate count agar (Biolife) at 30 °C for 48 h. Enterobacteriaceae were determined on Violet Red Bile Glucose (VRBG) (Biokar) at 37 °C for 24 h."

## 2.3. pH, moisture, weight loss, total lipid and total protein contents

"The pH values were measured in homogenates prepared by blending 10 g of sausage (Moulinex DPA141, Lyon, France) with 50 mL of distilled water for 2 min. Measurements were taken with a pH meter (microprocessor pH meter BT-500, Boeco, Hamburg, Germany). The moisture content was calculated by weight loss experimented by the sample (5 g) maintained in an oven (Memmert, UL 60, Schwabach, Germany) at 105 °C, until constant weight according to the ISO recommended method (ISO, 1973). Weight loss was expressed as the percentage of the initial weight (LIAROS *et al*., 2009)."
"Total lipids were extracted from 5 g of minced sausage according to the method of FOLCH *et al*. (1957). Total nitrogen was determined according to the Kjeldahl method and total protein estimated by multiplying the nitrogen content by 6.25."

## 2.4. Lipid oxidation analysis

"Lipid oxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) during the ripening period. This analysis was performed according to the method of GENOT (1996)" as described by EL ADAB *et al*. (2016).

## 2.5. Free amino acid (FAA) content

"Free amino acids were extracted and analyzed by reverse phase high-performance liquid chromatography (HPLC) (Agilent L 100 system, Province, Canada) equipped with a Hypersil ODS C18 column (250 mm × 4.6 mm dimensions of the column, 5 μm porosity)", as described by EL ADAB *et al*. (2016).

## 2.6. Biogenic amines analysis

Biogenic amines were determined using the method described by BOULARES *et al*. (2017). "Biogenic amines are first extracted from the test sample by chloridric acid (0.1 M) and then practice derivatization. Briefly, 4 mL of HCl (0.1 M) were added to 1 g of sausage. After homogenization, samples were centrifuged at 12.000 rpm for 20 min (Sigma, 6-16S, Munich, Germany) before being filtered. After that, 2 mL of the obtained aqueous fraction were homogenized with 1 mL of sodium bicarbonate and 2 mL of dansyl chloride, and then the mixture was heated during 1 h at 40 °C. After addition of 2 mL of diethyl ether, the organic fraction was collected and evaporated under nitrogen liquid stream. The mixture was then dissolved in 1 mL of acetonitrile. A standard solution of amines was prepared similarly and used as control. Finally, 20 $\mu$L of each derivatized solution were

injected onto HPLC column where the components will be retained unequally depending on their size and composition. A Knauer eurosphère 100-RP18 reversed-phase column (250 x 4.6 mm, 5 $\mu$m, Berlin, Germany) was used for chromatographic separation. Therefore, the detection was performed at 254 nm wavelength using Acetonitrile/water as mobile phase at a constant flow rate of 0.8 mL/min for 20 min."

### 2.7. Texture Profile Analysis (TPA)

"Texture profile analysis (TPA) of the samples was performed with a texture analyzer (TA-XT2 Stable Micro Systems, Haslemere, UK) equipped with a cylindrical probe of 50 mm in diameter. The sausages were cut in a cylinder 1 cm thick and 3 cm in diameter and compressed twice to 50% of their original thickness. Force-time curves were recorded at a crosshead speed of 1 mm/s. Texture profile parameters (Hardness, cohesiveness, springiness, gumminess and chewiness) were evaluated during the ripening of dry fermented sausages using the method of BOURNE (1978)."

### 2.8. Color measurement

"Color measurements were carried out using a CR-300 colorimeter (Minolta Chroma Meter CR-300, Tokyo, Japan). Each sausage was cut and the color of the slices was measured three times for each analytical point L*, a* and b* scale coordinates were obtained: L* (lightness), a* (redness) and b* (yellowness). Before each series of measurements, the instrument was calibrated using a white ceramic tile."

### 2.9. Sensory evaluation

"The sensory analysis was performed by a sensory panel of ten assessors who had undergone professional training. A slice of each sample batch (5 mm thick approximately) was served to the assessors. The sensory evaluation was based on a six point hedonic scale to determine red color (10 = red and shiny; 1 = dark and dull), after taste (10 = extremely desirable; 1 = extremely undesirable), fat intensity (10 = high; 1 = low), acidity (10 = strong acidity; 1 = light acidity), hardness (10 = firm; 1 = soft) and overall acceptability (10 = high; 1 = low)."

### 2.10. Statistical analysis

"Data were statistically analyzed using one-way analysis of variance (ANOVA) procedure of SPSS 17.0 (SPSS, Inc., Chicago, IL). Duncan's multiple range test was used to determine any significant difference between mean values and evaluations were based on a significance level of $p<0.05$."

## 3. RESULTS AND DISCUSSION

### 3.1. Microbiological analysis

The evolutions of microbial population during the ripening of control and inoculated sausages are shown in Fig. 1. The total viable counts (TVC) and LAB counts increased ($p<0.05$) during the ripening period. The numbers of LAB and TVC in starter-mediated

sausages were significantly higher than those in control ones ($p<0.05$), which could be explained by the prior inoculation of sausages by *Lactobacillus sakei*, *Staphylococcus carnosus* and *Staphylococcus xylosus*. Results showed that the number of lactic acid bacteria in dry fermented sausages increased ($p<0.05$) during the two first weeks of ripening. A decrease of their number ($p<0.05$) was observed during the two last weeks of ripening which could be due to the exhaustion of the sugar. These results were similar to those reported by QINXIU *et al*. (2016) and MEJRI *et al*. (2017).
Enterobacteriaceae counts decreased ($p<0.05$) during ripening in both control and starter-mediated sausages. This result was similar to that reported by LU *et al*. (2015). The number of Enterobacteriaceae is lower ($p>0.05$) in inoculated sausages than those measured on control ones. Enterobacteriaceae numbers dropped below 1 logarithmic unit in starter-mediated batches. This drop is due to bactericidal properties of starters (LORENZO *et al*., 2007; CIUCIU *et al*., 2014). Staphylococci profiles showed no significant differences ($p>0.05$). At day 0, staphylococci counts in starter-mediated sausages were more than five logarithmic units higher than those of the control samples. A decrease of the number of staphylococci in sausages was observed after seven days of ripening. Our results match with those found by ZHAO *et al*. (2011) in dry fermented mutton sausages. Yeast and molds counts increased ($p>0.05$) during the first seven days of ripening and then decreased ($p>0.05$) to reach at day 28 values of 4.15 and 3.77 log CFU/g, respectively, for control and inoculated batches.

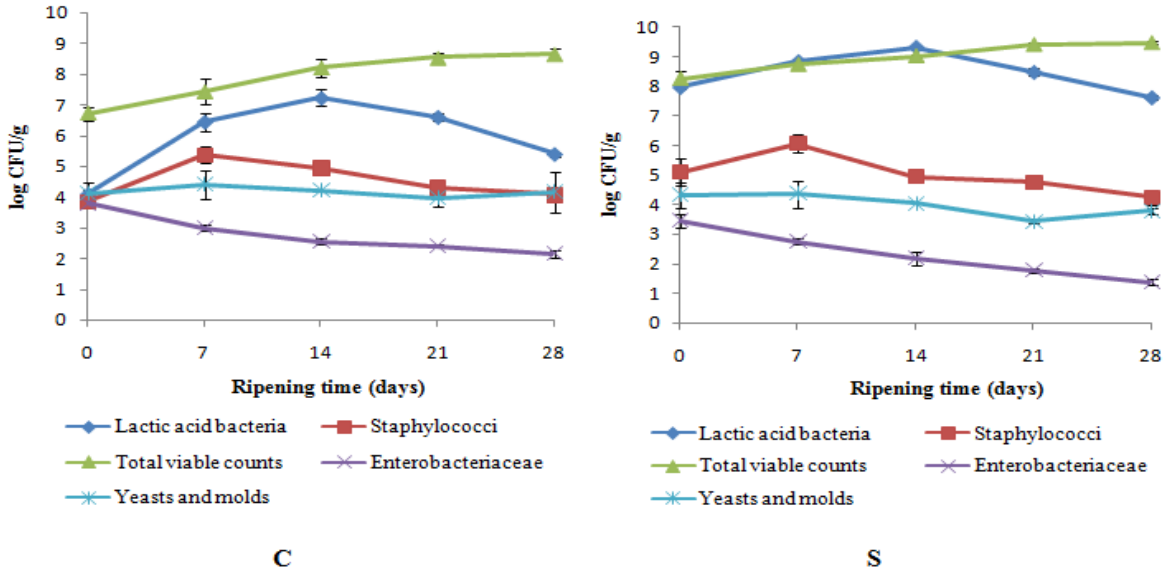


**Figure 1.** Evolution of microbial population during the ripening of control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures).

### 3.2. pH, moisture, weight loss, total lipid and total protein contents

The pH of control and starter-mediated sausages decreased ($p<0.05$) from 6.22 to 5.79 and 5.01 after 14 days of ripening, respectively (Fig. 2A). The pH decrease could be attributed to lactic acid production by LAB (NIE *et al*., 2014). Beyond the 14th day, pH values increased for both control and inoculated sausages. This may be caused by proteolytic

processes and mold growth on the sausage surface (ESSID and HASSOUNA, 2013). The weight of control sausages and those inoculated with mixed starter cultures decreased ($p<0.05$) during the ripening period (Fig. 2B). These results match with those found by JIN *et al*. (2010) and LIAROS *et al*. (2009). The moisture content decreased ($p<0.05$) in all the samples (Fig. 2C). However, no significant difference ($p>0.05$) was found between the different batches during the ripening process. This water loss is due to the elevated temperature of fermentation (24°C) and to the decrease of pH of sausages to their isoelectric pH (HAMOEN *et al*., 2013).

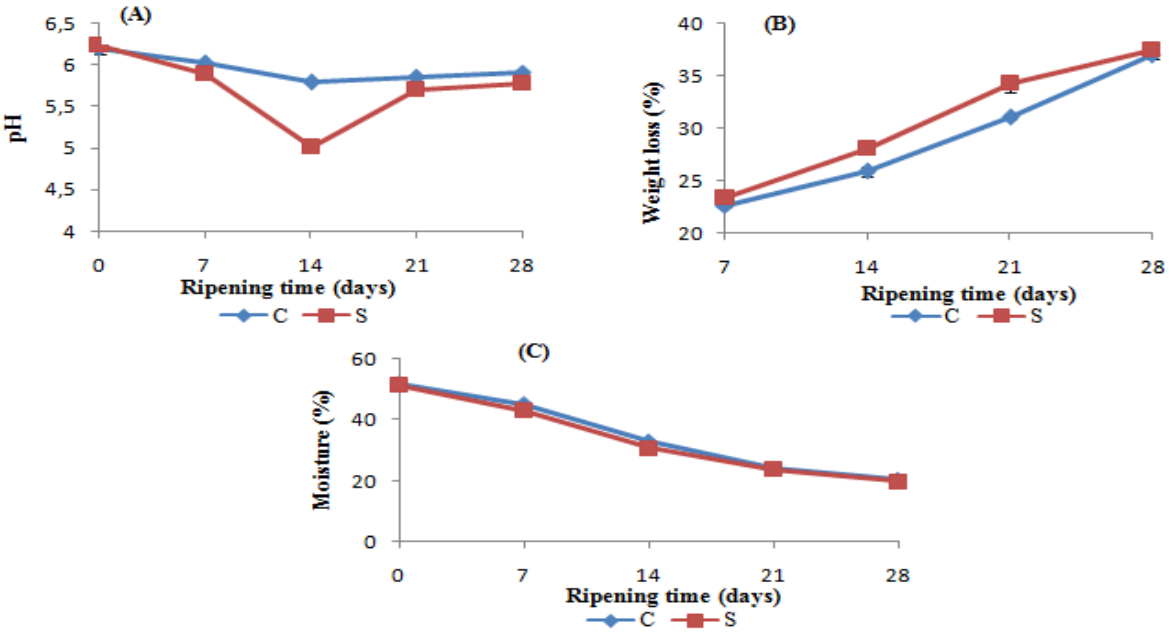


**Figure 2.** Evolution of pH (A), weight loss (B) and moisture (C) during the ripening of control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures).

Changes in total lipid and total protein contents during ripening of dry fermented sausages are summarized in Table 1.

**Table 1.** Evolution of the chemical composition during ripening of the control batch and starter-inoculated sausages.

| | Samples | Ripening time (days) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 21 | 28 |
| Chemical composition | | | | | | |
| Protein (%) | C | 15.75±0.21$^{a}$ | 19.25±0.44$^{b}$ | 20.13±0.88$^{b}$ | 21.02±0.88$^{b}$ | 27.78±0.22$^{c}$ |
| | S | 15.97±0.44$^{a}$ | 19.47±0.22$^{b}$ | 21.22±0.66$^{c}$ | 22.97±0.22$^{d}$ | 28.87±0.44$^{e}$ |
| Fat (%) | C | 18.1±0.1$^{a}$ | 21.50±0.1$^{b}$ | 26.65±0.05$^{c}$ | 34.7±0.5$^{d}$ | 36.1±0.7$^{e}$ |
| | S | 18.2±0.2$^{a}$ | 22.1±1.9$^{b}$ | 30.1±1.3$^{c}$ | 37.8±0.6$^{d}$ | 39.1±0.7$^{d}$ |

Samples: C, control camel meat sausage; S, sausage inoculated with mixed starter cultures. Data are means±standard deviation. Different letters in the same row indicate significant differences ($p<0.05$).

Results showed that protein content and lipid content increased ($p<0.05$) during ripening of control and inoculated sausages. However, protein content and lipid content showed no significant differences ($p>0.05$) between control batches and those inoculated with a mixture of strains. DALLA SANTA *et al*. (2014) reported that there was significant difference between control and inoculated Italian sausages in protein content at the end of ripening. However, they did not show any significant difference in lipid content.

### 3.3. Lipid oxidation analysis

The TBARS values increased ($p<0.05$) during ripening from 0.25±0.04 to 0.95±0.16 and 0.8±0.1 mg MDA/kg of sample, respectively, in control and inoculated sausages (Fig. 3). Results showed that there was no significant difference ($p>0.05$) between the different samples. Many factors could affect lipid oxidation such as "chemical composition of raw material, processing conditions, light, and access to oxygen (AHN *et al*., 2002)". The TBARS values are lower ($p>0.05$) in starter-mediated sausages than those found in control ones. Similar results were found by KARGOZARI *et al*. (2014) and EL ADAB *et al*. (2016) who reported that *S. xylosus* and *S. carnosus* could limit lipid oxidation in dry fermented sausages due to their antioxidant activity.

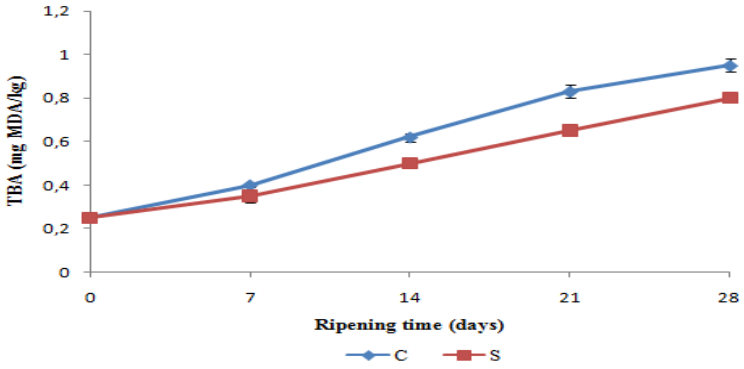


**Figure 3.** Changes in thiobarbituric acid (TBA) values during the ripening of control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures).

### 3.4. FAA content

The contents of total FAAs during ripening are shown in Table 2. The total FAAs increased through ripening ($p<0.05$); they reached at the day 28 values of 398.82 and 534.06 mg/100 g, respectively, for control and inoculated sausages. The highest total free amino acid content was found in inoculated samples. Similar results were found by ESSID and HASSOUNA (2013) and MEJRI *et al*. (2017). Many factors could affect the proteolysis in fermented sausages such as product formulation, technological ripening conditions and mixed starter cultures (HUGES *et al*., 2002).
The main amino acids present in the initial mixture were Arginine, threonine, glycine, alanine and glutamine with a concentration higher than 148.02 mg/100 g of dry matter. "The FAA content affects sensory properties impacting on fresh taste (glutamic acid and aspartic acid), sweet taste (glycine and alanine), bitter taste (arginine and leucine), sweet and bitter (lysine) and sour or salty (other FAA)" (DOMÍNGUEZ *et al*., 2016).

**Table 2.** Free amino acid content (mg amino acids/100 g of sausage) during ripening of the control batch and starter-inoculated sausages: C (control camel meat sausage), S (sausage inoculated with mixed starter culture).

| FAA | Ripening time (days) | | | | |
|---|---|---|---|---|---|
| | 0 | 14 | | 28 | |
| | C | C | S | C | S |
| Aspartic acid | 14.12±0.49$^{e}$ | 22.84±1.26$^{d}$ | 42.12±2.23$^{b}$ | 30.14±0.58$^{c}$ | 49.22±1.23$^{a}$ |
| Glutamic acid | 28.34±0.34$^{e}$ | 43.62±1.80$^{d}$ | 63.22±1.56$^{b}$ | 58.78±2.41$^{c}$ | 81.12±1.12$^{a}$ |
| Serine + Glutamine + Histidine | 8.62±1.30$^{d}$ | 9.02±2.31$^{cd}$ | 10.22±1.14$^{c}$ | 23.15±0.05$^{b}$ | 28.12±2.12$^{a}$ |
| Arginine + Threonine + Glycine | 84.32±2.50$^{e}$ | 95.56±2.14$^{d}$ | 130.02±0.77$^{b}$ | 120.45±1.36$^{c}$ | 145.02±0.89$^{a}$ |
| Alanine | 35.36±0.25$^{e}$ | 43.14±1.11$^{d}$ | 50.41±0.93$^{c}$ | 60.98±0.12$^{b}$ | 90.45±2.14$^{a}$ |
| Lysine | 10.65±1.18$^{e}$ | 22.34±2.23$^{d}$ | 25.17±1.25$^{c}$ | 26.23±0.04$^{b}$ | 28.78±1.45$^{a}$ |
| Tryptophane | 18.42±0.32$^{e}$ | 23.55±0.22$^{d}$ | 29.47±2.24$^{b}$ | 37.45±1.86$^{c}$ | 51.78±1.23$^{a}$ |
| Isoleucine | 3.12±0.45$^{e}$ | 4.65±1.01$^{d}$ | 9.12±0.88$^{b}$ | 7.52±1.03$^{c}$ | 13.12±0.58$^{a}$ |
| Leucine | 13.68±2.08$^{d}$ | 22.14±0.55$^{c}$ | 33.45±0.78$^{b}$ | 34.12±2.02$^{b}$ | 46.45±1.66$^{a}$ |
| Total | 216.63±0.79 | 286.86±0.71 | 392.2±0.58 | 398.82±0.85 | 534.06±0.49 |

Data are means±standard deviation.
Different letters in the same row indicate significant differences ($p<0.05$).

### 3.5. Biogenic amines contents

Total biogenic amine content in starter-mediated sausages was lower than in control ones during ripening ($p<0.05$) (Fig. 4). The total biogenic amine content was 285.67 mg/kg in sausages at the beginning of fermentation, which increased significantly during the first seven days of ripening to reach values of 338.28 and 310.14 mg/kg respectively, in the control sausages and sausages inoculated with *L. sakei*, *S. xylosus* and *S. carnosus*. Beyond the 7$^{th}$ day, total BA concentrations decreased significantly ($p<0.05$) to reach at the end of ripening respectively, values of 256.06 and 116.91 mg/kg. After 28 days of ripening, the total BA content in starter-mediated sausages was 54.3% lower than that in the control samples ($p<0.05$). The combination of three amine-negative bacteria, resulted in a drastic reduction of biogenic amine production. LEE *et al*. (2016) reported that *L. sakei* and *S. xylosus* could degrade BAs formed during fermentation through biogenic amine oxidases enzymes. These findings match with those found by HU *et al*. (2007) and NIE *et al*. (2014).
Changes in putrescine, cadaverine, spermine, spermidine and histamine concentrations during ripening of camel meat sausages are shown in Fig. 5. There was a decrease in all biogenic amines in the analyzed samples during the ripening. Spermine and spermidine were the predominant amine compounds in the sausages, followed by putrescine, histamine, and cadaverine. Our result is not in agreement with the study of GONZALEZ-FERNANDEZ *et al*. (2003) and LU *et al*. (2010), who reported that cadaverine and putrescine were the predominant amine compounds respectively, in Spanish pork sausage and traditional Chinese smoked horsemeat sausage. Many factors could contribute to the variability between the different types of products such as the microbiological quality of raw materials, ingredients, diameter of sausage, acidification, proteolysis and technological ripening conditions (BOVER-CID *et al*., 2001: BOZKURT and ERKMEN, 2002; LATORRE-MORATALLA *et al*., 2012).

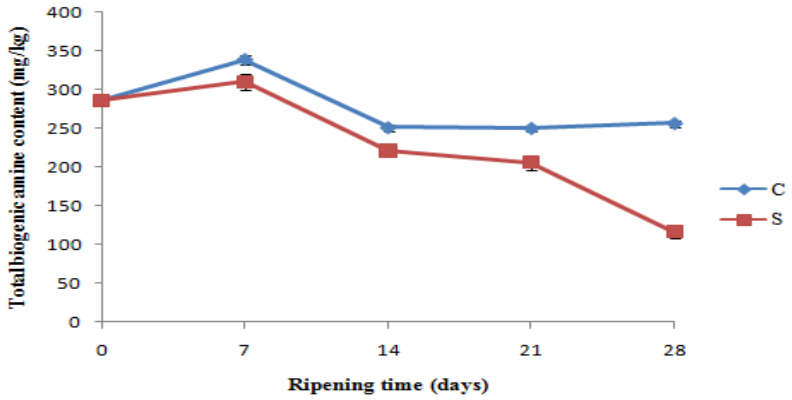


**Figure 4.** Changes in the total concentration of biogenic amines (mg/kg) in control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures) during ripening.

Spermidine and spermine are natural amines that always appear in fresh meat (HERÁNDEZ-JOVER *et al*., 1997). BOVER-CID *et al*. (2001) showed that these two BAs could be a source of nitrogen for some microorganisms, which could explain the decrease of spermidine and spermine concentrations during the ripening of dry fermented sausage.
The histamine concentrations were much lower in the starter-mediated sausages than that in the control batches (Fig. 5). At the end of ripening, the histamine content in inoculated sausages was 41.1% lower than that of the control ones ($p<0.05$). Many studies showed that histamine toxicity depended on the concentration upon absorption and it could be enhanced by the presence of cadaverine and putrescine (BOVER-CID *et al*., 2001; RENES *et al*., 2014). The drastic reduction of biogenic amine production is related to the drop of the pH of inoculated samples, which contribute to the decrease of the number of Enterobacteriaceae ($p<0.05$).
"Cadaverine can be used as an indicator of food hygiene (CHANG *et al*., 2012)." Cadaverine contents increased ($p<0.05$) during the first seven days of ripening from 0 mg/kg to 2.84 and 1.8 mg/kg for the control and starter-mediated sausages, respectively (Fig. 5). At the end of ripening, cadaverine accumulation was significantly ($p<0.05$) inhibited by 63.8% in starter-mediated sausages compared to the spontaneously fermented sausages. Results showed that there was a significant difference ($p<0.05$) between control batches and those inoculated with mixture of starter cultures. KOMPRDA *et al*. (2009) and RABIE *et al*. (2009) reported that the formation of cadaverine in fermented sausages is related to the presence of enterobacteria, which could be used as a chemical indicator of raw material and manufacturing practice hygiene. Values obtained for cadaverine indicate application of good hygiene in all phases of production.
The putrescine concentrations increased ($p<0.05$) during the initial 7 days. At the end of ripening, the putrescine concentration in inoculated sausages was 58.8% lower than that of the control ($p<0.05$) (Fig. 5).

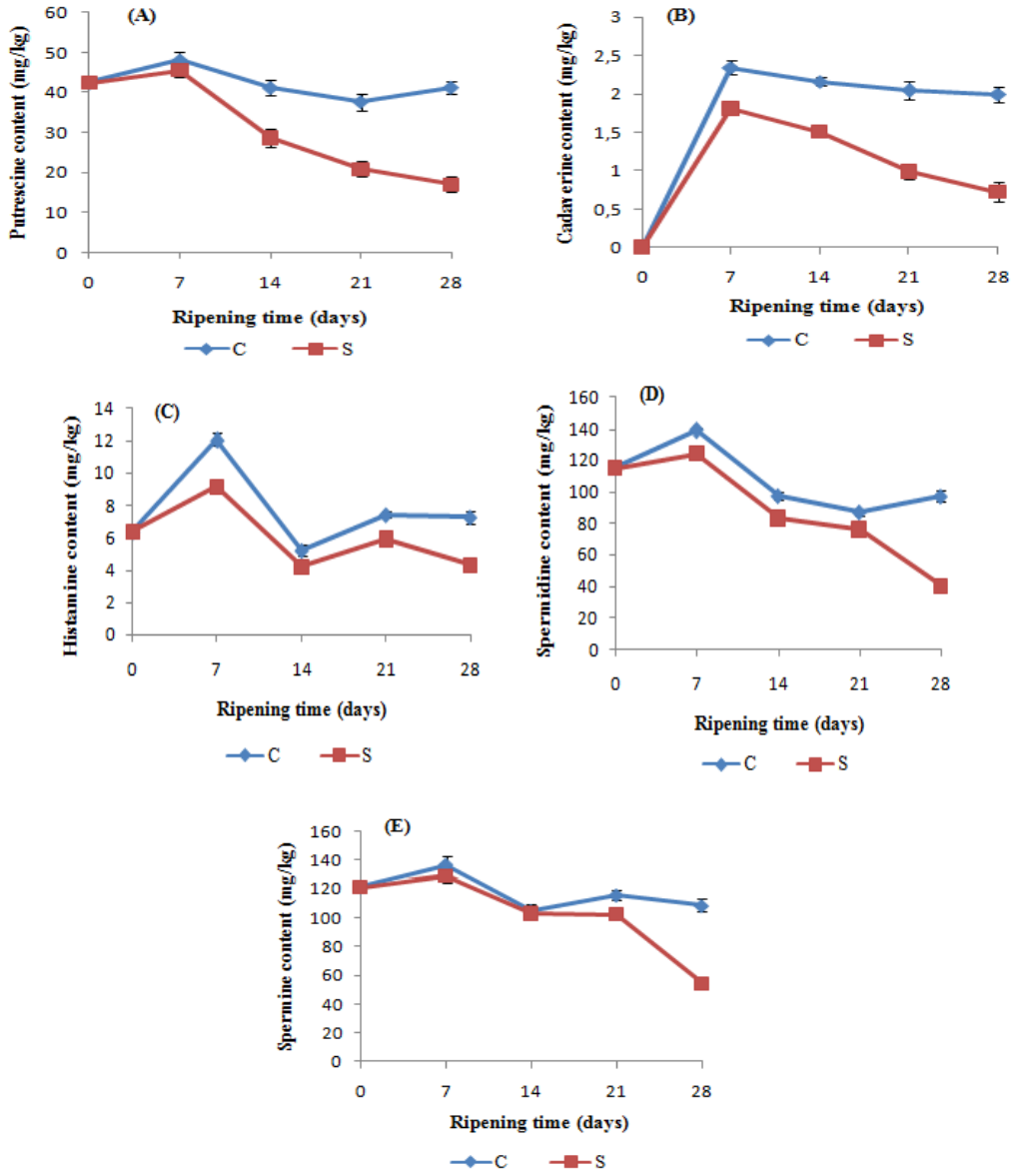


**Figure 5.** Changes in the amounts of putrescine (A), cadaverine (B), histamine (C), spermidine (D), and spermine (E) (mg/kg) in control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures) during ripening.

Our results showed that the combination of three bacterial strains could more effectively inhibit the formation of biogenic amines. Many studies reported the important role of *L. sakei* in the inhibition of the formation of biogenic amines (GENÇCELEP *et al*., 2012; LATORRE-MORATALLA *et al*., 2010; BAKA *et al*., 2011). "*L. sakei* is highly adapted to the fermented meat products and the optimal temperature of growth is between 15 and 25°C, which is the temperature range for sausages manufacture (BOVER-CID *et al*., 2001)." Moreover, GONZALEZ-FERNÁNDEZ *et al*. (2003) reported that *L. sakei* could reduce the formation of biogenic amine due to its strong acidifying activity. "BOVER-CID *et al*. (2001)

also reported that this specie is able to inhibit the production of biogenic amines in Spanish fermented sausage. However, when *L. sakei* was combined with *S. carnosus* or *S. xylosus* an even more effective reduction of amine accumulation was achieved compared with the effect of each strain used alone (LATORRE-MORATALLA *et al*., 2012)."
"MASSON *et al*. (1996) showed that *S. carnosus* and *S. xylosus* can be used as safe starter cultures. Whereas, STRAUB *et al*. (1995) found that *S. carnosus* contribute to the production of biogenic amines, but they did not find this for *S. xylosus*."

### 3.6. Texture profile analysis (TPA)

Fig. 6 shows the hardness, gumminess, chewiness, springiness, cohesiveness and resilience of control and inoculated sausages. Results showed that there were no significant differences between batches in any of the textural parameters studied.

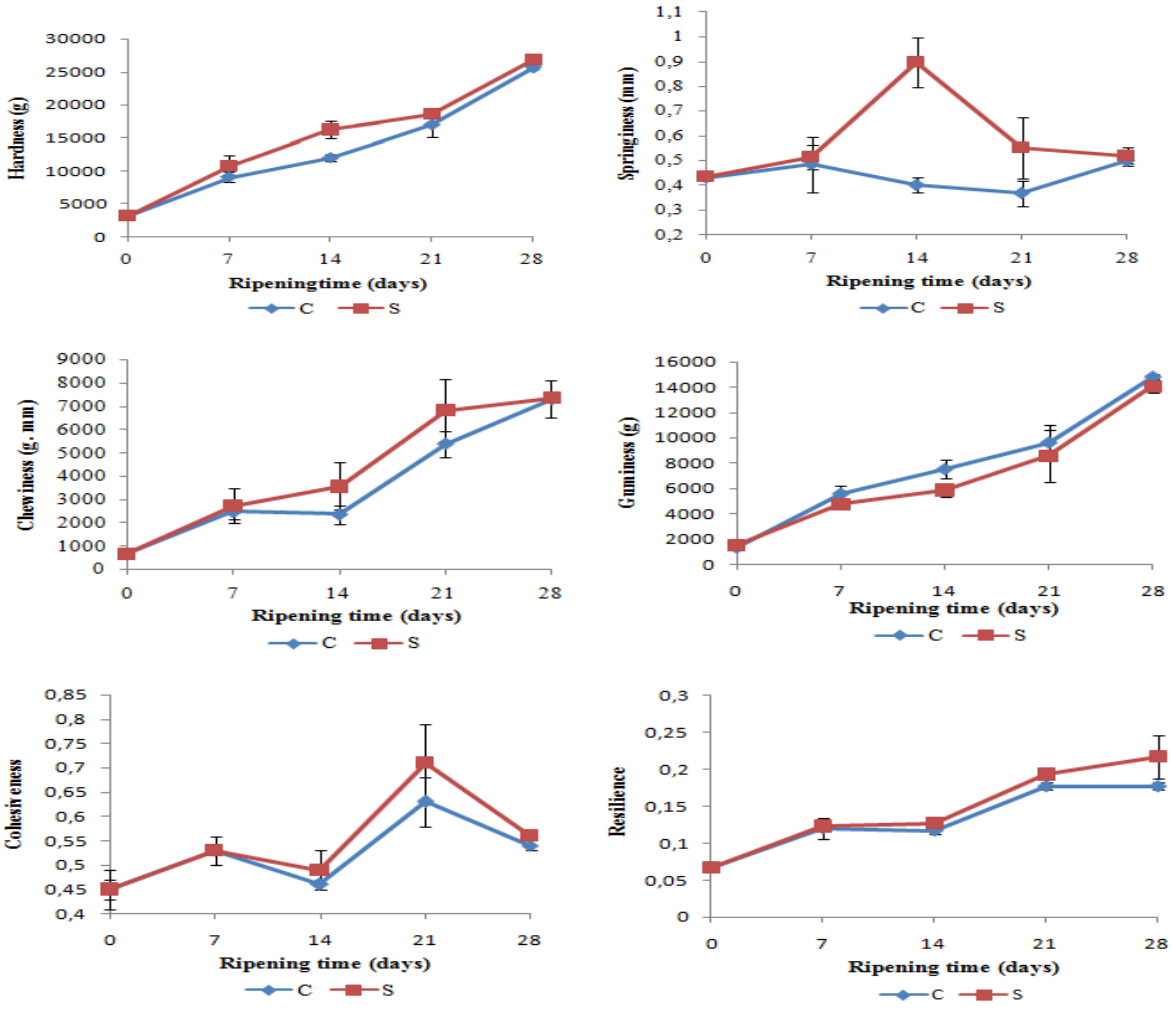


**Figure 6.** Changes in textural parameters during the ripening of control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures).

Hardness and springiness increased ($p<0.05$) during the ripening of control and inoculated sausages. Similar results were found by GONZALEZ-FERNANDEZ *et al*.

(2006) and BOZKURT and BAYRAM (2006) who reported that the increase of hardness could be explained by the elevated temperature during fermentation (24°C). The increase of springiness indicated that elasticity of camel meat sausage increased during the ripening period. Gumminess and chewiness values increased ($p<0.05$) during the whole process. These results are in agreement with those found by QINXIU *et al*. (2016).

### 3.7. Color properties

The color parameters, lightness (L*), redness (a*) and yellowness (b*) are shown in Fig. 7. L* values decreased ($p<0.05$) during ripening due to weight loss and higher myoglobin content (KADIM *et al*., 2008; OLIVARES *et al*., 2010). Moreover, results showed that L* values were significantly affected ($p<0.05$) by ripening time and not by the addition of mixed starter cultures ($p>0.05$). In relation to a* values, an increase ($p<0.05$) was observed during the first two weeks of ripening of dry fermented sausages followed by a significantly decrease which probably due to partial or total denaturation of nitrosomyoglobin because of the production of lactic acid (PEREZ-ALVAREZ *et al*., 1999; RUBIO *et al*., 2008). The evolution of a* and L* values found in this study was similar to that described by other authors (KAYAARDI *et al*., 2003; MEJRI *et al*., 2017). The b* values decreased ($p<0.05$) during ripening of both control and inoculated sausages. This finding was similar to that found by BOZKURT and BAYRAM (2006) during the ripening of sucuk.

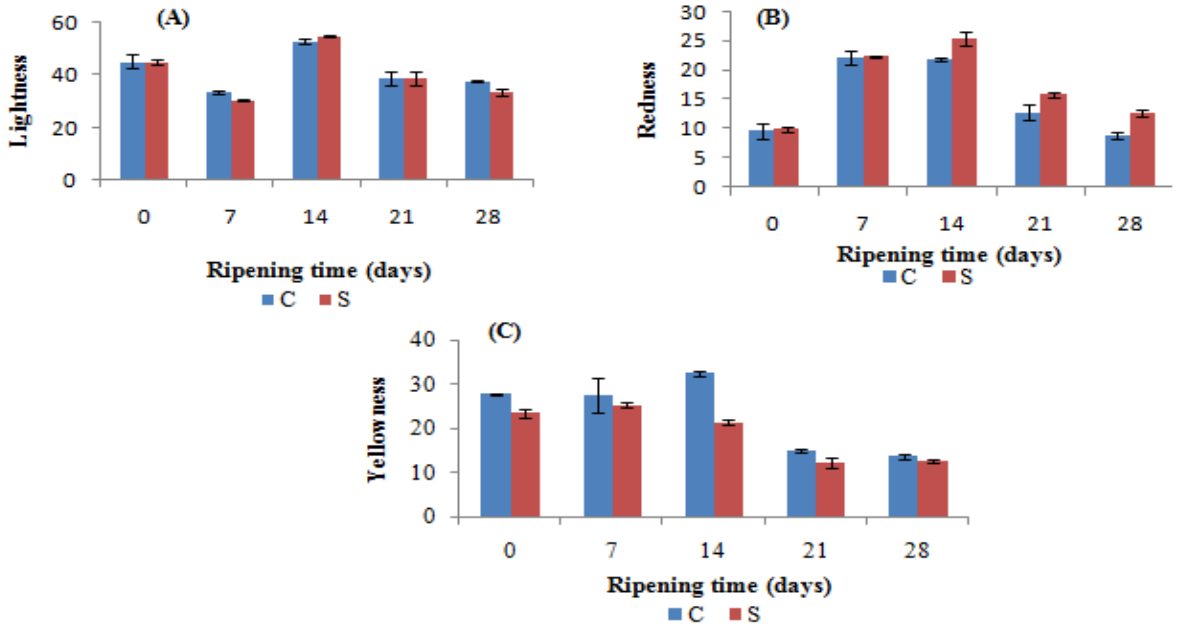


**Figure 7.** Changes in lightness (A), redness (B) and yellowness (C) during the ripening of control and inoculated sausages: C (control sausage), S (sausage inoculated with mixed starter cultures).

### 3.8. Sensory evaluation

The results of a sensorial evaluation of control and inoculated sausages are shown in Fig. 8. In fact, the starter-mediated sausages showed a more pronounced red color when

compared to control ones ($p<0.05$). RAVYTS *et al*. (2010) reported that the red color of fermented sausages was often related to the nitrate reductase activity of CNS. Moreover, inoculated sausages had higher scores of acidity ($p<0.05$) which could be explained by the lactic acid produced from bacterial carbohydrate metabolism. Additionally, sausages inoculated with mixed starter cultures showed a significantly greater overall aromatic intensity than that noted on control samples, which could be due to the lipolytic, acidifying and proteolytic activities of strains of *S. xylosus*, *S. carnosus* and *L. sakei* inoculated in meat products. Our results showed that there was no significant difference ($p>0.05$) in fat intensity. Inoculated sausages showed a significantly higher firmer texture ($p<0.05$) than those found on control samples. Similar results were found by FONSECA *et al*. (2013).

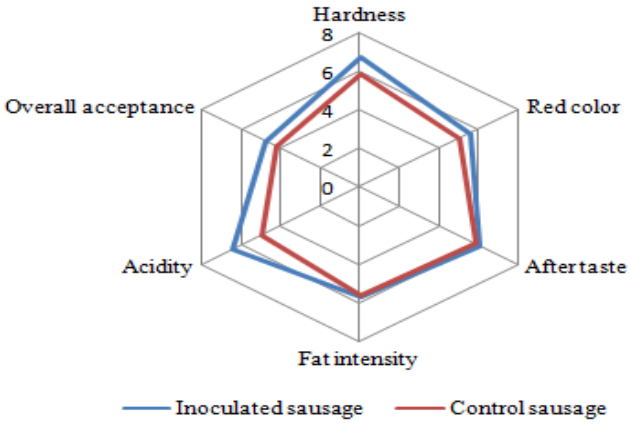


**Figure 8.** Sensory evaluation of control sausages and sausages inoculated with mixed starter cultures.

## 4. CONCLUSION

This study focused on the effect of mixed starter cultures on microbiological, biochemical and sensory characteristics of a dry fermented camel meat sausage. The bactericidal properties of *L. sakei* improved the hygienic quality of sausages by decreasing the number of Enterobacteriaceae. Inoculation of dry sausages with a mixture of strains, significantly delayed lipid oxidation and improved sensory characteristics. Moreover, the total biogenic amine concentration in starter-mediated sausages was much lower than that in the control samples. These results suggest that *L. sakei*, *S. xylosus* and *S. carnosus* could be used as safe mixed starter cultures in dry sausage production to inhibit biogenic amine formation and enhance sensory quality.

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PAPER

# CYCLIC PROANTHOCYANIDINS IN PINOT NOIR WINE

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## ABSTRACT

The identification of cyclic (or crown) B-type proanthocyanidins in wine was recently reported; this identification has unlocked new possibilities for their application to wine quality evaluation. Here, cyclic and non-cyclic B-type proanthocyanidins, along with other phenolic compounds as well as sensory and oenological parameters, were characterized in eleven Pinot Noir wines. The wines were produced from grapes harvested in different vineyards and under different winemaking conditions. With Principal Component Analysis (PCA) based on the cyclic proanthocyanidins or their relative proportions, it was possible to differentiate the wines according to specific winemaking conditions. Moreover, cyclic proanthocyanidins were related to the overall sensory quality of Pinot Noir wines.

*Keywords*: Pinot Noir, cyclic proanthocyanidins, winemaking, phenolic profile, high-resolution mass-spectrometry, sensory analysis

## 1. INTRODUCTION

Red Pinot Noir wine is a light-to-medium-bodied wine with a complex aroma profile (CASASSA *et al.*, 2018). It is produced in several viticultural areas as well as in South Tyrol (Italy). Several commercial frauds involving the marketing of Pinot Noir have been recorded. For instance, some producers were convicted in 2010 of mislabeling 13.5 million L of Pinot Noir wine that was replaced with cheaper wines made with Merlot and Syrah grape varieties (TAKEOKA *et al.*, 2011). For this reason, assessing the commercial quality of Pinot Noir wines and investigating a wider selection of authenticity markers became advisable. Several studies have been proposed for comparative authenticity assessments of Pinot Noir and other wines. For example, South Tyrolean Pinot Noir wines were differentiated from Cabernet Sauvignon using proton-transfer mass spectrometry analysis (SPITALER *et al.*, 2007). Furthermore, the polyphenol content and antioxidant activity of *nouveau* wines made from Pinot Noir and other grape varieties (PELLEGRINI *et al.*, 2000) were studied. In addition, the comparison of the phenolic and sensory profiles of organic wines made from Pinot Noir grapes and other varieties was performed (LANTE *et al.*, 2004). Pinot Noir showed a content of phenolic compounds (including phenolic acids) comparable to Cabernet Sauvignon and Cabernet Franc (VAN LEEUW *et al.*, 2014). However, Pinot Noir wines are lighter in color compared to other wines because of a lower total anthocyanin content (PETERLUNGER *et al.*, 2002). Also, the content of tannins in Pinot Noir grapes is lower compared to other red wines (CASASSA *et al.*, 2018; HARBERTSON *et al.*, 2008).

Phenolic compounds can be used to differentiate wines according to the winemaking technique (BAIANO *et al.*, 2009, SIREN *et al.*, 2015; ZHANG *et al.*, 2018), grape variety (BOSELLI *et al.*, 2004; PERESTRELO *et al.*, 2018; VAN LEEUW *et al.*, 2014), vintage (BELLOMARINO *et al.*, 2010; GEANA *et al.*, 2016; GIACOSA *et al.*, 2019), and geographical origin (GRANATO *et al.*, 2011; ROCCHETTI *et al.*, 2018; STOCKHAM *et al.*, 2013). The anthocyanin profile is currently one of the most employed parameters for authenticity assessment studies (OIV, 2007; VILLANO *et al.*, 2017). However, anthocyanins as chemical markers have a limited application for several reasons: they can be applied only to red wines, and furthermore, during the aging of wine, anthocyanins are oxidized or transformed into oligomeric and polymeric pigments through condensation reactions with flavanols (HE *et al.*, 2012; ZHANG *et al.*, 2018). Thus, the anthocyanin content decreases in aged wines, and the assessment of the grape varieties used to make red wine may be difficult. For this reason, more stable chemical markers should be identified and investigated for authenticity purposes with respect to the grape variety.

A recent study highlighted the presence of an unconventional cyclic B-type tetrameric procyanidin (also known as 'crown' procyanidin) in Cabernet Sauvignon, providing also its full structural characterization (ZENG *et al.*, 2019). Several studies have also identified the profiles of cyclic B-type tetrameric, pentameric, and hexameric procyanidins and prodelphinidins in red and white wines (LONGO *et al.*, 2018a,b,c; LONGO *et al.*, 2019; MERKYTE *et al.*, 2020), including Pinot Noir. The role of proanthocyanidins (PAC) as chemical markers to evaluate wine quality and authenticity is promising, as their profile and the relative proportions of the different congeners were preliminarily found to be dependent on the grape variety used for winemaking (LONGO *et al.*, 2018c; LONGO *et al.*, 2019). Besides, cyclic proanthocyanidins (C-PAC) showed greater stability towards strongly acidic and depolymerising conditions in comparison to (conventional) non-cyclic proanthocyanidins (NC-PAC) (ZENG *et al.*, 2019). These C-PAC compounds showed also

more resistance than their NC-PAC analogues towards fragmentation during mass spectrometric analysis (LONGO *et al.*, 2018a).
In this report, the profile of C-PAC was studied in eleven Pinot Noir wines from the same winery but produced with different winemaking practices. The aim of this study was to investigate the profile of PAC in these wines in relation to specific winemaking factors, such as the use of raisins or undesired stuck fermentations and the location of the vineyards. In addition, other phenolics and the sensory profiles were discussed. The results shed light on the possible role of C-PAC in relation to the effects of specific winemaking practices or geographical location of the vineyards.

## 2. MATERIALS AND METHODS

### 2.1. Wine samples, chemicals, and materials

Eleven red dry wines obtained from 100% Pinot Noir grapes were produced and donated by a local winery (Franz Haas, Montagna, BZ, Italy). The grapes were harvested in 2016 in different vineyards located between 350 and 800 m a.s.l. in Trentino-South Tyrol (Italy). The mass of grapes obtained for each vinification was 3.5 t. The maceration lasted eight days at a constant fermentation temperature of 26°C. The samples differed for aspects such as the altitude, location, and orientation of the vineyards and for the winemaking practices as described in Table 1.

**Table 1.** Description of the eleven Pinot Noir wines in terms of vineyard, altitude, location, orientation, and winemaking techniques.

| Wine | Vineyard | Altitude (a.s.l./m) | Location (orientation) | Winemaking technique |
|---|---|---|---|---|
| 1 | A | 400 | Pinzano (BZ) (South West) | Grape mass 3.5 t; 8 days maceration, 25-26°C fermentation temperature |
| 2 | A | 400 | Pinzano (BZ) (South West) | As wine 1, but a thermal maceration at 42°C was applied for 8 hours prior to alcoholic fermentation held at 20°C |
| 3 | A | 400 | Pinzano (BZ) (South West) | As wine 1, but it underwent a stuck fermentation followed by a second inoculation with supplementary addition of $SO_2$ |
| 4 | B | 780 | Trentino (South East) | As wine 1 |
| 5 | C | 750-800 | Aldino (BZ) (South) | As wine 1 (grapes have been treated with a leaf fertilizer) |
| 6 | C | 750-800 | Aldino (BZ) (South) | As wine 1 |
| 7 | D | 650 | Gleno (BZ) (South West) | As wine 1 |
| 8 | E | 350 | Mazzon (BZ) (North West) | As wine 1 |
| 9 | E | 350 | Mazzon (BZ) (North West) | As wine 1 |
| 10 | E | 350 | Mazzon (BZ) (North West) | As wine 1, but with 20% of non-destemmed grapes |
| 11 | E | 350 | Mazzon (BZ) (North West) | As wine 1, but using 100% raisins |

## 2.2. HPLC-DAD-HRMS/MS analysis

Solvents and standard compounds for the HPLC-HRMS/MS analysis were purchased from Sigma-Aldrich Ltd. All chemicals were LC-MS grade. The preparation of wine samples and the HPLC-HRMS/MS analysis were performed according to the procedure reported by LONGO *et al*., 2018a with slight modifications. Briefly, 20 mL of each wine were concentrated under low pressure (11 mbar) at 40°C. Then, a gentle $N_2$ flux was applied for 30 min and the samples were re-dissolved (with a sonication for 5 min) to a final concentration 10 times higher. Finally, all samples were filtered (0.2 $\mu$m) before HPLC injection.

A Q-Exactive HRMS instrument (Thermo Fisher Scientific, Rodano, Milano, Italy) was coupled to an Agilent 1260 HPLC (Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Milano, Italy) with a 16 channel DAD detector. The chromatographic separation was carried out using an ODS Hypersyl C18 LC column (125 mm × 4.6 mm i.d., 5 μm, Thermo Fisher Scientific), which was protected with a HPLC pre-column filter (ODS Hypersil, 5 $\mu$m pore size, 10 x 4 mm drop-in guards, Thermo Fisher Scientific) at a flow rate of 1 $mL \cdot min^{-1}$. The mobile phase consisted of solvent A (0.1% v/v formic acid in 0.02 $mol \cdot L^{-1}$ ammonium formate in water) and solvent B (0.1% v/v formic acid in saturated ammonium formate acetonitrile). The gradient program of solvent B was as follows: from 0 to 21 min 5%, 21 to 22 min 25%, 22 to 27 min 95%, 27 to 28 min 5%, followed by a re-equilibration step (5% B) from 28 to 35 min. The DAD spectra were recorded from 210 to 600 nm and provided real-time monitoring at 280 nm, 320 nm, 365 nm, 420 nm and 520 nm (+/- 4 nm). A post-column flow splitter valve (Upchurch Scientific) was used to feed both analyzers in parallel (DAD and HRMS) at a fixed ratio. For the Full MS analysis, the HESI source was operated in positive ionization mode for the analysis of proanthocyanidins and in negative ionization mode during the analysis of the phenolic profile. The following conditions were used: sheath gas at 20 (arbitrary units), auxiliary gas at 5 (arbitrary units), auxiliary gas temperature at 250°C, spray voltage at +3,500 kV, capillary temperature at 320°C and RF S-lens at 70 (arbitrary units). The mass range was from m/z 500 to 2,000 with the Full MS set resolution of 70,000 (@200 m/z), AGC target at 3.106, max injection time of 300. Full MS parameters were: MS/MS AGC target 106, max. injection time 300, FT-MS set resolution 35,000, loop count 5, isolation window 2 or 3 m/z with 1 m/z offset, normalized collision energy 15 eV (positive mode) and from 30 to 60 eV (negative mode). For data-dependent settings: minimum AGC target 3.103, apex trigger from 2 to 8 sec, charge exclusion from 3 to 8 and higher, dynamic exclusion 3 sec, "if idle" tool set to "pick others." Lock masses were constantly employed to correct mass deviations across the Full MS acquisition range throughout the experiments.

The HPLC-DAD data were collected and analyzed by the OpenLab software while the HPLC-MS data were collected and analyzed with Xcalibur 3.1 software and Compound Discoverer 2.0 (Thermo Fisher Scientific). Simple phenolic compounds quantitation was achieved at HPLC-DAD with external calibration and with injection of standard compounds (peaks integration at 280 nm).

## 2.3. Standard oenological characterization

Acetic acid, glucose and fructose, free and total $SO_2$ were measured using an automatic multi-parametric analyzer – Miura One (Exacta+Optech Labcenter S.p.A., San Prospero, Italy). All samples were filtered (0.2 $\mu$m, cellulose acetate filter) before the analysis without any specific sample preparation. Reagents for the enzymatic analysis of wines were

purchased from Exacta+Optech Labcenter S.p.A. (San Prospero, Italy). The total acidity was measured according to OIV (OIV, 2015a). The alcohol content was measured with a Malligand ebulliometer.

### 2.4. Sensory evaluation

A group of eight trained panelists (4 females and 4 males) aged from 30 to 50 years were recruited at Free University of Bozen-Bolzano, Faculty of Science and Technology. An initial qualitative analysis phase consisted in presenting the wine samples in order to define a common vocabulary of the sensory descriptors for Pinot Noir wines. Then, nineteen sensory descriptors were identified and evaluated with the procedure of the round table (YASAR *et al*., 2018). The visual descriptors were clarity, hue, and color intensity. The olfactory descriptors were olfactory intensity, floral, fruity, herbaceous, spicy, liquor, maderized, caramelized aromas, and solvent. The gustatory descriptors were alcoholic, softness, sweetness, acidity, sapidity, tannicity, and balance. Each descriptor was evaluated using a 10-point scale (1 = no perception, 10 = high intensity). The bottles were opened just before each sensory session and 30 mL of wine were offered randomly to the panelists in ISO glasses codified with 3-digit number at around 18°C. The presentation order of the samples was counterbalanced between and within participants. The participants were provided with mineral water to rinse their mouths between samples. At the end of the session, an overall quality judgment was also requested.

### 2.5. Statistical analysis

Principal Component Analysis was performed using XLStat (version 2019.2.2.59417, Addinsoft, Paris, France). NIPALS (Non-Linear Iterative Partial Least Squares) algorithm was preliminary applied to account for sparse missing values in the chemical datasets (WOLD *et al*., 1984). The relative abundances of non-cyclic and cyclic proanthocyanidins and their relative ratios were auto-scaled (mean-centered followed by division of each column - i.e. variable - by the standard deviation of that column). The average ratings of each sensory descriptor were instead only mean-centred as they all shared the same 10-point scale for the evaluation. 'Overall judgment' was used as supplementary variable (non-active) in the sensory analysis.

## 3. RESULTS AND DISCUSSION

In Table 1, the information on each analyzed Pinot Noir sample is reported. Samples 1, 4, 5, 6, 7, 8, and 9 were produced with the same winemaking procedure (mass of 3.5 t for each sample; 8 d maceration, 25-26°C fermentation temperature). The main differences among the cited samples were the altitude and the geographical orientation of the vineyards. Samples 1, 2, and 3 differed for the winemaking practice used: to produce wine 2, a thermal maceration at 42°C was applied for 8 h before the alcoholic fermentation; wine 3 instead underwent an unwanted stuck fermentation; thus, it was re-inoculated with selected yeast and then added with supplementary $SO_2$ to prevent off-fermentations (DI MATTIA *et al*., 2015). Wine 11 was obtained from grapes harvested in the same vineyard (E) of wines 8, 9, and 10, but using 100% raisin grapes obtained by cutting some vine shoots and leaving the clusters hanging on the plants for a few days. Wine 10 was made

with 20% of whole clusters (non-destemmed and uncrushed) that were left in the must during maceration/fermentation.

### 3.1. Oenological parameters

The standard oenological results are presented in Table 2. The alcohol content in Pinot Noir wines ranged from 12.8% (sample 4) to 15.4% (sample 11). As expected, wines 4, 5, and 6 obtained from the vineyards located in the highest sites showed the lowest alcohol content due to the lowest degree of grape ripeness whereas wines 1-3 and 8-11 showed the highest alcohol content since the grapes were cultivated in lower vineyards (Table 2). The highest alcohol content of sample 11 compared to the other Pinot Noir wines could be expected since this wine was made with 100% raisins (with higher sugar content). The pH ranged from 3.2 (sample 4) to 3.5 (sample 6). The first four wines had lower pH compared to the others. The pH fitted the usual pH range of red wines (3.0 – 4.0) (JACOBSON, 2006). The total acidity measured in samples 1-3, 5, and 7- 9 was 5.6 $gL^{-1}$ tartaric acid. Samples 4, 6, 10, and 11 had a higher total acidity (6.2 – 6.8 $gL^{-1}$ tartaric acid). All Pinot Noir wines had low acetic acid content (within the legal threshold of 1.2 $gL^{-1}$ acetic acid equivalents, OIV, 2015b and OIV, 2012). All the wines were dry and most of them showed a residual sugar content ranging from 0.06 $gL^{-1}$ (wines 3 and 6) to 0.44 $gL^{-1}$ (wine 7) (FERNANDEZ-NOVALES *et al.*, 2009). Wine 11 (made with 100% raisin grapes) contained the highest residual sugar content (1.63 $gL^{-1}$). Interestingly, wines 5 and 6 had the lowest glucose-fructose levels (0.07 and 0.06 $gL^{-1}$, respectively). The free $SO_2$ levels were relatively low (12 – 18 $mgL^{-1}$) and the total $SO_2$ (73 – 108 $mgL^{-1}$) was within the legal limits (OIV, 2012).

**Table 2.** Oenological parameters of the eleven Pinot Noir wines.

| Wine | [1]ABV (%) | pH | [2]total acidity ($gL^{-1}$) | acetic acid ($gL^{-1}$) | [3]Gl-Fr ($gL^{-1}$) | [4]$fSO_2$ ($mgL^{-1}$) | [5]$tSO_2$ ($mgL^{-1}$) |
|---|---|---|---|---|---|---|---|
| 1 | 14.4 | 3.38 | 5.6 | 0.21 | 0.19 | 14 | 107 |
| 2 | 14.4 | 3.33 | 5.6 | 0.24 | 0.17 | 14 | 93 |
| 3 | 13.7 | 3.25 | 5.6 | 0.41 | 0.06 | 13 | 108 |
| 4 | 12.8 | 3.21 | 6.8 | 0.40 | 0.14 | 12 | 88 |
| 5 | 13.1 | 3.48 | 5.6 | 0.25 | 0.07 | 14 | 79 |
| 6 | 13.4 | 3.54 | 6.2 | 0.32 | 0.06 | 13 | 82 |
| 7 | 14.7 | 3.42 | 5.6 | 0.36 | 0.44 | 12 | 83 |
| 8 | 14.8 | 3.41 | 5.6 | 0.31 | 0.31 | 15 | 73 |
| 9 | 14 | 3.48 | 5.6 | 0.30 | 0.22 | 14 | 90 |
| 10 | 14.5 | 3.46 | 6.5 | 0.39 | 0.30 | 18 | 91 |
| 11 | 15.4 | 3.50 | 6.8 | 0.40 | 1.63 | 18 | 90 |

[1]ABV: alcohol by volume (% v/v); [2]g/l tartaric acid; [3]gl-fr: glucose-fructose ($gL^{-1}$); [4]$fso_2$: free sulphur dioxide ($mgL^{-1}$); [5]$tso_2$: total sulphur dioxide ($mgL^{-1}$).

### 3.2. Profiles of proanthocyanidins

The proanthocyanidins (PAC) profile was analyzed by means of HPLC-HRMS and the results are reported in Table 3. Both non-cyclic procyanidins (NC-PC) and cyclic procyanidins (C-PC) were found in higher concentrations in Pinot Noir samples, compared to prodelphinidins (PD). All wines except sample 3 had a high content of dimeric procyanidins (NC-2 PCs). The abundances of NC-PC decreased at a higher degree of polymerization (DP). The highest amount of NC-6 PC (non-cyclic hexameric procyanidin) was present in wine 11. Wine 3 had instead the lowest amount of C-PAC. Also, wines 10 and 11 stood out with a higher content of C-6 PC (cyclic hexameric procyanidin) with respect to other samples. Furthermore, wine 11 had almost twice as much of C-5 PD (cyclic pentameric prodelphinidin) compared to wines 7 and 8.
Principal Component Analysis was performed using auto-scaled PAC variables, to highlight trends within the dataset that may suggest relationships between the PAC profiles and the different factors involved. In previous studies on the distribution of procyanidins (LONGO *et al.*, 2019) and prodelphinidins (LONGO *et al.*, 2018c) in wines, the relative (%) ratios were applied: these showed clear dependency upon the grape variety, but no study has yet addressed their relationship with the winemaking practices or the geographical origin. These ratios correspond to the proportions (%) of any cyclic congener over the total amount of cyclic + non-cyclic congeners by number and composition of monomers as reported in previous reports (LONGO *et al.*, 2018c; LONGO *et al.*, 2019). The PCA bi-plot of these ratios is shown in Fig. 1.
The total variance explained by the first two principal components is 84.0% (PC1: 69.6% + PC2: 14.4%). All variables are in positive correlation with the first principal component, except for the ratio of C-PD (cyclic prodelphinidins) with one and three (epi)gallocatechin units (indicated as %C-4-1-OH and %C-4-3-OH respectively). All %C-PC (relative (%) ratios of procyanidins) showed strong correlations among each other and also with most of the PD. Wine 3 is well separated from the other wines, which are clustered in the central area of the bi-plot. This is probably caused by the occurrence of a stuck fermentation: namely, as the fermentation halted prematurely, the extraction of the polyphenols from the berry skins was hampered, since the reached concentration of ethanol was lower in comparison to the other samples. After that event, sample 3 was racked before being re-inoculated with the yeast. Removing the skins at an early stage of maceration presumably prevented the completion of the extraction of polyphenols. However, this also slowed down the extraction of the non-cyclic congeners, since these are less polar compounds than the cyclic ones and require higher percentages of ethanol for their extraction. Instead, the cyclic compounds were still extracted in higher proportions (as evidenced in Fig. 1). Hence, the relative ratios (%) of cyclic congeners were "over-expressed" in sample 3. Notably, these percentages do not represent absolute concentrations, but instead they are just the relative proportions (%) of C-PAC over C-PAC *plus* NC-PAC (by DP and composition). Indeed, the data in Table 3 show that the peak areas in sample 3 are lower for all compounds than in the other samples. Notably, a recent study on the kinetics of skin extraction for C-PC in Cabernet Sauvignon showed that these compounds are extracted almost completely at the beginning of maceration (JOUIN *et al.*, 2019), while NC-PC are only extracted over time with the increasing formation of ethanol.

**Table 3.** Relative abundances (integrated total ion current) of non-cyclic and cyclic proanthocyanidins in the eleven Pinot Noir wines.

| PAC | | NC-2 PC I | NC-2 PC II | NC-2 PC III | NC-2 PC IV | NC-2 PC V | NC-3 PC | NC-4 PC | C-4 PC | NC-5 PC |
|---|---|---|---|---|---|---|---|---|---|---|
| m/z | | 579.1497 | 579.1497 | 579.1497 | 579.1497 | 579.1497 | 867.2124 | 1155.2760 | 1153.2604 | 1443.3392 |
| Wine samples | 1 | 22274956 | 22306888 | 22323575 | 22374250 | 22325218 | 7750638 | 2404025 | 162851 | 480128 |
| | 2 | 37425812 | 37336457 | 37336457 | 37354174 | 37354706 | 19392028 | 7135274 | 183528 | 1925495 |
| | 3 | 639543 | 639543 | 630124 | 639543 | 639543 | 79649 | 3475 | 25201 | 3698 |
| | 4 | 33026883 | 33012707 | 33004346 | 33012578 | 32999883 | 15520019 | 5166134 | 84876 | 1522658 |
| | 5 | 49790473 | 49787516 | 49753715 | 49788501 | 49788582 | 23452844 | 8328293 | 157309 | 2525998 |
| | 6 | 34515047 | 34495827 | 34503463 | 34515042 | 34499696 | 11871865 | 4004473 | 104562 | 967950 |
| | 7 | 58252407 | 58023701 | 58083779 | 58145139 | 58145139 | 26156359 | 7954429 | 316117 | 1873445 |
| | 8 | 45458622 | 45411956 | 45413310 | 45358853 | 45368291 | 25007492 | 8781802 | 301711 | 2467726 |
| | 9 | 12463012 | 12487106 | 12463722 | 12467415 | 12462662 | 4193665 | 1141355 | 131150 | 242130 |
| | 10 | 26662439 | 26665699 | 26664558 | 26668416 | 26646611 | 11066048 | 4442351 | 174339 | 1332144 |
| | 11 | 29720456 | 29692215 | 29720858 | 29720262 | 29720035 | 20466723 | 9604520 | 67151 | 3235409 |

| PAC | | C-5 PC | NC-6 PC | C-6 PC | NC-2 PD 1-galloc | NC-3 PD 1-galloc | NC-3 PD 2-galloc | NC-3 PD 3-galloc | NC-4 PD 1-galloc |
|---|---|---|---|---|---|---|---|---|---|
| m/z | | 1441.3213 | 1731.4010 | 1729.3870 | 595.1446 | 883.2072 | 899.2021 | 915.1970 | 1171.2710 |
| Wine samples | 1 | 168545 | 28905 | 8308 | 0 | 103469 | 12973 | 787 | 638805 |
| | 2 | 145329 | 348388 | 16802 | 565 | 71849 | 7113 | 0 | 1081774 |
| | 3 | 22871 | 1632 | 990 | 0 | 427 | 0 | 230 | 221433 |
| | 4 | 114903 | 251288 | 24875 | 0 | 80485 | 7434 | 0 | 1301338 |
| | 5 | 174167 | 467959 | 35912 | 575 | 231898 | 30436 | 514 | 2028349 |
| | 6 | 118992 | 155456 | 9765 | 0 | 157400 | 25428 | 5041 | 1023981 |
| | 7 | 263641 | 272430 | 13032 | 308 | 268426 | 45048 | 1024 | 1960693 |
| | 8 | 365229 | 446402 | 43924 | 0 | 164426 | 19409 | 1344 | 2336156 |
| | 9 | 117526 | 18048 | 4672 | 0 | 47356 | 6993 | 349 | 432706 |
| | 10 | 211433 | 307633 | 36639 | 0 | 107349 | 26470 | 1365 | 2719 |
| | 11 | 111594 | 784272 | 38554 | 0 | 39650 | 3466 | 0 | 1874952 |

| PAC | | NC-4 PD 2-galloc | NC-4 PD 3-galloc | NC-4 PD 4-galloc | C-4 PD 1-galloc | C-4 PD 2-galloc | C-4 PD 3-galloc | C-4 PD 4-galloc | NC-5 PD 1-galloc |
|---|---|---|---|---|---|---|---|---|---|
| m/z | | 1187.2660 | 1203.2605 | 1219.2550 | 1169.2557 | 1185.2507 | 1201.2456 | 1217.2405 | 1459.3343 |
| Wine samples | 1 | 40168 | 7470 | 1317 | 34465 | 2686 | 3725 | 0 | 60521 |
| | 2 | 45131 | 2996 | 735 | 60435 | 3717 | 14213 | 0 | 238163 |
| | 3 | 0 | 289 | 308 | 1277 | 198 | 0 | 0 | 0 |
| | 4 | 73318 | 10593 | 3446 | 61911 | 4280 | 21048 | 1941 | 369331 |
| | 5 | 331689 | 34976 | 4516 | 88724 | 8139 | 6740 | 1307 | 632792 |
| | 6 | 160249 | 20578 | 1964 | 42105 | 6604 | 5050 | 10487 | 232513 |
| | 7 | 225155 | 29024 | 3917 | 109277 | 22825 | 14161 | 3913 | 383685 |
| | 8 | 226537 | 17780 | 4891 | 99430 | 18169 | 4590 | 482 | 618359 |
| | 9 | 17701 | 10183 | 3886 | 21521 | 1577 | 18515 | 397 | 27818 |
| | 10 | 109507 | 17819 | 2251 | 49828 | 11919 | 17315 | 448 | 295178 |
| | 11 | 183308 | 4883 | 1081 | 210398 | 23102 | 3371 | 0 | 756973 |

| PAC | | NC-5 PD 2-galloc | NC-5 PD 3-galloc | NC-5 PD 4-galloc | C-5 PD 1-galloc | C-5 PD 2-galloc | C-5 PD 3-galloc | C-5 PD 4-galloc | C-5 PD 5-galloc |
|---|---|---|---|---|---|---|---|---|---|
| m/z | | 1475.3291 | 1491.3240 | 1507.3189 | 1457.3191 | 1473.3140 | 1489.3090 | 1505.3039 | 1521.2988 |
| Wine samples | 1 | 7874 | 0 | 0 | 49574 | 10550 | 1964 | 565 | 0 |
| | 2 | 9260 | 790 | 0 | 53667 | 5422 | 1422 | 294 | 0 |
| | 3 | 676 | 306 | 0 | 3030 | 886 | 0 | 0 | 0 |
| | 4 | 23878 | 3065 | 0 | 45778 | 5108 | 1889 | 0 | 0 |
| | 5 | 106745 | 16166 | 0 | 75154 | 16454 | 1808 | 0 | 0 |
| | 6 | 36370 | 7013 | 601 | 43342 | 8661 | 4357 | 0 | 0 |
| | 7 | 44404 | 3795 | 0 | 95602 | 26777 | 5586 | 295 | 0 |
| | 8 | 73686 | 4936 | 0 | 123419 | 30556 | 3078 | 0 | 257 |
| | 9 | 2255 | 1900 | 0 | 36030 | 6991 | 1400 | 0 | 0 |
| | 10 | 31967 | 4855 | 0 | 68776 | 19911 | 4301 | 0 | 0 |
| | 11 | 88643 | 7073 | 0 | 123989 | 13498 | 0 | 0 | 0 |

abbreviations: nc – non-cyclic; c – cyclic; numbers after nc or c indicates the number of monomer units of catechin or epicatechin (e.g. nc-2 is non-cyclic dimer); pc – procyanidins, pd – prodelphinidin; the last number in prodelphinidins indicates the number of gallocatechins in the oligomeric chain.

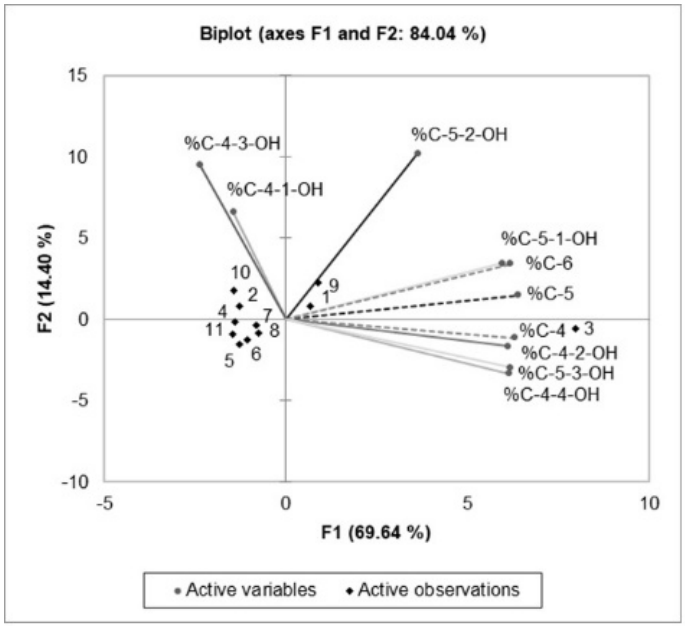


**Figure 1.** PCA bi-plot of the relative ratios of proanthocyanidins (%) of the eleven Pinot Noir wines. The vectors of the ratios of procyanidins are dashed, whereas the vectors for prodelphinidins are full. The first number in the abbreviations indicates the number of the monomers forming the proanthocyanidin. The second number shows the number of gallocatechin units in the oligomeric chain of prodelphinidins. F1 and F2, Principal Components. %C-N-M-X: ratio of relative abundance of a cyclic oligomer over the sum of relative abundances for cyclic and non-cyclic, considering the same relative compositions in (epi)catechins and (epi)gallocatechins and number of composing monomeric units. In the formula: C = cyclic, N = number of monomeric units, M = number of (epi)gallocatechins in the structure, X = -OH if the compound is a prodelphinidin or empty if it is a procyanidin.

In Fig. 2, the PCA models, which were elaborated over the relative abundances of NC-PAC (2A) and C-PAC (2B) are shown separately. The lack of NC-PAC in wine 3 is again confirmed in Fig. 2A (84.4% of total variance), where wine 3 is situated on the opposite side of PC1 with respect to all the variables. Wine 11 had higher concentrations of NC-PAC and the highest concentrations of residual sugars and alcohol (Table 2).
In fact, the grapes used for winemaking of sample 11 had been cut and left to dry hanging on the vine before the harvest, which had the effect of concentrating even further the polyphenols besides the sugars. Notably, in Figs. 2A and 2B the values used represent absolute abundances, as they are integrated peak values obtained with the HPLC-HRMS analysis (Table 3). Wines 3 and 11 are clearly separated from the others in 2A and 2B respectively, and the trends for the variables are shown: wine 3 was on the opposite part of most descriptors, while wine 11 was driven by the C-PD with one or two (epi)gallocatechin units.

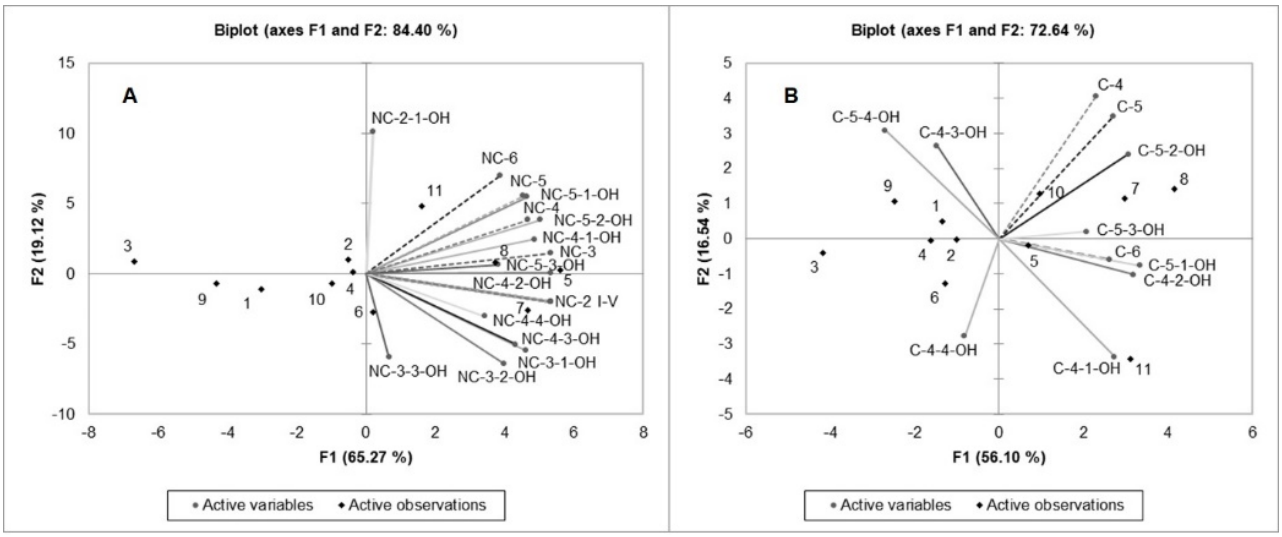


**Figure 2.** PCA bi-plots of non-cyclic proanthocyanidins (A) and cyclic proanthocyanidins (B) in Pinot Noir wines. NC - non-cyclic, C - cyclic. The vectors of procyanidins are dashed, whereas the vectors for prodelphinidins are full. The first number in the abbreviations indicates the number of the monomers. The second number shows the number of gallocatechin units in the oligomeric chain of prodelphinidins. F1 and F2, Principal Components.

### 3.3. Profiles of simple phenolics

Overall, none of the evaluated simple phenolic variables could distinguish significantly groups of samples; therefore, they were not included in the previous statistical analysis (*data not shown*). Instead, they are just mentioned qualitatively.
Seven monomeric phenolic compounds (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, catechin, caffeic acid, ferulic acid) were identified (Table 4), and concentrations were evaluated by standard injection according to LONGO *et al*. (2017) for phenolic compounds. Gallic, vanillic and caffeic acids were present in all samples. The highest amount of gallic acid was shown in wine 10, vanillic acid in wine 11, and caffeic acid in wines 7, 10 and 11. Wine 1 showed a higher content of protocatechuic acid; wine 3 was higher in ferulic acid; wines 10 and 11 in 4-hydroxybenzoic acid; wines 7 and 8 in catechin.

### 3.4. Sensory evaluation of Pinot Noir wines

Fig. 3 shows the PCA bi-plot for the sensory data. The first two components explained 46.2% of the total variance. The first principal component (26.6% of the total variance) was correlated with wine balance and the overall judgment on wine quality. Besides, PC1 was correlated with softness, sweetness, herbaceous, floral and fruity aromas. The second principal component (19.6%) was correlated with clarity, tannicity (astringency), and caramelized descriptors, which were inversely correlated with a maderized descriptor. As shown in Fig. 3**,** wines 1, 2, and 3 (vineyard A) were clustered on the left part of the graph. Samples 1 and 2 showed a very similar trend; thus the thermal maceration of wine 2 did not remarkably affect the sensory properties. However, wine 3 was characterized more by alcoholic, liquor, and maderized variables, and it was lacking in tannicity. Wines 5 and 6 (vineyard C) were situated in the center of the plot. Wines 8, 9, and 10 (vineyard E) were

situated on the same side as wine 11 (vineyard E). The wines 9, 10, and 11 were the most balanced and with a high overall judgment assigned by the panelists. Finally, the other two wines – 4 (vineyard B) and 7 (vineyard D) – were well separated from the other samples.

**Table 4.** Concentration of simple phenolic compounds in the eleven Pinot Noir wines evaluated by HPLC-DAD (280 nm) standard injections. Calibration curves with $R^2 = 0.999$ for evaluated compounds.

| Wine | Gallic acid (μM) | Protocatechuic acid (μM) | *p*-hydroxybenzoic acid (μM) | Vanillic acid (μM) | (+)-catechin (μM) | Caffeic acid (μM) | Ferulic acid (μM) |
|---|---|---|---|---|---|---|---|
| **1** | 171 | 672 | 0 | 206 | 5 | 14 | 2 |
| **2** | 229 | 1 | 0 | 440 | 2 | 24 | 1 |
| **3** | 225 | 63 | 0 | 304 | 5 | 20 | 4 |
| **4** | 203 | 56 | 2 | 300 | 3 | 22 | 0 |
| **5** | 220 | 40 | 0 | 319 | 2 | 21 | 0 |
| **6** | 213 | 47 | 0 | 308 | 1 | 20 | 0 |
| **7** | 221 | 1 | 2 | 348 | 21 | 30 | 0 |
| **8** | 153 | 1 | 0 | 404 | 31 | 29 | 0 |
| **9** | 253 | 1 | 4 | 256 | 3 | 16 | 0 |
| **10** | 311 | 1 | 6 | 314 | 0 | 31 | 1 |
| **11** | 180 | 1 | 6 | 482 | 0 | 30 | 1 |

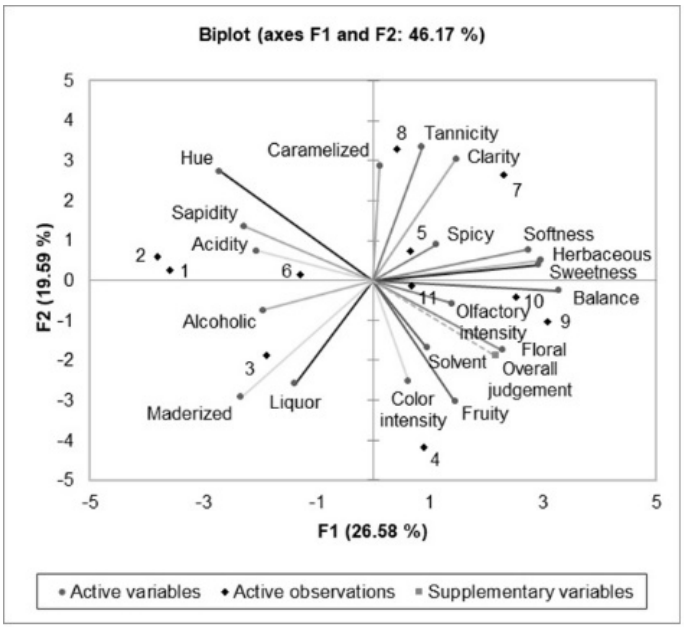


**Figure 3.** PCA bi-plot of the sensory data across the eleven Pinot Noir wines. Overall judgment was used as a supplementary variable. F1 and F2, Principal Components.

## 4. CONCLUSION

Using the profile of cyclic and non-cyclic proanthocyanidins, the separation of the most different samples of Pinot Noir wines, such as sample 3 (that had experienced a stuck fermentation) and sample 11 (that was produced using raisin grapes) was similar to that achieved with sensory analysis. Sample 3, with low proanthocyanidins concentration (including the cyclic ones), was described by the panel as highly maderized and lacking in tannins. Conversely, wine 11 (made with raisin grapes) contained the highest amount of cyclic tetrameric prodelphinidins and it was described as a balanced wine with a high overall quality judgment by the panel. The ratios between cyclic and non-cyclic proanthocyanidins confirmed the different solubility and extractability of these compounds and did reflect the occurrence of a stuck fermentation followed by racking and re-inoculation. Thus, the profile of cyclic and non-cyclic proanthocyanidins was affected by specific factors, such as the stuck fermentation or the use of 100% raisins. Both of these factors were related to the sensory quality judgement of Pinot Noir wines.

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## ABBREVIATIONS

ABV – alcohol by volume; PAC – proanthocyanidins; PC – procyanidin; PD – prodelphinidin; C- – cyclic oligomer; NC- – non-cyclic oligomer; C-*n* PC – cyclic *n*-meric (procyanidin); C-*n* PD – cyclic *n*-meric (prodelphinidins); C-*n* PD *m*-galloc – cyclic *n*-meric prodelphinidin with *m* (epi)gallocatechin units; PCA – Principal Component Analysis; NIPALS: Nonlinear Iterative Partial Least Square; PC*n*: *n* principal component; $fSO_2$: free $SO_2$; $tSO_2$: total $SO_2$.

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PAPER

# DIPPING SOLUTIONS OF NISIN AND BUFFERED SODIUM CITRATE SUPPLEMENTED WITH SODIUM DIACETATE FAIL TO PREVENT GROWTH OF *LISTERIA MONOCYTOGENES* ON VACUUM PACKAGED BEEF FRANKFURTERS STORED AT 4 AND 10°C UNDER MODEL CONDITIONS

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## ABSTRACT

This study evaluated commercially available dip solutions of nisin alone, buffered sodium citrate combined with sodium diacetate (BSCSD), and combined solution of the three antimicrobials for potential to control growth of surface-inoculated *Listeria monocytogenes* during vacuum packaged refrigerated storage of model beef frankfurters. None of the treatments prevented growth of *L. monocytogenes* during frankfurters storage. The combined treatments slowed growth of *L. monocytogenes* better than individual treatments. Failure to completely eliminate *L. monocytogenes* on frankfurters or inhibit outgrowth during storage might be attributed to high initial *L. monocytogenes* inoculum levels, insufficient quantities of antimicrobials to interact with all the target cells, low nisin activity at high pH, or presence of resistant subpopulations, such as nisin-resistant strains. The model conditions used in the experimental setup, such as elimination of natural microbiota from frankfurters and nutrient-rich diluent used for Listeria introduction on the surface, could also contributed to enhanced survival and growth of the pathogen.

*Keywords*: ready-to-eat meats, *Listeria monocytogenes*, nisin, sodium citrate, sodium diacetate, refrigerated storage

## 1. INTRODUCTION

Recontamination of cooked ready-to-eat (RTE) meat products with *L. monocytogenes* is a major food safety concern. In the U.S.A., *L. monocytogenes* caused an estimated 2,500 cases of foodborne illnesses with 20% mortality annually (MEAD *et al.,* 1999). A study performed over a decade later (SCALLAN *et al.*, 2011) also listed *L. monocytogenes* as the third leading cause of death due to foodborne illnesses at 19% mortality rate, only behind nontyphoidal *Salmonella* spp. and *Toxoplasma gondii*. In addition, a significant annual monetary loss is incurred by the food industry (USDA-ERS, 2014). U.S.-based food industries were forced to recall food products due to possible *L. monocytogenes* contamination on seven different occasions in December, 2018 alone (US FDA, 2018). The national health plan of reducing listeriosis cases to 0.25 person per 100,000 population has not yet been achieved since 2005 (BUCKNER, 2008). *L. monocytogenes* can survive under normally limiting and extreme physicochemical conditions (RYSER and DONNELLY, 2001). Moreover, *L. monocytogenes* is ubiquitous in nature. Though *L. monocytogenes* is susceptible to cooking, i.e. temperatures above 70°C (LUNDEN *et al.,* 2003), post-processing recontamination during cooked frankfurter cooling, case splitting, and packaging is difficult to avoid. At present, RTE meat facilities inspected by the U.S. Food Safety and Inspection Service (FSIS) operate under a 'zero tolerance' policy, which means any food contaminated with detectable levels of *L. monocytogenes* is deemed adulterated (USDA-FSIS, 2014). Therefore, hurdles are required to prevent initial contamination and inhibit growth of this pathogen during storage. One such hurdle is to use post-cooking application of antimicrobials to prevent growth of *L. monocytogenes*, which is accepted as one of three alternatives to control *L. monocytogenes* (U.S. Code of Federal Regulations, Title 9, Part 430, Section 430.4)

There are many potential methods to prevent spoilage and pathogenic bacteria from growth on RTE meats (TOKARSKYY and MARSHALL, 2010). For example, salts of organic acids (lactates, acetates, diacetates, sorbates, and benzoates) have been studied as antilisterial agents on meat products (BARMPALIA *et al.,* 2005; SAMELIS *et al.,* 2005; SIVAROOBAN *et al.,* 2007; STOPFORTH *et al.,* 2005; ZHU *et al.,* 2005). Activity of these compounds depends on agent concentration, product composition (pH, water activity, fat, nitrite, and salt content), storage temperature and packaging atmosphere (CLEVELAND *et al.,* 2001; GEORNARAS *et al.,* 2006a; LUNDEN *et al.,* 2003; MARTINIS *et al.,* 1997; NILSSON *et al.,* 1997). The contamination level of the food product is another factor that influences the activity of these antimicrobials (BEDIE *et al.,* 2001; WEDERQUIST and SOFOS, 1994).

Nisin is a Generally Recognized as Safe (Federal Register, 1988) bacteriocin that is produced by *Lactococcus lactis* subsp. *lactis* (NAIDU, 2000). Nisin has greatest antimicrobial activity in the pH range of 3.0-3.5 (FANG and LIN, 1994) with rapid activity loss at greater pH values (MONTVILLE and CHEN, 1998). Therefore, it is recommended to use nisin at a pH not greater than 5.5, especially in the presence of sodium chloride and nitrite in meat products (MARTINIS *et al.,* 1997; UKUKU and SHELEF, 1997). Studies have shown that nisin is effective in reducing *L. monocytogenes* counts on RTE meat products (DELVES-BROUGHTON, 2005; FANG and LIN, 1994; TOKARSKYY and MARSHALL, 2008; UKUKU and SHELEF, 1997). However, the use of nisin has been limited in food products due to the potential for emergence of nisin-resistant strains of *L. monocytogenes* (CRANDALL and MONTVILLE, 1998; LIU *et al.,* 2002). To overcome this problem, nisin has been combined with other treatments to achieve antilisterial effects (DELVES-

BROUGHTON, 2005; GEORNARAS et al., 2006a; SAMELIS *et al.*, 2005; TOKARSKYY and MARSHALL, 2008; ZHU *et al.*, 2005).
Buffered sodium citrate combined with sodium diacetate (BSCSD) is a mixture of citric acid, sodium citrate, and sodium diacetate (HULL, 2007). Buffered sodium citrate (BSC) is approved in the US for cured (9 CFR 318.7 [c] (4)) and uncured (9 CFR 381.7 [f] (4)) processed whole-muscle meat and poultry products (USDA-FSIS, 1996). BSC is used to enhance flavor in meat and poultry products and the recommended usage level is 1.0 to 1.3% by weight of total formulation (USDA-FSIS, 1996). In muscle-food products, BSC increases ionic strength, which in turn increases water holding capacity, lowers water activity, and causes less purge loss (CEYLAN *et al.*, 2003; HULL, 2007). Sodium diacetate is primarily used as flavor enhancer in meat products at no more than 0.25% of product formulation (USDA-FSIS, 2000). Sodium diacetate has been reported to have antilisterial properties in combination with lactates or nisin in meat and poultry products (SAMELIS *et al.*, 2002; SAMELIS *et al.*, 2005). BSCSD has been shown to be effective in controlling germination and outgrowth of *Clostridium perfringens* during cooling of cooked meat and poultry products (THIPPAREDDI *et al.*, 2003) and a single study showed possibility for inhibiting *L. monocytogenes* on beef frankfurters (CEYLAN *et al.*, 2003).
These antimicrobials have been used as individual solutions (PATEL *et al.*, 2006; SAMELIS *et al.*, 2005) or as separate solutions applied sequentially to food surfaces (GEORNARAS *et al.*, 2006a; GEORNARAS *et al.*, 2006b). Most previous studies have used salts of organic acids and nisin as additives in the meat formulation, while relatively fewer studies have evaluated their efficacy as dipping solutions post-processing. Little is known about the potential of these agents as processing aids applied as surface treatments rather than as ingredients. Therefore, the present study was designed to investigate dip solutions of nisin alone, BSCSD alone or the two in various combined treatment sequences on ability to control *L. monocytogenes* attached on RTE beef frankfurters during storage at 4 or 10°C.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of inoculum

A cocktail for inoculation was prepared using five strains of *L. monocytogenes*. Strains were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and included ATCC 15313 (rabbit isolate, England), ATCC 51414 (raw milk associated with listeriosis outbreak, Massachusetts, USA), ATCC 43256 (Mexican-style cheese isolate, California, USA) ATCC 19115 (human isolate), and ATCC 7644 (human isolate). Strains were maintained on trypticase soy agar slants with 0.6 % yeast extract (TSAYE; BD Diagnostic Systems, Sparks, MD) at 4°C with monthly transfers to fresh TSAYE slants. To prepare study inocula, each strain was streaked separately onto TSAYE plates and incubated 35°C for 24 h. A single colony of each strain from a TSAYE plate was transferred into separate 30 ml portions of trypticase soy broth with 0.6% yeast extract (TSBYE) and incubated at 35°C for 24 h to obtain stationary phase cultures (approximately 9 $\log_{10}$ CFU/ml). A multistrain cocktail was prepared by combining 2-ml portions of each turbid broth in a sterile test tube and vortexed for 10 sec. A 2-ml portion of this mixed-strain cocktail was then dispersed into 2 L of 0.1% peptone water (BD Diagnostic Systems) to achieve a final *L. monocytogenes* inoculum solution containing approximately 6 $\log_{10}$ CFU/ml.

## 2.2. Frankfurter inoculation

The experimental setup was rather a model than real-life in order to simulate the worst possible conditions for *Listeria* survival and growth, such as destruction of frankfurters natural microbiota to avoid competition, use of stationary culture of pathogen in nutrient-rich medium, modifying of frankfurters texture by autoclaving, overnight adaptation and attachment of *Listeria* to the meat surface before application of antimicrobials. Briefly, low-fat frankfurters without added antimicrobial agents were purchased from a commercial distributor and transported in a cooler on ice packs directly to the laboratory. The proximate composition of the frankfurters remained unknown. The frankfurters were immediately frozen and stored at -20°C until use but no longer than for a month.
For each replicate, 44 frankfurters (11 treatments x 4 sampling days) for 4°C storage and 66 frankfurters (11 treatments x 6 sampling days) for 10°C storage were thawed at 4°C overnight. After thawing, the frankfurters were aseptically cut in half perpendicular to the longitudinal axis (calculated: 2.5 cm diameter; 6 cm length; 57 $cm^2$ surface area; 22 ± 2 g). Each replicate portion of halved frankfurters were vacuum packaged (Multivac A300/16; Kansas City, MO) in vacuum bags (Vacuum Pouches; Prime Source, Kansas City, MO) at 999 mbar vacuum for 1 sec with a 2.5 sec seal. The vacuum-packaged frankfurters were autoclaved for 15 min at 121°C to destroy indigenous microorganisms (DORSA *et al.* 1993), and cooled for approximately 2 h at 25 ± 2°C in a laminar flow hood before inoculation with *L. monocytogenes*.
A solution of sterile 0.1 % peptone water was used as a negative control. Up to 20 halved frankfurters were placed into 4 L sterile steamer strainers (Progressive International, Kent, WA). Each batch of 20 was dipped in containers with either 2 L of negative control buffer or 2 L of the five-strain inoculum of *L. monocytogenes* for 5 min with constant agitation. After dipping, the strainers were removed and drained for 10 min into empty sterile containers. The inoculated frankfurters were aseptically transferred to plastic storage bags with a zip closure (Ziploc, S. C. Johnson and Son Inc., Racine, WI) and stored over night at 4°C to allow for low temperature adaptation and maximum adherence of *L. monocytogenes* to product surfaces. This practice is similar to commercial frankfurter manufacturing where cooked links can be hydrocooled after cooking or rack cooled overnight in a low temperature cooler.

## 2.3. Antimicrobial treatments

Commercially-available antimicrobials were secured from the U.S. food ingredients suppliers and prepared according to manufacturer's instructions but using sterilized distilled water for antimicrobial dissolution "as is". Nisin (1,000 IU/mg, ProFood International, Inc., Chicago, IL, USA) and buffered sodium citrate combined with sodium diacetate (BSCSD, World Technology Ingredients, Inc., Jefferson, GA, USA) were used to prepare eleven treatment dipping solutions. The exact composition of BSCSD was not declared by the supplier, but according to Hull (2007), it may have contained 65 to 95% sodium citrate and 5 to 35% of sodium diacetate. The inoculated frankfurter samples were dipped into 2-L portions of the following individual agent treatment solutions: 1) 0.1% sterile peptone water (no antimicrobials, *L. monocytogenes* positive control); 2) 2000 IU/ml nisin; 3) 4000 IU/ml nisin; 4) 6000 IU/ml nisin; 5) 2.5% (w/v) BSCSD; 6) 3.0% (w/v) BSCSD; 7) 3.5% (w/v) BSCSD. In addition, two sequential treatment solution treatments were conducted; 8) 6000 IU/ml nisin followed by 3.5% (w/v) BSCSD (nisin-BSCSD) and 9) 3.5 % (w/v) BSCSD followed by 6000 IU/ml nisin (BSCSD-nisin). Finally, a combined

solution treatment was used: 10) 6000 IU/ml nisin and 3.5% (w/v) BSCSD; along with a negative control: 11) non-inoculated samples dipped in 0.1% peptone water.
Dipping was conducted by using sterile steamer strainers as used earlier for frankfurter inoculation. For a single treatment, not more than 20 frankfurter samples were dipped into 2.0 L of treatment solution for 5 min and drained for 10 min before vacuum packaging. For sequential treatments, samples were dipped into the first solution for 5 min, drained for 10 min, and then dipped into another bucket containing the second solution for 5 min followed by draining for 10 min.
Treated half-frankfurter samples were aseptically individually placed into separate vacuum bags (Prime Source, 15.24 cm x 21.59 cm) and vacuum packaged (Multivac A300 /16). The packages from each treatment were randomly selected into two batches, which were stored at 4 or 10°C for 42 and 20 days, respectively.

**2.4. Bacterial enumeration**

Frankfurters stored at 4°C were analyzed on days 0, 14, 28 and 42, and those stored at 10°C were analyzed on days 0, 4, 8, 12, 16 and 20. Twelve hours post-treatment was designated as day 0. On each sampling day, two packages from each treatment were randomly selected and analyzed for microbial counts. Twenty-three milliliters of 0.1% peptone water was added to each frankfurter sample (1:1 ratio) in a stomacher filter bag (Thermo-Fisher Scientific, Fairlawn, NJ). The samples were homogenized for 2 min using a stomacher (Stomacher 400 Lab Blender, Seward Medical, London, UK) and the homogenate was serially diluted with 0.1% peptone water. An aliquot of 0.1 ml was taken from each dilution, and spread plated on duplicate modified Oxford agar (BD Diagnostic Systems) plates followed by incubation at 35°C for 48 h. Colonies that appeared 2 to 3 mm in diameter and grayish black with a halo were enumerated as *L. **monocytogenes*.

**2.5. pH measurements**

The surface pH of treated frankfurters was determined using Corning Pinnacle 530 pH meter (Corning, NY). Half-cut frankfurter samples were dipped into treatment solutions for 5 min and then drained for 10 min before pH measurement. The pH values of the antimicrobial solutions were also measured prior to application.

**2.6. Statistical analysis**

Three replicate experiments were performed for each storage temperature. At each sampling point, duplicate samples from each treatment were removed from storage. Plate counts were converted to $\log_{10}$ CFU/g. Least-square means of bacterial counts for each treatment were estimated and significance of differences were determined using analysis of variance using general linear model of Statistical Analysis System 9.1 (SAS 2002). All differences were reported at a significance level of $P \leq 0.05$.
In addition, the logarithm of the *L. monocytogenes* counts from each of the two storage temperatures were modeled as a function of time using the Baranyi model (BARANYI AND ROBERTS, 1994). For curve fitting, the program DMFit (provided by Dr. J. Baranyi, IFR (Institute of Food Research, Reading, UK) was used (BARANYI, 2005). Four Baranyi model parameters were measured: 1) lag phase; 2) $\mu_{max}$, which expresses the maximum specific growth rate (per day); 3) $Y_0$, which represents the lower asymptote, corresponding to the initial bacterial counts ($\log_{10}$ CFU/g); and 4) $Y_{end}$, represents the upper asymptote,

corresponding to the maximum bacterial counts ($\log_{10}$ CFU/g) when the growth curve forms an upper plateau at the stationary phase of growth. The lag phase is formally separated from the exponential and the stationary phase, which can be regarded as part of the potential growth curve defined by the model. The main difference between this model and other sigmoid curves is the mid-phase curve is similar to a linear curve, unlike other classical sigmoid curves that have a pronounced curvature.

## 3. RESULTS AND DISCUSSION

### 3.1. pH of frankfurters dipped in antimicrobial solutions

Table 1 shows treatment solution and frankfurter surface pH values. BSCSD solutions had pH values above 5.6, which is not considered inhibitory to growth of *L. monocytogenes* (USDA-ARS, 2019, Pathogen Modeling Program Online). Nisin solutions had growth inhibitory pH values below 4.0 (USDA-ARS, Pathogen Modeling Program Online). Despite the low pH of the nisin solutions, no significant ($P$>0.05) change in the surface pH of frankfurters was observed after dipping in any of the treatment solutions (Table 1).

**Table 1.** pH values of antimicrobial solutions and treated frankfurters.

| | pH mean ±SD [a] | |
|---|---|---|
| **Treatment** | **Treatment solution** | **Surface pH of treated frankfurters** |
| Positive control | 6.3±0.07 | 6.0±0.12 ab |
| Nisin (2000 IU/ml) | 3.9±0.02 | 5.8±0.03 b |
| Nisin (4000 IU/ml) | 3.5±0.42 | 5.8±0.11 b |
| Nisin (6000 IU/ml) | 3.7±0.07 | 5.8±0.03 b |
| BSCSD (2.5%) | 5.8±0.03 | 5.8±0.05 b |
| BSCSD (3.0%) | 5.8±0.01 | 5.8±0.10 b |
| BSCSD (3.5%) | 5.6±0.19 | 6.1±0.06 a |
| Nisin-BSCSD | 3.9±0.10 - 5.6±0.03 | 5.8±0.02 b |
| BSCSD-Nisin | 5.8± 0.02 - 3.7±0.11 | 5.7±0.12 b |
| Combined | 5.6±0.11 | 5.8±0.08 b |

[a]All means are duplicate measurements from three different experiments. Means within a column followed by the same letter(s) are not significantly different ($P$>0.05).

Similar pH results were seen by PATEL *et al.* (2006) after dipping turkey frankfurters in solutions of nisin, sodium lactate, or sodium diacetate, either alone or in combination. BEDIE *et al.* (2001) also did not observe significant changes in the pH of frankfurters when sodium acetate, sodium diacetate, or sodium lactate were added as ingredients. These and other studies (SAMELIS *et al.*, 2005; SCHLYTER *et al.*, 1993; SHELEF and ADDALA, 1994) reveal that pH reduction is not a major contributor to the antilisterial property of these antimicrobials. Instead, activity within the meat system accounts for antilisterial activity (DOORES *et al.*, 2005; TOKARSKYY and MARSHALL, 2008).

### 3.2. Effects of nisin, BSCSD, and combined solutions

Tables 2 and 3 show effects of 2000, 4000, or 6000 IU nisin/ml, 2.5, 3.0, or 3.5% BSCSD, sequential nisin-BSCSD treatment, sequential BSCSD-nisin treatment, and a combined nisin-BSCSD treatment solution on surface inoculated frankfurters stored at 4 and 10°C.

**Table 2.** Effect of nisin, BSCSD, and combined solutions on growth of *L. monocytogenes* on beef frankfurters stored at 4°C*.

| Treatment | *L. monocytogenes* population (mean $\log_{10}$ CFU/g ± SD) Time (day) | | | |
|---|---|---|---|---|
| | 0 | 14 | 28 | 42 |
| Positive control | 4.4±0.4 a | 8.0±0.1 a | 8.5±0.2 a | 9.0±0.2 a |
| Nisin (2000 IU) | 2.2±1.1 b | 7.0±0.1 bc | 8.2±0.3 a | 9.0±0.1 a |
| Nisin (4000 IU) | 2.1±0.8 b | 7.2±0.3 bc | 8.1±0.1ab | 9.0±0.2 a |
| Nisin (6000 IU) | 2.3±0.2 b | 7.0±0.1 bc | 8.2±0.2 a | 9.0±0.2 a |
| BSCSD (2.5%) | 4.0±0.5 ab | 7.3±0.1 bc | 8.2±0.2 a | 8.9±0.3ab |
| BSCSD (3.0%) | 3.2±1.1 ab | 7.3±0.0 b | 8.4±0.3 a | 8.8±0.2ab |
| BSCSD (3.5%) | 3.3±0.8 ab | 7.1±0.1 bc | 8.3±0.3 a | 8.7±0.0 b |
| Nisin - BSCSD | 3.1±0.8 ab | 6.9±0.3 c | 7.5±0.4 bc | 8.7±0.1 b |
| BSCSD - Nisin | 2.7±1.2 ab | 7.3±0.2 bc | 7.5±0.4 bc | 9.0±0.1 a |
| Combined | 2.7±1.3 ab | 6.3±0.2 d | 7.4±0.3 c | 8.9±0.3 ab |

*Means within a column followed by the same letter(s) are not significantly different ($P$>0.05). Minimum detection limit was 2.0 $\log_{10}$ CFU/g.

**Table 3.** Effects of nisin, BSCSD, and combined solutions on the growth of *L. monocytogenes* on beef frankfurters stored at 10°C*.

| Treatment | *L. monocytogenes* population (mean $\log_{10}$ CFU/g ± SD) Time (day) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 4 | 8 | 12 | 16 | 20 |
| Positive control | 4.7±0.5 a | 7.7±0.5 a | 8.6±0.1 abc | 8.7±0.1 ab | 8.8±0.1 a | 9.0±0.1 abc |
| Nisin (2000 IU/ml) | 2.9±0.3 bc | 5.6±0.1 d | 7.9±0.5 abcd | 8.3±0.2 bc | 8.4±0.2 b | 8.6±0.1 d |
| Nisin (4000 IU/ml) | 2.8±0.2 c | 6.5±0.2 bcd | 7.9±0.4 abcd | 8.3±0.3 bc | 8.5±0.3 ab | 8.7±0.1 cd |
| Nisin (6000 IU/ml) | 2.2±1.0 c | 6.1±0.7 d | 8.0±0.1 abcd | 8.0±0.1 cd | 7.9±0.1 c | 8.5±0.3 d |
| BSCSD (2.5%) | 4.2±0.9 ab | 7.5±0.7 ab | 8.9±0.2 a | 9.0±0.2 a | 8.6±0.1 ab | 9.1±0.2 ab |
| BSCSD (3.0%) | 4.5±0.5 a | 7.4±0.6 abc | 8.5±0.8 abc | 9.0±0.2 a | 8.6±0.1 ab | 9.3±0.2 a |
| BSCSD (3.5%) | 4.5±0.4 a | 7.4±0.5 abc | 8.8±0.6 ab | 8.8±0.3 a | 8.6±0.1 ab | 9.0±0.2 abc |
| Nisin - BSCSD | 3.0±0.5 bc | 6.3±0.4 cd | 7.7±0.3 cd | 7.9±0.3 cd | 8.4±0.1 b | 8.8±0.1 bcd |
| BSCSD -Nisin | 2.7±0.5 c | 6.3±0.2 cd | 7.9±0.3 bcd | 8.0±0.1 cd | 8.6±0.2 ab | 8.9±0.1 bc |
| Combined | 2.7±0.6 c | 6.1±0.3 d | 7.5±0.1 d | 7.7±0.1 d | 8.3±0.2 b | 8.5±0.1 d |

*Means within a column followed by the same letter(s) are not significantly different ($P$>0.05). Minimum detection limit was 2.0 $\log_{10}$ CFU/g.

Treatment and sampling of negative control beef frankfurters followed the same procedures as inoculated samples and showed no *L. monocytogenes* contamination (results not shown). Positive-control untreated samples supported *L. monocytogenes* growth to populations at or exceeding 8 $\log_{10}$ CFU/g by 14 days of storage at 4°C (Table 2) and 8 days at 10°C (Table 3). Treatment with nisin alone initially reduced ($P<0.05$) *L. monocytogenes* counts by approximately 2 logs at both storage temperatures; however, there was no observed ($P>0.05$) dose-response relationship. Although treatment with BSCSD alone appeared to reduce counts initially at both temperatures, these reductions were not significant ($P>0.05$). Sequential nisin-BSCSD and BSCSD-nisin treatments and combined nisin-BSCSD treatment initially reduced ($P<0.05$) counts by approximately 2 logs at 10°C (Table 3) but not ($P>0.05$) at 4°C (Table 2). This is suggestive that warmer temperature application may increase antimicrobial activity of the combined treatments.

Despite some initial count reductions, none of the treatments were able to prevent growth of *L. monocytogenes* during frankfurter storage at 4 or 10°C under proposed model conditions (Tables 2 and 3). By 14 days at 4°C, all treatments were able to keep *L. monocytogenes* counts significantly lower ($P<0.05$) than the untreated control, but the count reduction was small (around 1 log) (Table 2). The combined nisin-BSCSD treatment achieved a larger 1.7 log reduction ($P<0.05$) at 14 days of storage at 4°C. Sequential and combined nisin-BSCSD treatments were able to keep counts around 1 log lower ($P<0.05$) than the untreated control for up to 28 days at 4°C. No meaningful count differences were observed among the treatments on the last day of testing, 42 days at 4°C. Similar trends were observed when frankfurters were stored at 10°C, with no meaningful treatment differences observed by 16 days of storage.

Based on previous reports we expected that sequential or combined nisin-BSCSD treatments would be more effective than treatment with either antimicrobial preparation alone (CEYLAN *et al.,* 2003; CLEVELAND *et al.,* 2001; DELVES-BROUGHTON, 2005; JUNEJA AND THIPPAREDDI, 2004; SAMELIS *et al.,* 2001; SAMELIS *et al.,* 2005; THIPPAREDDI *et al.,* 2003; ZHU *et al.,* 2005). The combined application of antimicrobials (either sequential or as a single solution) did not influence *L. monocytogenes* counts, except day 14 at 4°C, where nisin-BSCSD yielded significantly lower counts. Perhaps application of low-pH nisin solution before BSCSD application influenced activity at this time point.

It is noteworthy that surviving *L. monocytogenes* populations after antimicrobial treatments were able to initiate growth under model conditions. Baranyi model growth curves at 4 and 10°C are shown in Figures 1 and 2, respectively.

Growth rate kinetics of the bacterium at the two storage temperatures are shown in Tables 4 and 5. There were no lag-phase growth differences observed with any treatment at 4 or 10°C based on the Baranyi model (results not shown). In pork bologna formulated with 1.8% sodium lactate and 0.125% sodium diacetate, BARMPALIA *et al.* (2005) observed a *L. monocytogenes* lag phase of 13.78 days and 5.02 days at 4 and 10°C, respectively, using the same Baranyi model. GEORNARAS *et al.* (2006b) found a 10.2 day lag phase at 10°C for commercial cooked sausages treated with 1.5% potassium lactate plus 0.05% sodium diacetate and no lag phase for untreated sausages. Result differences in lag phase observations may be due to variations in experimental protocol including number of data points taken during the lag phase, different starting inocula populations, type of product, presence or absence of indigenous bacteria that could be inhibitory to *L. monocytogenes*, or differences in absorption rates of antimicrobial solutions due to specific surface characteristics (BARMPALIA *et al.,* 2004; GEORNARAS *et al.,* 2006a; GEORNARAS *et al.,* 2006b; SAMELIS *et al.,* 2001, 2002, 2005).

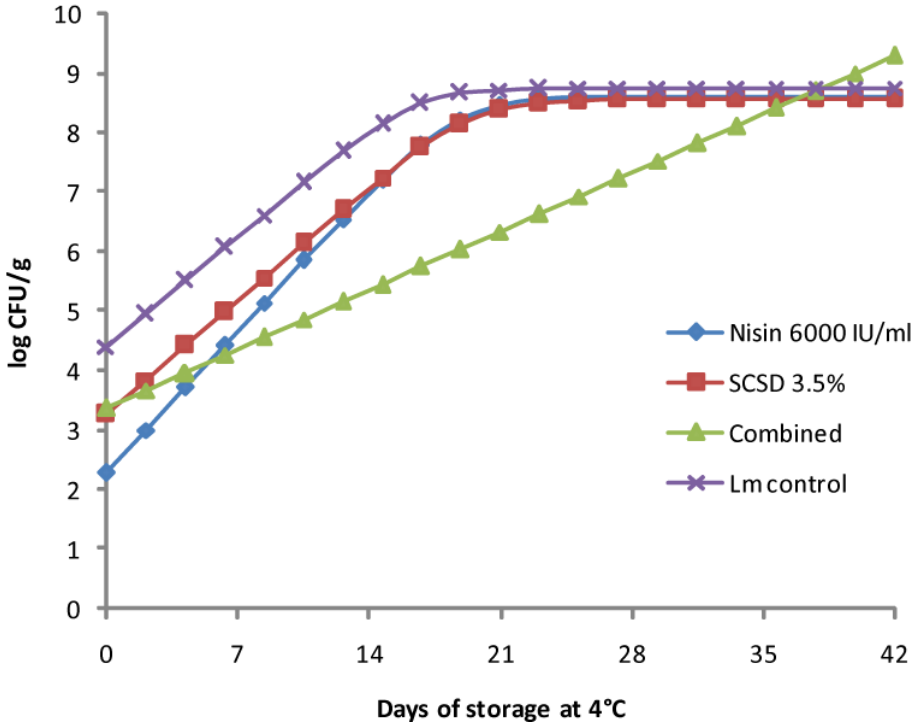


**Figure 1.** Growth of *L. monocytogenes* on vacuum packaged beef frankfurters dipped in solutions of 6000 IU/ml nisin, 3.5% BSCSD, and combined solution, stored at 4°C. Lm control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials. Data points are not actual experimental values but aids for better visualization of Baranyi graphs.

**Table 4.** Growth kinetics of *L. monocytogenes* inoculated on the surface of beef frankfurters treated with nisin alone, BSCSD alone, nisin-BSCSD in sequence, BSCSD-nisin in sequence, both combined, then vacuum packaged and stored at 4°C for 42 days.

| Treatment | Maximum specific growth rate ($\mu_{max}$; per day ±standard error) | $Y_0$ [a] ($log_{10}$ CFU/g) | $Y_{end}$ [b] ($log_{10}$ CFU/g) | $R^2$ |
|---|---|---|---|---|
| Lm control | 0.26±0.030 | 4.4 | 8.7 | 0.98 |
| Nisin (2000 IU/ml) | 0.35±0.045 | 2.2 | 8.6 | 0.93 |
| Nisin (4000 IU/ml) | 0.37±0.046 | 2.1 | 8.5 | 0.93 |
| Nisin (6000 IU/ml) | 0.34±0.041 | 2.2 | 8.6 | 0.94 |
| SCSD (2.5%) | 0.23±0.025 | 4.0 | 8.5 | 0.96 |
| SCSD (3.0%) | 0.30±0.057 | 3.1 | 8.6 | 0.86 |
| SCSD (3.5%) | 0.27±0.059 | 3.2 | 8.6 | 0.84 |
| Nisin + SCSD | 0.28±0.039 | 3.1 | 8.0 | 0.91 |
| SCSD + Nisin | 0.34±0.046 | 2.7 | 8.2 | 0.90 |
| Combined | 0.14±0.046 | 3.3 | -c | 0.89 |

[a]Lower asymptote estimated by the Baranyi model.

[b]Upper asymptote estimated by the Baranyi model.

[c]No value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to the stationary phase.

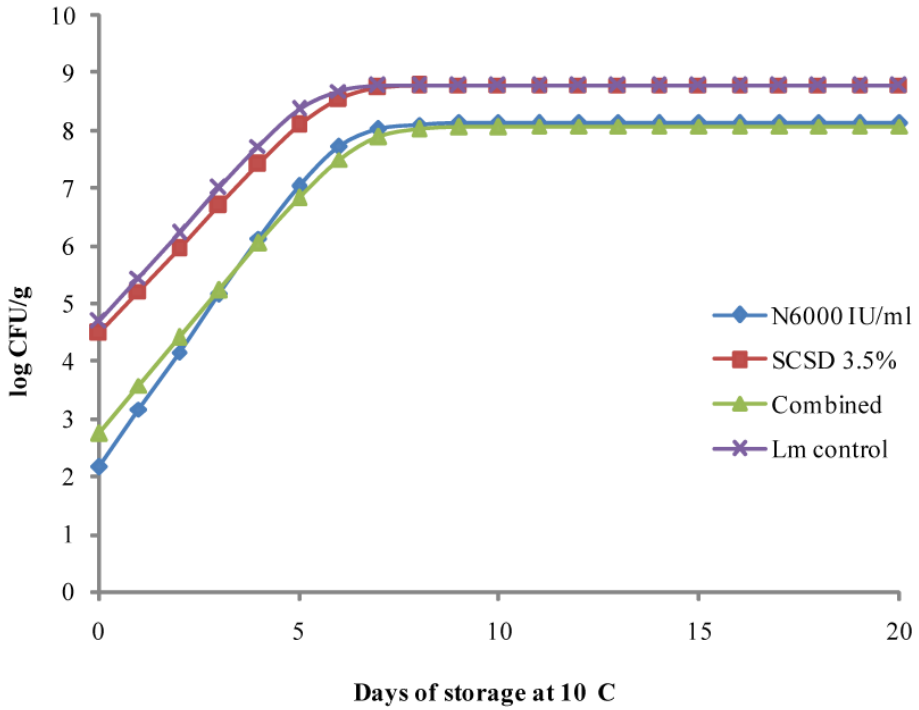


**Figure 2.** Growth of *L. monocytogenes* on vacuum packaged beef frankfurters dipped in solutions of 6000 IU/ml nisin, 3.5% BSCSD, and combined solution, stored at 4°C. Lm control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials. Data points are not actual experimental values but aids for better visualization of Baranyi graphs.

**Table 5.** Growth kinetics of *L. monocytogenes* inoculated on the surface of beef frankfurters treated with nisin, BSCSD, alone or in sequence or combination, then vacuum packaged and stored at 10°C for 20 days.

| Treatment | Maximum specific growth rate ($\mu_{max}$; per day ±standard error) | $Y_0$[a] ($log_{10}$ CFU/g) | $Y_{end}$[b] ($log_{10}$ CFU/g) | $R^2$ |
|---|---|---|---|---|
| Lm control | 0.77±0.020 | 4.7 | 8.8 | 0.95 |
| Nisin (2000 IU/ml) | 0.66±0.017 | 2.9 | 8.4 | 0.98 |
| Nisin (4000 IU/ml) | 0.95±0.021 | 2.8 | 8.4 | 0.97 |
| Nisin (6000 IU/ml) | 1.00±0.04 | 2.1 | 8.1 | 0.92 |
| SCSD (2.5%) | 0.84±0.037 | 4.2 | 8.9 | 0.89 |
| SCSD (3.0%) | 0.70±0.037 | 4.6 | 8.9 | 0.87 |
| SCSD (3.5%) | 0.74±0.027 | 4.5 | 8.8 | 0.92 |
| Nisin + SCSD | 0.79±0.031 | 3.0 | 8.2 | 0.93 |
| SCSD + Nisin | 0.89±0.027 | 2.7 | 8.4 | 0.96 |
| Combined | 0.83±0.29 | 2.7 | 8.1 | 0.94 |

[a]Lower asymptote estimated by the Baranyi model.
[b]Upper asymptote estimated by the Baranyi model.
[c]No value could be estimated when the upper part of the growth curve ceased without forming an upper asymptote that corresponds to the stationary phase.

The $Y_0$ values of control samples were similar, 4.7 and 4.4 $\log_{10}$ CFU/g at 4 and 10°C, respectively (Tables 4 and 5). Treatment count reductions were addressed earlier when discussing results shown in Tables 2 and 3.

Maximum specific growth rate ($\mu_{max}$) of nisin-treated frankfurters ranged from 0.339-0.368 $day^{-1}$ while that of BSCSD was 0.230-0.274 $day^{-1}$ and control was 0.263 $day^{-1}$ at 4°C. This implies that surviving nisin-treated *L. monocytogenes* grew faster than non-stressed populations. Others (LUCHANSKY and CALL, 2004; RUTHERFORD, 2004; SAMELIS *et al*, 2005) found similar results of greater $\mu_{max}$ values with treated samples than with control samples. Those researchers postulated that nisin could have slowed the growth of other bacteria thus reducing the bacterial competition against *L. monocytogenes*, which allowed it to grow at a faster rate than when in the presence of competitor organisms. In contrast, the present study used sterile frankfurters to avoid possible confounding effects of any indigenous microflora, which implies another potential, as yet unproven, reason for nisin-resistant growth stimulation. For example, it is possible that a fast-growing, nisin-resistant strain was in the inoculum mix. Also, perhaps nisin-exposed cells are primed to multiply quickly as a stress-response survival strategy, especially in case of absence of indigenous microbiota.

Nisin was more effective than BSCSD in both initial population size reduction and growth inhibition during storage (Tables 1 and 2). Combining nisin with BSCSD did not improve nisin activity. Similar results were reported when nisin was used with sodium diacetate (FANG and LIN, 1994; SAMELIS *et al.*, 2005; STOPFORTH *et al.*, 2005), sodium acetate (SAMELIS *et al.*, 2002), potassium benzoate (GEORNARAS *et al.*, 2006b; SAMELIS *et al.*, 2005), potassium sorbate (SAMELIS *et al.*, 2005), sodium lactate (FANG and LIN, 1994), lactic acid (STOPFORTH *et al.*, 2005), acetic acid (STOPFORTH *et al.*, 2005) or grape seed extract (SIVAROOBAN *et al.*, 2007). Consistent with our findings in most combinations, nisin appeared to be the main contributing antimicrobial factor and lacked significant combined activity with other antimicrobials.

The mode of action of nisin involves pore formation in the cytoplasmic membrane, which leads to rapid removal of free amino acids, adenosine triphosphate, and cations from the cell (ABEE *et al.*, 1994). The antilisterial effect of nisin occurs immediately after cells are exposed to the bacteriocin resulting in cell death (EL-KHATEIB *et al.*, 1993). Present results are in agreement with previous studies (GEORNARAS *et al.*, 2005; NILSSON *et al.*, 1997; SAMELIS *et al.*, 2002; SAMELIS *et al.*, 2005) showing initial reductions of *L. monocytogenes* populations following treatment with nisin and subsequent cell recovery and growth during storage. The inability of nisin to maintain its antilisterial activity has previously been observed in RTE meat products (LUCHANSKY and CALL 2004; SAMELIS *et al.*, 2005). Some apparent reasons for loss of nisin activity include insufficient quantities of nisin to interact with all the target cells, nisin activity dependent on low pH, increased resistance of some strains of *L. monocytogenes*, or uneven distribution of nisin within the food (BOUTTEFROY *et al.*, 2000; HENNING *et al.*, 1986; MURIANA, 1996; SAMELIS *et al.*, 2005, MARTINIS *et al.*, 1997). It is known that *L. monocytogenes* is prone to spontaneous development of resistance to nisin due to mutations (CRANDALL and MONTVILLE, 1998; MARTINIS *et al.*, 1997; LIU *et al.*, 2002); however, the strains used in the present study are not believed to be nisin resistant. Addressing these limitations with nisin, TOKARSKYY and MARSHALL (2008) reported synergistic activity between nisin, lactic acid and monolaurin against *L. monocytogenes* growth when lactic acid was able to increase membrane fluidity and hence increase nisin activity. Additionally, model conditions used in this study were favorable for *Listeria* growth and different from real-life situations. Model conditions included destruction of frankfurters natural microbiota to avoid

competition, use of stationary culture of pathogen in nutrient-rich medium for inoculation, modifying of frankfurters texture by autoclaving, overnight adaptation and attachment of *Listeria* to the meat surface before application of antimicrobials.
Dipping in 2.5, 3.0 and 3.5% BSCSD had little impact on *L. monocytogenes* growth at 4 or 10°C (Tables 4 and 5). THIPPAREDDI *et al.* (2003) reported that BSCSD of ≥ 1.0 % was effective in reducing *C. perfringens* populations in roast beef or injected pork. The antimicrobial mechanism of BSCSD may be similar to organic acid esters, which lower the intracellular pH within the microbial cells, alters cell membrane permeability that affect substrate transport, and inhibits the electron transport system required for energy regeneration (THIPPAREDDI *et al.*, 2003). BSCSD activity increases as product pH decreases (CEYLAN *et al.*, 2003; HULL, 2007).
Previous studies with BSCSD against *L. monocytogenes* in meat products are limited. One study observed inhibition of *L. monocytogenes* by 0.2% sodium diacetate, and stimulated growth with 1% buffered sodium citrate (15 parts sodium citrate, 1 part citric acid w/w) in cooked cured ham products (STEKELENBURG and KANT-MUERMANS, 2001). In contrast to present results, CEYLAN *et al.* (2003) found significant inhibition of *L. monocytogenes* using 1% buffered sodium citrate in combination with 0.1% sodium diacetate in beef frankfurters stored at 3.9°C. The most likely explanation for disparate results is whether agents are added to product formulation (growth inhibition) compared to use as dipping agents (not inhibitory). The present protocol also allowed for *L. monocytogenes* to attach and adapt during overnight storage at 4°C, a process not unlike commercial practice where frankfurter links potentially exposed to *L. monocytogenes* environmental contamination are often cooled overnight prior to packaging the next day.

## 4. CONCLUSION

The results of this study demonstrate that dip antimicrobial treatments with nisin and/or BSCSD were not effective in preventing low-temperature growth of *L. monocytogenes* on the surface of frankfurters under proposed model conditions. Traditional use of such antimicrobials either as direct product ingredients or as in-package solutions remain as more appropriate application methods. Effort to increase activity by combining the different antimicrobial agents did not increase activity.

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PAPER

# VALORIZATION OF RED GRAPE (*VITIS VINIFERA* CV. SANGIOVESE) POMACE AS FUNCTIONAL FOOD INGREDIENT

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## ABSTRACT

The winemaking process generates large amounts of by-products that are still a potential source of bioactive compounds to be used as functional food ingredients. This study is aimed to develop a cow milk kefir fortified with Sangiovese red grape pomace. The phenolic extracts of skins and seeds, which were obtained after infusion and ultrasound-assisted extraction (UAE), were rich in (+)-catechin (105.0±147.0 mg/L) and (-)-epicatechin (76.0±364.0 mg/L) based on HPLC-DAD analysis. The UAE-derived skin extracts were selected for their best antioxidant effectiveness and incorporated into kefir, whose functional properties have been investigated through spectrophotometric assays. The addition of UAE-derived skin extract to kefir was significantly effective in enhancing the total phenolic content (43.2 %) thus increasing the total antioxidant capacity (+47.7 %) and lowering $IC_{50}$ ABTS (-36.0%) and DPPH (-31.45%). An increased inhibitory activity towards $\alpha$-amylase, $\alpha$-glucosidase and pancreatic lipase was also achieved by decreasing the corresponding IC50 values (69.39 and 73.01 μg/mL, respectively).

*Keywords*: antioxidant activity, kefir, grape pomace, ultrasound-assisted extraction, HPLC-DAD, metabolic syndrome, lipase inhibition

## 1. INTRODUCTION

Grape pomace is the most abundant by-product during the winemaking process, mainly composed by the grape skin and seeds. Huge amounts of this winery waste are generated every year, posing environmental and economic issues for their polluting load and disposal costs (DWYER *et al*., 2014; BERES *et al*., 2017; GARCÍA-LOMILLO *et al*., 2017.). Possible uses set by the European Regulation No. 555/2008 (EU, 2008) consider the exploitation of winery by-products for energy production, animal feeding, soil fertilization or ingredients recovery to be included in pharmaceutical, cosmetic and food processes. In particular, the latter aspect has gained much attention in recent years as many applications have been reported dealing with the enhancement of bioactive molecules from grape pomace for functional food design (GALANAKIS, 2012; YU and AHMEDNA, 2013; RESTUCCIA *et al*., 2019). Polyphenols from winery wastes represent one of the most attractive phytochemicals due to their health-promoting properties including effect on metabolic syndrome (MetS) (XIA *et al*., 2010; YU and AHMEDNA, 2013; XIA *et al*., 2014; TEIXEIRA *et al*., 2014; JARA-PALACIOS *et al*., 2015; CHIVA-BLANCH and BADIMON, 2017). Phenolics are usually used for fortification of many food products, including fish and seafood, meat, juices as well as bakery and dairy products (FERNÀNDEZ-MARÌN *et al.,* 2013; FONTANA *et al.,* 2013; BERES *et al.,* 2017; GARCÍA-LOMILLO *et al.,* 2017).

MetS is characterized by several medical conditions including high blood glucose levels, high serum triglycerides, low value of high-density lipoprotein (HDL), and high blood pressure. MetS is associated with several diseases including cardiovascular diseases, heart failure, stroke, hyperuricemia, fatty liver, polycystic ovarian syndrome, erectile dysfunction, and diabetes (MENDRICK *et al*., 2018). Frequently, patients with MetS are overweight or obese. In these patients a progressive reduction in insulin secretion coupled with a progressive rise in insulin resistance are present. Moreover, a persistent blood hyperglycaemia determined, at cytosolic and mitochondrial level, an over production of Reactive Oxygen Species (ROS) (KANETO *et al*., 2010). To this regard, a topic of great interest is represented by the diet supplementation with functional foods. In fact, different studies indicated their positive effect on the body weight reduction; their action seemed to be related to the inhibition of the digestive enzymes interfering with the hydrolysis and absorption of lipids and carbohydrates (i.e. α-amylase, α-glucosidase and pancreatic lipase) (TUNDIS *et al*., 2018; LOIZZO *et al*., 2019).

Recently, AKABERI and HOSSEINZADEH (2016) proposed the use of grapes for MetS syndrome treatment. It is well know that grapes are rich in proanthocyanidins that have been used for diverse dedications such as antioxidants, nutritional supplements, preventing atherosclerosis, managing cardiovascular complications, to treat dyslipidaemia, other than as free radical scavenging and lipid lowering. Furthermore, several scientific studies and applications, confirmed the absence of toxicity of the tested grape pomace extracts (BLADÉ *et al*., 2016; COSTABILE *et al*., 2019; CARULLO *et al*., 2019).

In this context, the goal of this research was focused on the realization of a new milk kefir, obtained by the addition of a Sangiovese pomace extract during the kefir preparation. The supplementation was accomplished in order to improve the antioxidant profile and the impact on metabolism. For this purpose, the grape skin and seeds extracts, obtained by two different procedures, infusion (a classical method) and ultrasound-assisted method (innovative and time-saving), were chemically investigated by HPLC analyses and the antioxidant activity was also monitored. Skin extract obtained by ultrasound-assisted extraction (UAE) resulted the most interesting extract and it was added to kefir grains and skimmed milk. Successively, the enriched product was tested for its antioxidant and

hypoglycaemic features by *in vitro* colorimetric assays, α-amylase and α-glucosidase inhibition tests. The hypolipidemic effects by pancreatic lipase inhibition were also assessed.

## 2. MATERIALS AND METHODS

### 2.1. Materials and chemicals

Sangiovese pomace was kindly given by Le Moire farm (Catanzaro, Italy) during September 2019. Cell culture and cell culture materials were obtained from Sigma-Aldrich Chemical Co. Ltd (Milan, Italy). All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Co. Ltd (Milan, Italy) and VWR International (Milan, Italy).

### 2.2. Preparation of the grape extracts

Skins and milled seeds (30 g), obtained by manually separation from Sangiovese pomace, were added of 200 mL ethanol/water mixture (50:50 v/v) acidified at pH=2 with HCl 37% (v/v) (CARRERA *et al.*, 2012; GONÇALVES *et al.*, 2017). The mixture was then subjected to ultrasound assisted extraction (UAE) at 30°C for 15 minutes (10 cycles/sec), at an ultrasonic frequency of 40 kHz using the ultrasound-bath Branson model 3800-CPXH (Milan, Italy). Alternatively, skins and seeds were immersed in a solution ethanol/water (80:20 v/v) and heated at 60°C for 60 minutes. The two procedures were repeated three times. The mixtures were then filtered out and concentrated under reduced pressure using a rotary evaporator Buchi RII®. Ration extraction between grape sample and solvent mixture was 1:6. All the extracts were stored at -18°C until analyses. The obtained samples were labelled as A (skin extract obtained after UAE); B (skin extract obtained after infusion); C (seed extract obtained after UAE) and D (seed extract obtained after infusion).

### 2.3. Determination of D-(+)-glucose, D-(−)-fructose and organic acids

The sugars level in Sangiovese skin and seed extracts was performed using a Knauer high liquid chromatography system (Asi Advanced Scientific Instruments, Berlin, Germany) equipped with a Knauer HPLC Pump K-120 (Asi Advanced Scientific Instruments, Berlin, Germany), a Rheodyne injection valve with loop of 20 μL and a Smartiline RI detector 2300. Elution was obtained on a VARIAN Meta Carb H Plus column (300 mm× 7.8 I.D., 5 μm). The column temperature was 55°C and the flow rate was 0.25 mL/min. The mobile phase consisted of 0.01 N $H_2SO_4$ solution. The HPLC analyses of organic acids were performed on a Knauer (Asi Advanced Scientific Instruments, Berlin, Germany) system equipped with two pumps Smartiline Pump 1000, a Rheodyne injection valve (20 μL) and a photodiode array detector UV/VIS equipped with a semi-microcell. Separation was obtained using an Acclaim OA column (250 mm×4.0 I.D., 5 μm) at T = 30°C. The mobile phase consisted of 100 mM $Na_2SO_4$ (pH =2.65 with methane sulfonic acid) and the flow rate was 0.6 mL /min. Stock solutions of each standard, in different diluted concentration ranging from 0.2-2 g/L, were prepared in ultra-pure water provided by a Milli-Q system (Millipore Co., Bedford, MA). All solutions were filtered through 0.45 μm glass-microfiber GMF Whatman chromatographic filter (HAWP Millipore Co., Bedford), before analysis.

## 2.4. Phenolic characterization of Sangiovese skin and seed extracts

High performance liquid chromatography coupled to a diode array detector (HPLC-DAD) was applied to determine the phenolic profile of Sangiovese grape skin and seed extract (KAMMERER *et al*., 2004). The analysis was performed on a Knauer system (ASI - Advanced Scientific Instruments, Berlin, Germany) equipped with two Smartiline Pump 1000 pumps, a Rheodyne injection valve (20 $\mu$L) and a UV - VIS photodiode series detector equipped with a semi-microcell. Compounds were separated according the procedure previously described by LOIZZO *et al*. (2019) and monitored at 280, 254, 330 and 305 nm. Compounds identification and quantification were carried out by comparing the spectra and relative retention times of Sangiovese extracts peaks with those obtained by injecting pure standards as selected marker (gallic acid, (+)-catechin and (-)-epicatechin, catechin, caffeic acid, rutin, *trans*-resveratrol, and quercetin).

## 2.5. Measurement of the total phenolic content (TPC) and total anthocyanins (TA)

The total phenolic content (TPC) was detected in the phenolic extracts of grape seeds and skins and kefir or fortified kefir, according to the Folin-Ciocalteu colorimetric method of RESTUCCIA *et al*. (2011). Briefly, a 6.0 mL volume of each properly diluted sample was added with 1.0 mL of Folin-Ciocalteu reagent and after 3 min with 3.0 mL of 5.0% w/v $Na_2CO_3$. Positive control and blank solutions was also prepared by substituting the sample with the same volume of 0.1 (w/v) ascorbic acid and hydro alcoholic solution (50:50 v/v), respectively. After shaking for 2 h, the absorbance value of each mixture was measured at 760 with a Jasco V-530 UV/Vis spectrometer (Jasco, Tokyo, Japan). The TPC was expressed as mg of gallic acid equivalents (GAE) *per* g of extract for samples A-D and mg GAE *per* L for kefir (M2) and fortified kefir (M2A10).
The total anthocyanins (TA) was determined using the differential pH method (LOIZZO *et al*., 2019). Anthocyanins undergo a reversible modification of the structure with a change in the pH that occurs with a variation in the absorbance spectrum. Seven and half mg of extract were added to 5 mL of distilled water. For each sample two dilutions were prepared, one with a 0.025 M hydrochloric acid buffer solution at pH 1, and the other with a 0.4 M sodium acetate buffer solution at pH 4.5, corrected with hydrochloric acid. The solutions were left to equilibrate for 15 min. Spectrophotometric reading was per-formed at 510 nm and 700 nm. The results were expressed as cyanidine-3-*O*-glucoside equivalent (CGE) *per* g of extract.

## 2.6. Determination of total antioxidant capacity (TAC)

A literature protocol, with few changes was employed to determine total antioxidant capacity (TAC) of each extract, kefir or fortified kefir (CIRILLO *et al*., 2012). Briefly, 0.3 mL of hydro alcoholic solution (50:50 v/v) of sample were added to 1.2 mL of reagent solution (0.6 M $H_2SO_4$, 28.0 M $Na_3PO_4$, and 4.0 M $(NH_4)_2MoO_4$). The reaction mixture was incubated at 95°C for 150 min and after cooling to room temperature, the absorbance of the mixture was measured at 695 nm. Positive control and blank solutions was also prepared by substituting the sample with the same volume of 0.1 (w/v) ascorbic acid and hydro alcoholic solution (50:50 v/v), respectively. The total antioxidant activity of each extract was expressed as mg of catechin equivalent (CTE) *per* g of extract for samples A-D and mg CTE *per* L for kefir (M2) and fortified kefir (M2A10).

## 2.7. Determination of scavenging activity on DPPH radicals

Free radical scavenging properties of the extracts were estimated towards DPPH (2,2-diphenyl-1-picrylhydrazyl)acid)) radical (SPIZZIRRI *et al*., 2011). Briefly, in a volumetric flask (10 mL) were placed 1.0 mL of hydro alcoholic solution (50:50 v/v) of each extract, kefir or fortified kefir, 4.0 mL of hydroalcoholic solution (50:50 v/v) and 5.0 mL of ethanol solution of DPPH (200 μM), obtaining a solution of DPPH with a final concentration of 100 μM. The solution was incubated at 25°C and, after 24 h, the absorbance of the remaining DPPH was determined at 517 nm. Ascorbic acid 0.1 (w/v) was used as positive control. The scavenging activity of the tested matrices was measured as the decrease in absorbance of the DPPH and it was expressed as $IC_{50}$, defined as the concentration of sample that causes a decrease in the initial DPPH concentration by 50%.

## 2.8. Determination of scavenging effect on the ABTS radical cation

Free radical scavenging properties of the extracts were estimated in aqueous media towards ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical (RESTUCCIA *et al*., 2017) ABTS radical cation ($ABTS^{\cdot+}$) solution (7.0 mM) remains in the dark at room temperature for 12-16 h, and then the concentration was adjusted to an absorbance of 0.70±0.02 at 734 nm. In a general procedure, the scavenging effect of the samples was evaluated by adding 500 μL of hydroalcoholic solution (50:50 v/v) on each extract, kefir or fortified kefir at 2.0 mL of the $ABTS^{\cdot+}$ radical solution. The mixture was incubated in a water bath at 37°C and protected from light. The decrease of absorbance at 734 nm was measured at the endpoint of 5 min. Ascorbic acid 0.1 (w/v) was used as positive control. The antioxidant activity was measured as the decrease in absorbance of the ABTS and it was expressed as $IC_{50}$, defined as the concentration of sample that causes a decrease in the initial ABTS concentration by 50%.

## 2.9. Kefir preparation and enrichment

Three mg of fresh kefir grains (Kefiralia, Burumart Commerce S.L, Spain) were added in a glass flask at room temperature, containing 3 mL, of six type of commercially available milks. The container not-hermetically closed, was posed at 20-25°C for 24 hours with or without the addition of the extract. The six types of milk, purchased in a local market, were labeled Ultra High Temperature (UHT) whole milk (M1), skimmed milk (M2) and partially skimmed milk (M3), pasteurized fresh whole milk (M4), skimmed milk (M5), and partially skimmed milk (M6). The kefir grains obtained by each milk type were finally weighted and the pH value measured by using the pH 211 Microprocessor pH Meter (HANNA Instruments Italia, Milan, Italy). The enriched kefir was obtained mixing 1.0, 5.0 and 10.0 mg of the A extract with 10.0 mL of M2 in the same experimental conditions in which pH was measured.

## 2.10. Carbohydrate hydrolysing enzymes inhibition assay

The carbohydrate-hydrolyzing enzymes inhibition was detected following the procedure previously described by LEPORINI *et al.*, (2020). α-Amylase (0.0253 g in 100 mL of cold water) was mixed with starch solution (0.125 g of potato starch in 25 mL of 20 mM sodium phosphate buffer and 6.7 mM sodium chloride. The mixture was stirred for 15 min at 65°C. Sangiovese pomace samples, freeze-dried kefir and enriched kefir at concentrations

ranging from 25 to 1000 μg/mL were added to starch solution and left to react with enzyme at 25°C for 5 min. The absorbance was read at 540 nm. In the α-glucosidase assay a maltose solution (12 g of maltose in 300 mL of 50 mM sodium acetate buffer) was mixed with α-glucosidase (1 mg of enzyme in 10 mL of ice cold distilled water) and *o*-dianisidine (DIAN) (LOIZZO *et al*., 2019). The peroxidase/glucose oxidase (PGO) system-colour reagent solution was obtained by dissolving one capsule in 100 mL of ice-cold distilled water. Samples (pomace extracts, freeze-dried kefir and freeze-dried enriched kefir) at concentrations ranging from 25 to 1000 μg/mL were added to 250 μL maltose solution and 5 μL enzyme and left to incubate at 37°C for 30 min. Then, perchloric acid was added and the mixture was centrifuged. The supernatant was collected and mixed with DIAN and PGO, and left to incubate at 37°C for 30 min. The absorbance was read at 500 nm. Acarbose was used as positive control in both assays. The $IC_{50}$ value for each sample, defined as the concentration of sample causing 50% enzyme inhibition was determined from the curves plotted and tabulated.

### 2.11. Pancreatic lipase inhibitory activity

Pancreatic lipase inhibitory activity was determined by 96-well plate method based on the procedure proposed by EL-SHIEKH *et al*. (2019). 4-Nitrophenyl octanoate (NPC), 5 mM in dimethylsulfoxide solution and an aqueous solution of porcine pancreatic lipase enzyme (1 mg/mL), and Tris-HCl buffer (pH 8.5) were prepared. Pomace extracts, freeze-dried kefir and freeze-dried enriched kefir at concentrations ranging from 2.5 to 40 mg/mL were added in a well with 6 μL of the enzyme, 6 μL of NPC and 279 μL of buffer. The mixture was incubated at 37°C for 30 min. The absorbance was measured at 405 nm. Orlistat was used as a positive control. The $IC_{50}$ value for each sample, defined as the concentration of sample causing 50% enzyme inhibition, was determined from the curves plotted and tabulated.

### 2.12. Statistical analysis

All of the data obtained from three replicates (n=3) were presented as mean±standard deviation (SD) were subjected to One-way analysis of variance (ANOVA) using Prism GraphPad Prism version 4.0 for Windows, GraphPad Software (San Diego, CA, USA). After that, the Tukey's test was performed to compare all means. Differences among means with $p<0.05$ were accepted as representing statistically significant. Dunnett's test was used to compare each mean of biological data to a positive control mean. Differences among means with ****$p<0.0001$; ***$p<0.001$; **$p<0.01$ were accepted as representing statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. Chemical composition of Sangiovese pomace skin and seeds extracts

Grape sugar and acid were monitored by HPLC-RI. Glucose was particularly abundant in sample A followed by B. Differently, fructose was more present in skin extract obtained after infusion (sample B) (13.98 g/100g) (Table 1). Tartaric acid was the most abundant acid in all samples except seed extract obtained after infusion (sample D).

**Table 1.** Sugar and acids in Sangiovese skins and seeds extracts.

| | Glucose (g /100 g) | Fructose (g/100 g) | Tartaric acid (g/L) | Malic acid (g/L) | Lactic acid (g/L) | Acetic acid (g/L) | Fumaric acid (g/L) |
|---|---|---|---|---|---|---|---|
| A | 13.79±0.11$^{a}$ | 6.01±0.11$^{b}$ | 7.81±0.10$^{a}$ | 4.86±0.04$^{a}$ | 1.82±0.05$^{a}$ | 2.68±0.04$^{d}$ | 0.005±0.004$^{a}$ |
| B | 13.14±0.12$^{b}$ | 13.98±0.20$^{a}$ | 4.64±0.09$^{c}$ | 1.67±0.03$^{d}$ | 0.09±0.03$^{d}$ | 3.25±0.01$^{b}$ | 0.006±0.002$^{a}$ |
| C | 0.71±0.07$^{d}$ | 0.32±0.07$^{d}$ | 6.71±0.14$^{b}$ | 2.61±0.04$^{b}$ | 0.80±0.04$^{b}$ | 4.51±0.04$^{a}$ | 0.003±0.001$^{a}$ |
| D | 1.17±0.08$^{c}$ | 1.19±0.24$^{c}$ | 2.74±0.11$^{d}$ | 1.78±0.06$^{c}$ | 0.46±0.02$^{c}$ | 3.05±0.04$^{c}$ | 0.003±0.003$^{b}$ |

A (skin extract obtained after UAE); B (skin extract obtained after infusion); C (seed extract obtained after UAE) and D (seed extract obtained after infusion). Data are expressed as mean ± standard deviation (SD) ($n$= 3). Means within each column and with different lowercase letters are statistically different according to Tukey's test ($p$<0.05).

*Vitis vinifera* genotypes, environmental factors, postharvest treatments and applied extraction procedure for recovery of polyphenolic compounds influenced their amount in the grapes extracts (SPIGNO and DE FAVERI, 2007; BUCIĆ-KOJIĆ *et al*., 2009). Grape skins and seeds TPC was evaluated by Folin-Ciocalteu procedure, an analytical methodology based on the electrons transferring from phenolic compounds to the Folin-Ciocalteu reagent in an alkaline medium. The TPC values of grape skins and seeds appear strictly related both to raw material (seeds or skins) and extraction procedure (infusion or UAE), as reported in the Table 2.

The TPC in the grape extracts examined in this study ranged from 130.65 to 259.26 mg GAE *per* g of extract. Generally, for both raw materials, UAE procedure appeared more useful than the infusion technique and higher amounts of TPC were detected in the skins (259.26 mg GAE *per* g of extract, for A), compared with the seed extracts (207.55 mg GAE *per* g of extract, for C).

The TA content ranged from 39.31 to 10.24 CGE *per* g of extract for skin extract obtained after UAE and seed extract obtained after infusion, respectively. Our data are in the same order of magnitude of those reported by MENDOZA *et al*. (2012).

From HPLC analysis gallic acid, (+)-catechin and (-)-epicatechin, catechin, caffeic acid, rutin, *trans*-resveratrol, and quercetin were identified (Table 2). Different levels of phenolic compounds could be detected from seed and skin.

**Table 2.** TPC and phenolic profile of Sangiovese skins and seeds extracts.

| | TPC (mg GAE *per* g extract) | TA (CGE *per* g of extract) | Gallic acid (mg/L) | (+)-Catechin (mg/L) | Caffeic acid (mg/L) | (-)-Epicatechin (mg/L) | Rutin (mg/L) | *trans*-Resveratrol (mg/L) | Quercetin (mg/L) |
|---|---|---|---|---|---|---|---|---|---|
| A | 259.26±2.12$^{a}$ | 39.31±2.89$^{a}$ | 2.18±1.04$^{b}$ | 147.0±1.15$^{a}$ | 4.27±0.07$^{b}$ | 76.0±1.15$^{d}$ | 13.12±0.14$^{a}$ | 21.44±0.18$^{a}$ | 247.0±1.39$^{a}$ |
| B | 171.48±2.71$^{c}$ | 22.48±2.77$^{b}$ | 1.64±0.05$^{bc}$ | 105.0±1.90$^{c}$ | 3.48±0.06$^{c}$ | 126.0±1.02$^{c}$ | 9.11±0.07$^{ab}$ | 14.66±0.11$^{b}$ | 210.0±1.30$^{b}$ |
| C | 207.55±3.22$^{b}$ | 21.65±2.56$^{b}$ | 104.0±1.08$^{c}$ | 124.0±1.17$^{b}$ | 5.87±0.07$^{a}$ | 364.0±1.17$^{a}$ | 2.17±0.09$^{b}$ | N.D. | 3.35±1.37$^{c}$ |
| D | 130.65±1.10$^{d}$ | 10.24±1.13$^{c}$ | 88.20±1.16$^{a}$ | 122.0±1.12$^{b}$ | 2.43±0.06$^{d}$ | 232.0±1.12$^{b}$ | 2.41±0.05$^{b}$ | N.D | 1.66±1.24$^{c}$ |

A (skin extract obtained after UAE); B (skin extract obtained after infusion); C (seed extract obtained after UAE) and D (seed extract obtained after infusion). N.D.: Not detected. Data are expressed as mean ± standard deviation (SD) ($n$= 3). Means within each column and with different lowercase letters are statistically different according to Tukey's test ($p$<0.05).

Generally, (+)-catechin and (-)-epicatechin represent the main abundant compounds with values in the range105.0-147.0 and 76-364 mg/L, respectively. The applied extraction procedure, influenced a lot the amount of gallic acid. In fact, both C and D are characterized by the highest amount with values of 104.0 and 88.20 mg/L, respectively. A similar consideration could be done for *trans*-resveratrol and quercetin that are concentrated only in B and A samples (values of 14.66 and 21.44 mg/L, and 210.0 and 247.0 mg/L, respectively). According to our data, both catechin and epicatechin were the main abundant compounds of seeds from *V. vinifera* cv. Frankovka as reported by BUCIĆ-KOJIĆ *et al*., 2009. Significant amount of gallic acid were also found by Doshi *et al*., (2015) that evaluated the phenolic profile of Pusa Navarang and Merlot seeds and skin. In particular, Pusa Navarang seed extract showed high amounts of catechin and epicatechin whereas quercetin was abundant in its skin extract. According to our data, *trans*-resveratrol was detected only in skin with values of 34.5 and 37.5 mg/L for Pusa Navarang and Merlot varieties, respectively. These values are twofold higher than that found in Sangiovese samples. According to GODEVAC *et al*., (2010) the phenolic composition of grape seed extracts from *V. vinifera* cv., Prokupac, Smederevka, Italian Riesling, Traminer, Black Burgundy, Gamay Noir, Muscat Hamburg and Gamay Bojadiser, showed the flavan-3-ol monomers as the main abundant compounds especially in seeds derived from red wine grapes. Regarding rutin, our data showed that this flavonoid glycoside was more concentrated in skin than seeds. However, GENGAIHI *et al*., (2014) evidenced a main content of rutin in both Red romy and Grenache noir seeds. Quercetin-3-*O*-glucoside (39.86 μg/g dry sample), rutin (37.01 μg/g dry sample) and *trans*-resveratrol (32.88 μg/g dry sample) were the most abundant compounds identified in *V. tiliifolia* skin (JIMÉNEZ *et al*., 2018). The results recorded in the TPC determination are partially in agreement with the concentration of polyphenols recorded by HPLC-DAD. However, in this comparison it is necessary to consider the contribute of the single class of molecules to TPC value and the contribution of others compounds, such as pigments like anthocyanin, present at high concentration in the grape skins (BABBAR *et al*., 2011). Furthermore, it should be pointed out that some non-phenolic components also having reducing capacity, such as organic sugars and acids, affect the TPC value, leading to overestimated total phenolic contents. Finally, phenolic compounds differently react with the Folin-Ciocalteu reagent and several flavonoid molecules produce poor responses, leading to an underestimated TPC value (CIRILLO *et al.*, 2016).

### 3.2. Evaluation of the antioxidant properties of Sangiovese skin and seed extracts

The results concerning the antioxidant activity expressed as total antioxidant capacity (TAC) and radical scavenging activity in aqueous and organic environments appear quite related to TPC values. Specifically, TAC values, as reported in Fig. 1, highlighted as sample A returned the most significant result (326.54 mg CTE *per* g extract), emphasizing the existence of a positive relationship between TPC and TAC.
Some recorded dissimilarities can be explained invoking structural differences in the phenolic molecules that affected antioxidant power of the extracts. Literature data indicate that the presence of others interfering molecules, often prevent a linear correlation between TPE and antioxidant activity (FERNANDO *et al*., 2018). The ability of the extracts as scavenger of lipophilic DPPH radical was expressed in terms of $IC_{50}$ and reported in Table 3.

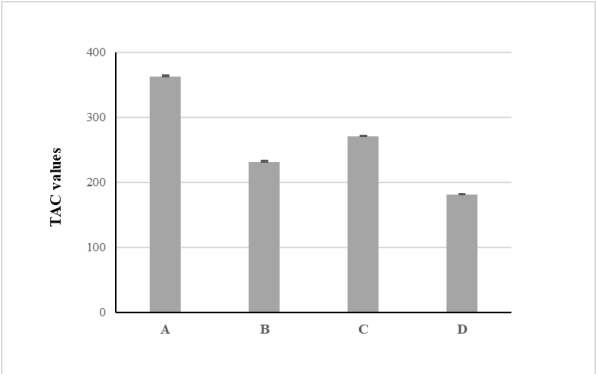


**Figure 1.** TAC values of Sangiovese pomace samples. A (skin extract obtained after UAE); B (skin extract obtained after infusion); C (seed extract obtained after UAE) and D (seed extract obtained after infusion).

A comparison of $IC_{50}$ values shows that skins extracts (7.6 and 12.0 μg/mL for A and B, respectively) returned 2-times lower values compared to the seeds extracts. A comparison of this data with the HPLC-DAD analyses displayed as the solubility of recorded polyphenol plays an important role in the lipophilic scavenger activity. In particular, the high concentration of quercetin recorded in the skins extracts (both UAE and infusion extraction methodologies) justified the better performances recorded for A and B. Moreover, UAE process appears in both cases the better technique leading to lower $IC_{50}$ values. The recorded data showed quite correspondence with the results of TAC and TPE. This trend is evident for both the samples and some discrepancies could be justified by considering the different environment (organic and aqueous) in which the assays were performed. The scavenging capacity of the extracts in the aqueous environment against the ABTS radical was expressed in terms of $IC_{50}$ value, and Table 3 displayed the recorded data.

**Table 3.** Radical scavenging activity of extracts from skins and seeds of Sangiovese cv grape.

| Sample | DPPH $IC_{50}$ (μg/mL) | ABTS $IC_{50}$ (μg/mL) |
|---|---|---|
| A | 7.6±0.7** | 38.5±0.5**** |
| B | 12.0±0.3**** | 42.0±1.2**** |
| C | 14.8±0.6**** | 42.2±2.1**** |
| D | 24.5±0.5**** | 43.5±1.1**** |
| Positive control | | |
| Ascorbic acid | 5.0±0.8 | 1.7±0.3 |

A (skin extract obtained after UAE); B (skin extract obtained after infusion); C (seed extract obtained after UAE) and D (seed extract obtained after infusion). Data are expressed as mean ± S.D. ($n$ = 3). Differences within and between groups were evaluated by One-way analysis of variance test followed by a multicomparison Dunnett's test compared with the positive control (****$p$<0.0001; ** $p$<0.01).

The analysis of the $IC_{50}$ values exhibited comparable values for both matrices and the extraction methodologies did not deeply affect the result. However, the scavenging

activity recorded in the aqueous environment appeared more than three times lower compared with the organic one. Lack of data concerning extracts antioxidant capacity of the investigated cultivars made very hard to compare the collected values with the literature analyses. In addition, many factors, such as fruit ripening, weather conditions, soil and place of growth, largely affect the distribution of the antioxidants in the vegetable matrix, further complicating whatever qualitative-quantitative comparison (TANG *et al*., 2018). The juxtaposition of these data with the HPLC-DAD analyses underlined also that the solubility of the recorded polyphenol plays an important role in the lipophilic scavenger activity. In particular, the high concentration of quercetin recorded in the skins extracts (UAE or infusion extraction method), justified the better performances recorded for sample B and A.

### 3.3. Kefir enrichment

As already stated, six types of milk have been considered for kefir preparation to underline the effect of different raw materials on kefir production (*i.e* grains growth and pH decrease). As summarized in Table 4, after 24 hours, all the considered samples produced a positive variation of the kefir grains mass as well as a decrease in pH values. Considering the obtained data (mass and pH) in the same experimental conditions, we decided to enrich only the skimmed milk (M2), which showed the lower kefir grains mass variation, in order to evaluate the improved effect of extract addition. Noteworthy, skimmed milk is mostly requested by female population and health fanatics, due to the poor fats content. Comparison between M2, M5 and M6, in terms of mass variation and pH decrease, supported the choice to use the former to obtain the functional beverage by adding 1, 5, 10 mg of extract A to 10 mL of M2, before grains addition (DOS SANTOS *et al*., 2017). Addition levels have been carefully selected to avoid possible antimicrobial effects exerted by wine polyphenols on kefir microorganisms (GARCÍA-LOMILLO *et al*., 2014; KATALINIĆ *et al*., 2010). The highest addition level, in fact corresponded to 0.1% w/w or to a supplementation equivalent to 25.9 mg GAE/100g of kefir.

**Table 4.** Milk type screening.

| Sample | mass (g) | | Mass variation | pH | | pH decrease |
|---|---|---|---|---|---|---|
| | t (h) = 0 | t (h) = 24 | | t (h) = 0 | t (h) =24 | |
| M1 | 0.30±0.01 | 0.37±0.02$^{b}$ | 0.07±0.01$^{c}$ | 6.98±0.02 | 4.71±0.01$^{d}$ | 2.27±0.01$^{c}$ |
| M2 | 0.29±0.01 | 0.31±0.01$^{c}$ | 0.02±0.01$^{d}$ | 6.99±0.01 | 4.42±0.01$^{f}$ | 2.57±0.01$^{a}$ |
| M3 | 0.29±0.02 | 0.44±0.01$^{a}$ | 0.15±0.02$^{a}$ | 7.01±0.02 | 5.54±0.02$^{a}$ | 1.47±0.02$^{e}$ |
| M4 | 0.30±0.02 | 0.44±0.02$^{a}$ | 0.14±0.02$^{a}$ | 7.02±0.01 | 4.76±0.02$^{c}$ | 2.26±0.01$^{c}$ |
| M5 | 0.29±0.01 | 0.39±0.01$^{b}$ | 0.10±0.01$^{b}$ | 6.97±0.02 | 4.98±0.01$^{b}$ | 1.99±0.01$^{d}$ |
| M6 | 0.31±0.02 | 0.41±0.02$^{b}$ | 0.10±0.02$^{bc}$ | 7.00±0.01 | 4.65±0.01$^{e}$ | 2.36±0.01$^{b}$ |

Data are expressed as mean ± standard deviation (SD) ($n$= 3). Means within each column and with different lowercase letters are statistically different according to Tukey's test ($p$<0.05).

As we recently reported (CARULLO *et al*., 2020) for the same kind of samples, the applied fortification level ensured proper activity of LAB and yeasts, at the same time, improving antioxidant features and avoiding possible drawbacks. Metabolomics analyses of the kefir

extracts, in fact, revealed the same compounds produced during fermentation with and without addition, demonstrating that metabolic pathways of LAB and yeasts were not influenced by the presence of the wine pomace extracts, at least under the qualitative point of view (CARULLO *et al*., 2020). Under the technological point of view, TSENG and ZHAO (2013) found that the addition of grape pomace flour to milk at levels higher than 3%, produced an excessive syneresis of yogurt while at levels higher than 5% the coagulation was inhibited. Considering sensory features, grape extract at 1% improved the acceptability of yogurt (KARAASLAN *et al*., 2011) and only reaching fortification with skin wine pomace at 6% induced decrease in the liking score, especially for the taste and flavor (MARCHIANI *et al*., 2016). All considered, the supplementation levels employed in this research are far below those generally found in literature and regarded as producing adverse effects.

The kefir grains (i.e. M2A1, M2A5 and M2A10) were weighted and their pH was measured after fortification and after 24 h of activity. As reported in Table 5, the kefir grains presented different weights, in particular M2A10 resulted the heaviest, although pH values resulted similar among the three kefirs. For this reason, considering that a similar pH value agrees with a similar chemical composition, we decided to investigate only the heaviest kefir (M2A10) for their properties in metabolic syndrome (CARULLO *et al*., 2020; SETTANNI *et al*., 2019). By the comparison between unfortified kefir (M2) with M2A1, M2A5 and M2A10 showed a lower pH decrease probably in relation with high sugars and organic concentration found in the wine pomace extract. More sugars in the environment mean more lactic acid in kefir, thus producing the strong pH decrease. At the same time, wine organic acids can be metabolized and/or partially remain after fermentation. In the latter case, they obviously contribute to the pH decrease. A significant increase in TPC concentrations was observed in M2A10 that showed a TPC of 393.32 mg GAE *per* L in comparison to unfortified kefir (M2) that showed a TPC of 274.74 mg GAE *per* L, as a consequence of the addition of the extract to the unfermented milk before the kefir production. In general, this value appears in accordance with literature data confirming that during the fermentation process, irregular changes in the total phenol content of kefir samples can be observed, due to the biotransformation of phenolic compounds (KARAASLAN *et al.*, 2011; NAJGEBAUER-LEJKO and SADY, 2015; PERNA *et al.*, 2019; OZCAN *et al.*, 2019).

**Table 5.** Screening of the enriched kefir samples.

| Sample | mass (g) | | Mass variation | pH | | pH decrease |
|---|---|---|---|---|---|---|
| | t (h) = 0 | t (h)=24 | | t (h) = 0 | t (h)=24 | |
| M2A1 | 0.29±0.01 | 0.34±0.01$^{b}$ | 0.05±0.01$^{b}$ | 7.00±0.01 | 2.90±0.01$^{c}$ | 4.10±0.01$^{a}$ |
| M2A5 | 0.30±0.01 | 0.37±0.01$^{b}$ | 0.07±0.01$^{b}$ | 6.99±0.01 | 3.00±0.01$^{a}$ | 3.99±0.01$^{b}$ |
| M2A10 | 0.30±0.02 | 0.59±0.01$^{a}$ | 0.29±0.02$^{a}$ | 7.01±0.01 | 2.95±0.02$^{b}$ | 4.06±0.02$^{a}$ |

Data are expressed as mean ± standard deviation (SD) ($n$= 3). Means within each column and with different lowercase letters are statistically different according to Tukey's test ($p$<0.05).

### 3.4. Evaluation of the functional properties of kefir fortified with UAE-derived Sangiovese skin extract

Polyphenol compounds influence the composition of microbiota by the inhibition of pathogen growth and the stimulation of the commensal bacteria growth and these changes appear deeply related to their chemical structure and concentration (CUEVA *et al*., 2010). The action of fermentative enzyme, such as β-glycosidase, produces the hydrolysis of complex phenolic compounds to simpler types producing a significant increase of the total phenolic content (CODA *et al*., 2012). A further increase of the antioxidant activity can be obtained by milk supplementation, before the fermentation process, with a source of bioactive molecules. These compounds can interact with the milk proteins determining changes in the microbiological quality and oxidative stability of the dairy products. The results of antioxidant activity (TAC and DPPH free radical scavenging assays) showed that enriched kefir had higher antioxidant activity than unfortified kefir (Fig. 2 and Table 6).

**Table 6.** Radical scavenging activity of kefir from skins and seeds of Sangiovese cv grape.

| Kefir sample | DPPH $IC_{50}$ (μg/mL) | ABTS $IC_{50}$ (μg/mL) |
|---|---|---|
| M2 | 107.0± 2.9**** | 498.3±8.2**** |
| M2A10 | 73.4±1.7**** | 318.7±7.4**** |
| Positive control | | |
| Ascorbic acid | 5.0±0.8 | 1.7±0.3 |

Data are expressed as mean ± SD ($n$ = 3). Differences within and between groups were evaluated by One-way analysis of variance test followed by a multicomparison Dunnett's test compared with the positive control (****$p$<0.0001).

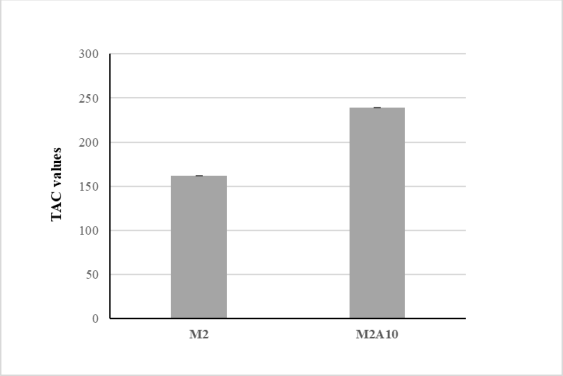


**Figure 2.** TAC values of kefir (M2) and fortified kefir (M2A10).

Data clearly showed significant increase of TAC value (+47.7% in the fortified kefir respect to the unfortified one), while $IC_{50}$ value against DPPH radical displayed a value that is 14.7% lower compared to control kefir.

Obtained results highlighted that the fortified kefir displayed improved antioxidant performances, compared to the unfortified sample.

### 3.5. Effect of kefir and its fortified derived products on enzymes linked to MetS

Both kefir and its derived fortified product are able to inhibit the enzymes linked to metabolic syndrome in a concentration- dependent manner. Generally, α-amylase resulted more sensible to the action of our investigated samples and the obtained $IC_{50}$ values are quite similar than that found with the positive control acarbose (Table 7).

**Table 7.** Effect of fortified kefir on enzyme linked to MetS.

| Sample | α-Amylase $IC_{50}$ (μg/mL) | α-Glucosidase $IC_{50}$ (μg/mL) | Lipase $IC_{50}$ (μg/mL) |
|---|---|---|---|
| M2 | 71.73±2.5** | 86.18±2.8*** | 79.15±2.6*** |
| M2A10 | 69.39±2.2** | 84.47±2.7*** | 73.01±2.5*** |
| Positive Control | | | |
| Acarbose | 50.0±0.9 | 35.5±1.2 | |
| Orlistat | | | 37.5±0.92 |

Data are expressed as means ± SD ($n$= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Orlistat used as positive control in pancreatic lipase test. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test compared with the positive control (***$p$<0.001, **$p$<0.01).

Fortified kefir (M2A10) showed an $IC_{50}$ value of 69.39 mg/mL. Value of 84.47 mg/mL was found against α-glucosidase. Moreover, M2A10 exerted a promising activity against lipase enzyme ($IC_{50}$ value of 73.01 mg/mL). Previously MORENO *et al*., (2003) demonstrated that grape seed extract (GSE) was able to inhibit fat-metabolizing enzyme pancreatic lipase. More recently, HASSAN *et al*., (2014) reported the ability of water and ethanol grape seed extracts (WGSE and EGSE) against some pancreatic enzymes including α-amylase and lipase. Analysis of data revealed that EGSE had higher α-amylase inhibitory activity in comparison with WGSE. Moreover, daily administration of Kefir (3.6 mL/200 g) in alloxan-induced diabetes mellitus rats for 20 days, showed a significant reduction in blood glucose, total cholesterol, triglycerides, as well as in low density lipoprotein (LDL) and very low density lipoprotein (VLDL) whereas HDL was effectively increased (GHAZI *et al*., 2018). The positive effect of kefir on MetS was confirmed, also, by a randomized double-blind placebo-controlled clinical trial on diabetic patients treated with kefir containing *Lactobacillus casei* (600 mL/day) for 8 weeks (OSTADRAHIMI *et al*., 2015).

## 4. CONCLUSIONS

The present study assessed the total phenolic and anthocyanin content, HPLC-DAD phenolic profile, antioxidant activity, α-amylase, α-glucosidase, and lipase inhibitory activities of kefir and Sangiovese pomace fortified kefir. Between the two extractive methods, UAE showed the better performances in the recovery of the bioactive

compounds. Moreover, UAE skin extract (A) characterized by the highest TPC and TA content was selected to be added to Skimmed Milk (M2) and kefir grains. The amount of sample A added to kefir grains did not inhibit the growth of microorganisms as revealed by the increase weight of fortified kefir grains after 24 hours. Fortified kefir showed a better antioxidant activity than unfortified one as evidenced by the comparison of data obtained in all performed assays (TAC, DPPH, and ABTS). Despite the new beverage demonstrated a good performance in the inhibition of key enzymes linked to metabolic syndrome (α-amylase, α-glucosidase and lipase) this activity is not significantly different from unfortified kefir. In conclusion, our data showed a chemical characterization of winery wastes (Sangiovese skins and seeds) also proposing a useful extraction of bioactive molecules to be used as functional ingredients for fermented milk fortification.

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## ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) |
| BHT | Butylated Hydroxytoluene |
| CGE | Cyanidine-3-*O*-glucoside equivalent |
| CTE | Catechin equivalent |
| DIAN | *o*-Dianisidine |
| DM2 | Diabetes Mellitus Type 2 |
| DPPH | 2,2-Diphenyl-1Picrylhydrazyl |
| GAE | Gallic acid equivalent |
| HDL | High-Density Lipoprotein |
| HPLC-DAD | High-Performance Liquid Chromatography/Diode Array Detector |
| $IC_{50}$ | Half Maximal Inhibitory Concentration |
| MET | Metabolic syndrome |
| PGO | Peroxidase/Glucose Oxidase |
| ROS | Reactive Oxygen Species |
| SD | Standard Deviation |
| TA | Total Anthocyanins |
| TAC | Total antioxidant capacity |
| TPC | Total phenolic content |

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PAPER

# EFFECT OF EXTRUSION ON THE TOTAL ANTIOXIDANT CAPACITY AND FREE PHENOLIC COMPOUNDS OF WHEAT BRAN BY RESPONSE SURFACE METHODOLOGY

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## ABSTRACT

There are some antioxidants in wheat bran provides health benefits. But the influence of extrusion pre-treatment on the antioxidant capacity and free phenolic compounds of wheat bran is not clear. Herein, it was investigated by response surface methodology (RSM). Within the experimental range, free phenolic compounds (FPC) increased gradually with feed moisture and extrusion temperature. And the total antioxidant capacity of extruded wheat bran increased gradually with rising feed moisture and screw speed. The optimized extrusion parameters were extrusion temperature at 86°C, feed moisture at 22% and screw speed at 160 rpm. The total FPA reached 3136.9 μg GAE $g^{-1}$ and the ferulic acid content was 93.4 $\mu g.g^{-1}$. Extrusion treatment for wheat bran significantly improved the antioxidant properties and increased the concentration of gallic acid and ferulic acid. The effect of extrusion temperature on total free phenol content is extremely significant.

*Keywords*: wheat bran, extrusion, response surface methodology, Trolox equivalent antioxidant capacity, free phenolic compounds

## 1. INTRODUCTION

Wheat bran accounts for approximately 14% of the whole wheat grain. It is consisted of multiple layers, including aleurone layer, the nucellar epidermis, the inner pericarp, and the outer pericarp (from inside to outside) (MATEO *et al*., 2012), (PANDEY and RIZVI, 2009; PERALES-SÁNCHEZ *et al*., 2014). Numerous literatures have been found to make a thorough inquiry species of the phenolic compounds exist in wheat, especially in wheat bran fraction (NEACSU *et al*., 2017; ROSICKA-KACZMAREK *et al*., 2018). In plants, phenolics compounds incorporate a wide variety of compounds including flavonoids, tannins, coumarins, and phenolic acids (HOSENEY, 2010). What's more, phenolics compounds including a benzene ring bearing one or lots of hydroxyl groups and phenolic acids that derivatives of either hydroxybenzoic or hydroxycinnamic acid are usually being all cereals (KIM *et al*., 2006; ZHENG *et al*., 2015). Generally, many beneficial compounds in cereals dedicate to their antioxidant characteristics, such as tocols, carotenoids, polyphenols, flavonoids, anthocyanins, lignans. The comprehensive antioxidant ability of all antioxidants in the cereals is generally represents as total antioxidant capacity (TAC) (RE *et al*., 1999; RICE-EVANS *et al*., 1999).

Phenolic acids in wheat bran usually can be divided into three type of existing forms: soluble free phenolic acids, conjugated phenolic acids, and insoluble-bound phenolic acids (ROSICKA-KACZMAREK *et al*., 2018). The free and conjugated phenolic acids make up only a small section, while most of the phenolic acids are bound phenolic compounds by ester and ether linkages with cell wall components such as arabinoxylans and lignin (LIU *et al*., 2016). The aleurone layer is mostly composed of arabinoxylan with a high content of ferulic acid (FA) monomers and low levels of FA dimers (RAMOS-ENRÍQUEZ *et al*., 2018a). In fact, different form of phenolic acid worked on various impacts on human health. When the dietary contain bound forms were intake by human, it would be useful in the precaution of colon cancer and other cancers (LEI *et al*., 2012; RAMOS-ENRÍQUEZ *et al*., 2018b). However, the intake of soluble free and conjugated forms is attributed to quickly absorption in the stomach and small intestine as well as distribution throughout the body.

Extrusion technology is a combination of mechanical shearing action, pressure action and thermal energy, which causes the material to be suddenly released from the high temperature and huge pressure state to the normal temperature and pressure and the internal structure and physical and chemical properties of the extruded material would be changed and extrusion technology also is a kind of processing methods to force materials at a predetermined feed rate, to flow through materials and through certain die holes to obtain products of different shapes and properties, and the food is called extruded food (AAM *et al*., 2017). As a high-tech in the field of food processing, extrusion technology opens -up to a new way of production that is simple, mechanized and highly automated for the development of convenience food. Besides, it has a high efficiency, and low cost in processing, as well as the products are easy to digest, keep the nutrient at a maximum degree, and is conducive to long-term storage.

Some evidence has been reported the activities of lipoxygenase and polyphenol oxidase were greatly reduced, and the shelf life was prolonged compared to the wheat bran, which has not been extruded (PILLI *et al*., 2010). What's more, the content of free phenolic compound in the product was obviously improved, and the antioxidant capacity was also enhanced. Therefore, it can be used as a excellent material for making whole wheat food or health-benefit products for improving the nutritional value (BRENNAN *et al*., 2011).

In our previous single factor study, it was found that the antioxidant ability and to phenolic content of extruded wheat bran were higher when the feed moisture was 22-24% and the screw speed was 130-190 rpm at the extrusion temperature of 80-100°C. To improve the antioxidant ability and shelf life of wheat bran, the optimum extrusion condition on the antioxidant capacity and free phenolic compounds of wheat bran was investigated by RSM. And the phenolic compounds extracted from wheat bran before and after extrusion were also identified by ultra-high performance liquid chromatography (UPLC).

## 2. MATERIALS AND METHODS

### 2.1. Materials and reagents

Wheat brans were obtained from Hubei Sanjie Agricultural Industrialization Co. Ltd (Hubei province, China); 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma–Aldrich. Other chemicals and solvents for chromatographic grade analysis were obtained from Merck (Darmstadt, Germany). All the other chemicals and solvents were of analytical grade.

### 2.2. Sample preparation

The wheat brans were milled and sieved to a 60 mesh size. The powder was put in to two-screw extruder (FMHE36-24, Hunan Fuma Branch Food Engineering Technology Co, Ltd) and then the extruded wheat brans were dried at 50°C for 18 hours to cut down the moisture content, and then extruded wheat brans were milled and sieved to a 60 mesh size. The powder of extruded wheat bran was kept in a black laboratory bottle, and the bottles were placed at -20°C in refrigerator.

### 2.3. Ultrasound extraction

0.5 g of the dried powder of extruded wheat brans were thoroughly mixed with 10 mL of 60% ethanol and placed in a 50 mL amber laboratory bottle (DHANANI *et al*., 2017). The operating extraction was last for 1.5 hours at 60°C. The supernatants were combined after centrifugation (3622×g, 20 min). After centrifugation the supernatant were evaporated to 2 mL at 45°C and placed in amber laboratory bottle at -20°C until used.

### 2.4. Determination of Trolox equivalent Antioxidant activity

ABTS assay was implemented the concordat of Pellegrini N (RE *et al*., 1999) with a little modifications by condition. $ABTS^{\cdot+}$ radical solution was prepared to mix 10 mL of ABTS stock solution (7mM ABTS in water) with 176 μL of potassium persulfate (140 mmol/L), which was kept in darkness 12-16 h at 4°C. The $ABTS^{\cdot+}$ reagent was diluted with anhydrous ethanol to detect the absorbance of 0.700 ±0.02 at 734 nm (Berg *et al*., 1999).
The 0.1 mL of the sample solution and 3.9 mL of diluted ABTS•+ were thoroughly mixed and placed in a 10 mL amber glass tube and shook at room temperature for 6 minutes. The absorbance of the reaction was measured at 734 nm ($A_{734} = A_s$) using glass cuvettes. Then 0.1 mL of sample and 3.9 mL of anhydrous ethanol or 3.9 mL of diluted $ABTS^{\cdot+}$ were operated as the above mention and the absorbance $A_{734}$ remarked as $A_t$ and $A_0$ respectively.

The calibration curve was set using Trolox at the consistence range of 50–1000μmol/L in ultrapure water.
The total antioxidant capacity of wheat bran extruded material was expressed as a TEAC per 1 g of dry matter of a sample (μmol/g).

$$\%\ \text{inhibition rate of ABTS} = \left(1 - \frac{(A_S - A_r)}{A_0}\right) \times 100\% \qquad (1)$$

**2.5. Determination of extruded wheat bran and raw wheat bran total free phenolic content**

The total free phenolic content was analyzed as determined following described previously (LI *et al*., 2008; VÁZQUEZ *et al*., 2015a) with some modification. Briefly, a stock solution of gallic acid with pipette gallic acid control solutions was prepared at a concentration of 0.5 mg/ml. With this solution, calibration curve was prepared for the different dilutions. In the dim light, 1 mL of the extract obtained from raw wheat bran and extruded wheat bran were placed in each well of a burette and 9 mL of deionized water, 1 mL of Folin-Ciocalteu reagent, and 2 mL of sodium carbonate solution (w/w=1/4) were added in orderly. All blanks except the extract was also prepared. Then these burettes were put in water bath at 50°C for 0.5 h. Secondly, 12 mL of deionized water was added in the burette and put at room temperature for 0.5 h. Subsequently, the burettes were read on a spectrophotometer at an absorbance of 745 nm (VÁZQUEZ *et al*., 2015a).
Above all results were represented as gallic acid equivalent (mg gallic acid/g of extruded wheat bran material GAE/g of dried sample).

**2.6. UPLC analysis**

UPLC-PDA was used to determine free phenolic compounds extracted from raw and extruded wheat bran. The chromatographic system was made up an Acquity UPLC (Waters, US) equipped with PDA. Samples were separated using a Waters Column (ACQUITY UPLC@HSS T3 1.8 μm, 2.1×150 mm Column). The column temperature was maintained at 40°C. Methanol-acetonitrile solution (1:1, v/v) and acetic acid (2.50%, v/v) were used as mobile phase A and B, respectively. The gradient program was as follows: 5-20% A (0-6 min), 20-40% A (6-15 min), 40-70% A (15-18 min), and 70-5% A (18-24 min). Phenolic acids were detected at 280 nm. The phenolic acid content was calculated from the peak area according to the calibration curve by using the external standard method and expressed as μg/g DW.

**2.7. Experimental design**

The effect of factors such as extrusion temperature (80°C, 90°C, 100°C), screw speed (130 rpm, 160 rpm, 190 rpm), and feed moisture (20%, 22%, 24%) on free phenolic compounds and Trolox equivalent antioxidant capacity (TEAC) were tested. A three-level-three-factor and seven central point factorial design were employed requiring a total of 19 experiments. The BBD was used to determine the optimal extrusion conditions that maximum of TEAC and FPC of extruded wheat bran.
The three independent variables of extrusion temperature (°C, $X_1$), feed moisture (%, $X_2$) and screw speed (rpm, $X_3$) at three levels (-1, 0, +1) were set. The coded and actual values of variables were shown in Table 1.

**Table 1.** Level of coded and real values for factorial design.

| Factors | Level | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| Extrusion temperature (°C) ($X_1$) | 80 | 90 | 100 |
| Feed moisture (%) ($X_2$) | 20 | 22 | 24 |
| Screw speed (rpm) ($X_3$) | 130 | 160 | 190 |

### 2.8. Statistical analysis

All experiments were carried out in triplicates and results were expressed as means ± standard deviation (n=3). ANOVA was carried out to determine any significant differences ($p < 0.05$). Response surface plots were generated using Design-Expert 6.0.

## 3. RESULTS

### 3.1. Effect of extrusion condition on TEAC content of extruded wheat bran

The mean values of TEAC content and the content of FPC each of the 19 treatments at the different extrusion conditions were shown in Table 2. The highest of TEAC content of 14.1143 μmol/g was obtained in experimental run number 7 with an extrusion temperature of 90°C, feed moisture of 22% and a screw speed of 160 rpm. While the lowest TEAC content of 12.7929 μmol/g was observed in experimental run number 16 with an extrusion temperature of 90°C, feed moisture of 22% and screw speed at 190 rpm.
In Tables 3 and 4, the estimated regression coefficients and ANOVA of TEAC of extruded wheat bran was observed. The quadratic regression model was extremely significant ($p=0.0004< 0.001$) and the lack of fit was not significant ($p=0.1609>0.05$) at the same time, showing that the model was in good agreement with the experimental data of TEAC content (BANNOUR *et al*., 2017; Berg *et al*., 1999; Zhen *et al*., 2016). The regression coefficient ($R^2 = 0.9264$) suggested the experimental and predicted content data had been a good fit in the experiment. The linear and quadratic of feed moisture showed a significant difference, indicating the effect on TEAC content. Moreover, the interactive variables between feed moisture and screw speed showed a significant difference ($p=0.0286< 0.05$), suggesting the effect on TEAC content. And then also the interactive variables between extrusion temperature and screw speed showed a significant difference ($p=0.0210< 0.05$), suggesting the effect on TEAC content.
In order to analyze the effect of interaction of the different variables, the response surface curves were plotted. Meanwhile, for the purpose of determining the optimal extrusion condition of the responses of the independent variables with maximized TEAC content of extruded wheat bran. The concentration of TEAC content of extruded wheat bran increased with increasing feed moisture and screw speed. However, when the feed moisture was added to nearly 22% and the screw speed added to 160 rpm, the TEAC content slowly dropped, as described in Fig. 1C. TEAC values also enhanced with the enhanced of extrusion temperature and feed moisture data as shown in Fig. 1A. Fig. 1C

revealed the increasing TEAC content with an increase of screw speed and extrusion temperature.

**Table 2.** Values for TEAC and FPC of extruded wheat bran extracts under different extrusion conditions.

| | Factorial design | | | Determination | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Total free phenolic content (mg GAE/g) | TEAC (μmol/g) |
| Run number | Extrusion temperature (°C) | Feed moisture (%) | Screw speed (rpm) | Experimental | Experimental |
| 1 | 90 | 24 | 130 | 3.04±0.06 | 13.03±0.01 |
| 2 | 100 | 22 | 130 | 2.83±0.05 | 13.23±0.01 |
| 3 | 90 | 22 | 160 | 3.14±0.04 | 13.80±0.01 |
| 4 | 90 | 20 | 130 | 2.94±0.01 | 13.03±0.05 |
| 5 | 90 | 24 | 190 | 2.78±0.06 | 13.55±0.02 |
| 6 | 80 | 24 | 160 | 3.04±0.02 | 13.56±0.02 |
| 7 | 90 | 22 | 160 | 3.18±0.02 | 14.11±0.01 |
| 8 | 80 | 22 | 130 | 3.14±0.03 | 13.49±0.01 |
| 9 | 90 | 22 | 160 | 3.17±0.03 | 13.80±0.01 |
| 10 | 100 | 22 | 190 | 2.99±0.01 | 13.64±0.03 |
| 11 | 90 | 22 | 160 | 3.15±0.03 | 13.88±0.04 |
| 12 | 90 | 22 | 160 | 3.21±0.04 | 13.84±0.01 |
| 13 | 100 | 20 | 160 | 2.88±0.04 | 13.08±0.01 |
| 14 | 90 | 22 | 160 | 3.21±0.01 | 13.91±0.07 |
| 15 | 80 | 20 | 160 | 3.07±0.01 | 13.23±0.01 |
| 16 | 90 | 20 | 190 | 2.92±0.01 | 12.79±0.01 |
| 17 | 100 | 24 | 160 | 2.90±0.01 | 13.21±0.03 |
| 18 | 80 | 22 | 190 | 2.92±0.02 | 13.08±0.05 |
| 19 | 90 | 22 | 160 | 3.13±0.05 | 13.76±0.02 |

**Table 3.** ANOVA.

| Source | TEAC ($R^2$=0.9264) | | | | | Total FPC ($R^2$=0.9579) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SS | DF | MS | F-value | p-value | SS | DF | MS | F-value | p-value |
| Model | 2.40 | 9 | 0.27 | 12.58 | 0.0004 | 0.00 | 9 | 0.00 | 22.74 | < 0.0001 |
| Lack of fit | 0.11 | 3 | 0.04 | 2.45 | 0.1609 | 0 | 3 | 0.00 | 2.77 | 0.1329 |
| Pure error | 0.09 | 6 | 0.01 | | | 0 | 6 | 0.00 | | |

**Table 4.** ANOVA for Quadratic model.
Response 1: TEAC of extruded wheat bran.

| Source | SS | df | MS | F-value | p-value | |
|---|---|---|---|---|---|---|
| Model | 2.40 | 9 | 0.2662 | 12.58 | 0.0004 | significant |
| A-extrusion temperature | 0.0051 | 1 | 0.0051 | 0.2407 | 0.6354 | |
| B-feed moisture | 0.1834 | 1 | 0.1834 | 8.67 | 0.0164 | |
| C-screw speed | 0.0108 | 1 | 0.0108 | 0.5093 | 0.4935 | |
| AB | 0.0107 | 1 | 0.0107 | 0.5035 | 0.4959 | |
| AC | 0.1649 | 1 | 0.1649 | 7.79 | 0.0210 | |
| BC | 0.1433 | 1 | 0.1433 | 6.77 | 0.0286 | |
| $A^2$ | 0.1277 | 1 | 0.1277 | 6.04 | 0.0364 | |
| $B^2$ | 0.8298 | 1 | 0.8298 | 39.21 | 0.0001 | |
| $C^2$ | 0.5207 | 1 | 0.5207 | 24.60 | 0.0008 | |
| Residual | 0.1905 | 9 | 0.0212 | | | |
| Lack of Fit | 0.1050 | 3 | 0.0350 | 2.45 | 0.1609 | not significant |
| Pure Error | 0.0855 | 6 | 0.0143 | | | |
| Cor Total | 2.59 | 18 | | | | |

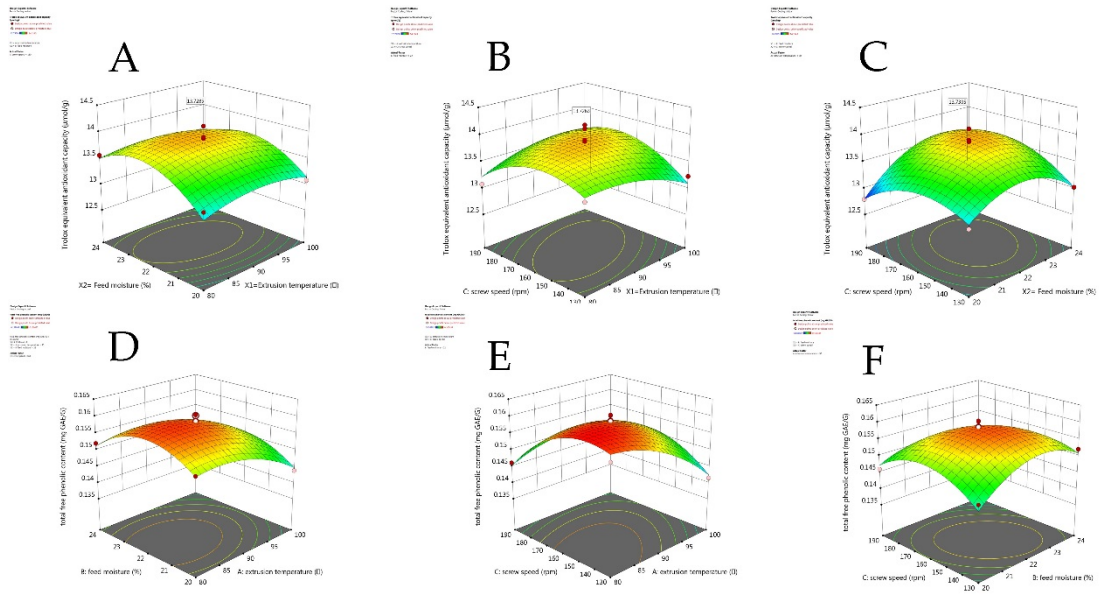


**Figure 1.** Effect of different extrusion conditions on TEAC and FPC : (A) TEAC effect of extrusion temperature and feed moisture; (B) TEAC effect of extrusion temperature and screw speed; (C) TEAC effect of feed moisture and screw speed; (D) FPC contents effect of extrusion temperature and feed moisture; (E) FPC contents effect of extrusion temperature and screw speed for extrusion; (F) FPC contents effect of screw speed and feed moisture for extrusion.

### 3.2. Effect of extrusion condition on FPC contents of extruded wheat bran

Table 5 shown that FPC contents of extruded wheat bran quadratic regression model was extremely significant ($p< 0.0001$). What's more, the lack of fit had a p-value higher ($p=0.1392>0.05$). In Table 3, the total FPC value of $R^2 = 0.9579$ demonstrated the model to be a well fit for the experimental data of total FPC of extruded wheat bran.

Individual independent variables extrusion temperature had an extremely significant effect on the total FPC content which was indicated by the linear data model ($p=0.0006<0.01$), and screw speed had major impact effect on the total phenolic content ($p=0.0133<0.05$), but feed moisture didn't show significant effect on the total phenolic content. The interaction between extrusion temperature and feed moisture showed a not significant effect on the total FPC content ($p=0.5254> 0.05$), and interaction between feed moisture and screw speed also showed a significant effect on the total phenolic content ($p=0.0136<0.05$). Meanwhile, the interplay between extrusion temperature and screw speed also expressed an extremely significant effect on the total FPC content ($p=0.001< 0.01$).

When the extruded temperature was over 100°C, the extruded wheat bran's antioxidant capacity and total free phenolic contents might decrease, which it may be that the phenolic compounds might be degraded. The results showed that the effect of extrusion temperature on total free phenol content is extremely significant ($p=0.0006<0.01$). Meanwhile, screw speed also indicated an obviously effect on the FPC content ($p=0.0133<0.05$).The interaction of the three independent variables of extrusion temperature, feed moisture, and screw speed was used to plot the response surface curves for the total phenolic content as shown in Fig. 1D, Fig. 1E, Fig. 1F. Increasing the extrusion

temperature from 80°C to 100°C at constant feed moisture (Fig. 1D) and screw speed (Fig. 1E) did change total phenolic content. Although, with the extrusion temperature, feed moisture and screw speed increasing, the total FPC contents of extruded wheat bran, as shown in Fig. 1D, Fig. 1E, Fig. 1F. But, when extrusion temperature and screw speed at nearly 100°C and 190 rpm, respectively, a small cut down in total phenolic content was observed (Fig. 1E). This expressed that a portion of phenolic compounds would be degraded by over high extrusion temperature and screw speed in extrusion progress.

**Table 5.** ANOVA for Quadratic model.
Response 2: FPC of extruded wheat bran.

| Source | Sum of Squares | df | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| Model | 0.0008 | 9 | 0.0001 | 22.74 | < 0.0001 | significant |
| A-extrusion temperature | 0.0001 | 1 | 0.0001 | 26.32 | 0.0006 | |
| B-feed moisture | 5.234E-07 | 1 | 5.234E-07 | 0.1360 | 0.7208 | |
| C-screw speed | 0.0000 | 1 | 0.0000 | 9.45 | 0.0133 | |
| AB | 1.680E-06 | 1 | 1.680E-06 | 0.4365 | 0.5254 | |
| AC | 0.0001 | 1 | 0.0001 | 23.14 | 0.0010 | |
| BC | 0.0000 | 1 | 0.0000 | 9.36 | 0.0136 | |
| $A^2$ | 0.0001 | 1 | 0.0001 | 15.42 | 0.0035 | |
| $B^2$ | 0.0002 | 1 | 0.0002 | 44.71 | < 0.0001 | |
| $C^2$ | 0.0002 | 1 | 0.0002 | 44.71 | < 0.0001 | |
| Residual | 0.0000 | 9 | 3.848E-06 | | | |
| Lack of Fit | 0.0000 | 3 | 6.709E-06 | 2.77 | 0.1329 | not significant |
| Pure Error | 0.0000 | 6 | 2.418E-06 | | | |
| Cor Total | 0.0008 | 18 | | | | |

### 3.3. Verification of predictive optimal extrusion conditions

The predicted extrusion conditions of wheat bran extruded material at 85.85°C of extrusion temperature, 22.19% of feed moisture and 154 rpm of screw speed provided the maximum TEAC content of extruded wheat bran, and the maximum total phenolic content was reached at the predicted conditions of 85.85°C of extrusion temperature, 22.19% of feed moisture and 154 rpm of screw speed. The predicted extruded conditions of wheat bran were the same for the TEAC content and total phenolic content for convenient operation, and considering the experiment in practice the optimal extraction parameters were adjusted to be 86°C of extrusion temperature, 22% of feed moisture and 160 rpm of screw speed at which that the predicted TEAC content was 13.8472 μmol $g^{-1}$

and total phenolic content was 13.19754 mg GAE $g^{-1}$ DW. This strongly suggests that the model is suitable to predict TEAC content, total phenolic content using extrusion at selected conditions.

### 3.4 Identification and quantification phenolic compounds of extruded wheat bran and raw wheat bran by UPLC

Table 6 showed results about the concentration of each phenolic compound obtained for the extruded wheat bran and raw wheat bran hydroalcoholic extracts. Two kinds of phenolic compounds (11.39 μg.$g^{-1}$ gallic acid and 78.40 μg.$g^{-1}$ ferulic acid) were identified in raw wheat bran and 4 kinds of phenolic compounds (12.58 μg.$g^{-1}$ gallic acid, 61.06μg.$g^{-1}$ caffeic acid, 93.40 μg.$g^{-1}$ ferulic acid, and 183.64μg.$g^{-1}$ rutin) in the extruded wheat bran. The compounds caffeic acid and rutin were not identified in the raw wheat bran. So those two phenolic compounds became the significant differences between the raw wheat bran and extruded wheat bran. Meanwhile, the raw wheat bran's TEAC was 12.38±0.22 μmol.$g^{-1}$ and extruded wheat bran's TEAC was is 13.91±0.04 μmol.$g^{-1}$. And then the raw wheat bran's total free phenolic compounds was 2.88±0.09 mg GAE.$g^{-1}$, while extruded wheat bran's was 3.14±0.07 mg GAE.$g^{-1}$ the extruded wheat bran. Above all, the reason why that extruded wheat bran antioxidant capacity and free phenolic compounds significantly higher than the raw wheat bran could be explained by these dates.

**Table 6.** Characterization of extruded wheat bran extracts.

| Determination | This study | |
|---|---|---|
| | The raw wheat bran | The extruded wheat bran |
| Total free phenolic compounds (mg GAE.$g^{-1}$) | $2.88\pm0.09^{Aa}$ | $3.14\pm0.07^{Bb}$ |
| TEAC (μmol. $g^{-1}$) | $12.38\pm0.22^{Aa}$ | $13.91\pm0.04^{Bb}$ |
| Ferulic acid (μg.$g^{-1}$) | $78.40\pm0.001^{Aa}$ | $93.40\pm0.000^{Bb}$ |
| Gallic acid (μg.$g^{-1}$) | $11.39\pm0.002^{Aa}$ | $12.58\pm0.001^{Bb}$ |
| Caffeic acid (μg.$g^{-1}$) | 0 | 61.06±0.001 |
| Rutin (μg.$g^{-1}$) | 0 | 183.64±0.001 |

The content of gallic acid (12.58 μg $g^{-1}$ extruded wheat bran), ferulic acid (93.4 μg $g^{-1}$ extruded wheat bran) in extruded wheat bran were higher than the raw wheat bran. The extrusion technology helped the wheat bran to break the cell structure of wheat bran and then the phenolic compounds were released by high temperature, strong pressure, and great powerful shear force.
Phenolic compounds have one or more hydroxyl groups conjugated to an aromatic hydrocarbon group, which characterizes the phenolic structure (CHAIYASUT *et al*., 2017; GUTIÉRREZ-GRIJALVA *et al*., 2017; HILBIG *et al*., 2018). The phenolic compounds specially structure bring about these compounds antioxidant activity to a certain degree, which may be higher or lower depending on the position and number of hydroxyls (APEABAH *et al*., 2017; VÁZQUEZ *et al*., 2015b). The presence of several phenolic compounds in extruded wheat bran extracts might explicate the antioxidant activity demonstrated for the extruded wheat bran.

## 4. CONCLUSION

In the present work, BBD was successfully carried out to set the extrusion conditions optimized parameters for the antioxidant capacity and free phenolic compounds of extruded wheat bran. In comparison to raw wheat bran, the extruded wheat bran resulted in the higher recoveries of both total free phenolic compounds and Trolox equivalent antioxidant capacity, which showed that extrusion could enhance the antioxidant capacity and free phenolic compounds of wheat bran.
Moreover, both the raw wheat bran and extruded wheat bran, yielded the same phenolic compounds, namely gallic acid, ferulic acid caffeic acid and rutin, were determined by UPLC. The study suggested that the use of extrusion pre-treatment in enhancing contents the of desired bioactive components from food industry by-product was a numerous potential extraction technology.From the data of the response surface, the extruded technology is efficient, economic and environmental process technology. Because the antioxidant capacity and free phenolic compounds were significantly improved by extrusion treatment. Thus, the extruded wheat brans would be a good source of natural antioxidants.

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## ABBREVIATIONS

| | |
|---|---|
| RSM | response surface methodology |
| FPC | free phenolic compounds |
| GAE | gallic acid equivalent |
| FA | ferulic acid |
| LDL | low-density lipoprotein |
| PDA | photo-diode array |
| UPLC | ultra performance liquid chromatography |
| TEAC | Trolox equivalent antioxidant capacity |
| BBD | Box-Behnken design |

DW dry weight
ANOVA analysis of variance
ABTS 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
SS sum of squares
DF degree of freedom
MS mean square

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PAPER

# MICROBIOLOGICAL QUALITY AND ANTIMICROBIAL EFFICACY OF COMBINED OREGANO ESSENTIAL OIL AND ACETIC ACID ON FRESH LETTUCE

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## ABSTRACT

This study determined the microbiological quality of lettuce purchased at Durban markets, and evaluated the antimicrobial effects of oregano essential oil (OEO), acetic acid (AA) and combination (OEO+AA)] on the survival of *Escherichia coli* and *L. monocytogenes* on lettuce for 6 days. Aerobic and anaerobic spore formers, *Staphylococcus aureus*, *Escherichia coli* and *L. monocytogenes* were microscopically and phenotypically identified from the lettuce Decontamination was higher and significantly different ($p>0.05$) at 5ºC with combined 0.3% AA+0.1% EOE, and complete inhibition of pathogens was observed day 2. This formulation can increase antimicrobial efficacy and balance sensory attributes of treated lettuce.

*Keywords*: acetic acid, combined treatments, decontamination, oregano essential oil

## 1. INTRODUCTION

There has been a huge increase in the consumption of fresh, minimally processed fruits and vegetables in the last decade. This is due to the minimal labor that is required to prepare these food items and are a great source of a variety of vitamins, minerals and other phytochemicals which are beneficial to health (RAMOS *et al.*, 2013). The increase of consumption of minimally processed ready- to- eat vegetables such as lettuce has however led to an increase in the number of reported cases of foodborne outbreaks linked to the consumption of contaminated vegetables (MURRAY *et al.*, 2017). However, information on outbreaks or presence of pathogens in or on fresh produce leading to foodborne outbreaks in South Africa is scarce due to the absence of an efficient reporting system (JORDAAN 2013). Most prevalent pathogenic microorganisms reported in contaminated vegetables include bacteria such as *E. coli* O157:H7, *Listeria monocytogenes* and some *Salmonella* species. These are able to survive under adverse environmental conditions and form biofilms (CALLEJÓN *et al.*, 2015).
Decontamination methods used for vegetables in fresh produce industry aim to decrease the microbial populations without necessarily eliminating them (de MEDEIROS BARBOSA *et al.*, 2016) Consumers are aware of the limitations of disinfectants used in fresh-cut produce in terms of taste and freshness (PONCE *et al.*, 2004). Oregano essential oil (OEO) and acetic acid (AA) have proven to be effective against food borne pathogens such *as Escherichia coli O157:H7, Camphylobacter jejuni, Salmonella enterica*, and *Listeria monocytogenes* (RAEISI *et al.*, 2015). However, the use of these antimicrobials individually requires that food be exposed to large doses for effective inhibition of pathogens. EOE and AA have very strong odors and could impair sensory qualities at high concentrations, which is generally not accepted by customers. Therefore, the combination of preservatives serves a promising method to be able to achieve optimum pathogen inhibition without affecting the quality of food (MIYAGUE *et al.*, 2015).
The combination of technologies with antimicrobial/preservative effects has been used in the food industry to maintain food quality and ascertain that pathogens can be eradicated or controlled (NAZER *et al.*, 2005). Hence, the objective of this study is to evaluate the effectiveness of individual and combined oregano essential oil and acetic acid on inoculated iceberg lettuce.

## 2. MATERIALS AND METHODS

### 2.1. Collection of samples

A total of 60 samples of lettuce and spinach (30 samples each) were purchased from two different retail markets; the open market and fresh retail market (local supermarket). Samples were collected in sterile LDPE zip lock bags and stored at 4 ºC until testing. The test pathogens (*E. coli* O157:H7 ATCC 4388 and *L. monocytogenes* ATCC 7644) were collected from the Department of Biotechnology and Food Technology, Durban University of Technology, South Africa. EOE and AA were purchased Nautica Organic's in Durban, South Africa.

## 2.2. Experimental design

For the microbiological quality of leafy vegetables, 60 samples (30 lettuce and 30 spinach samples each purchased equally from retail and open markets) were evaluated for the presence of aerobic and anaerobic spore formers, *Staphylococcus aureus*, *E. coli* and *L. monocytogenes*. The assay with oregano essential oil and acetic acid followed a 3x3x2x2 factorial combination. The effect of four factors: type of treatments (oregano EO, acetic acid, oregano EO+ acetic acid); level of concentration (0.05%, 0.1%, 0.3%), contact times (2 min, 5 min) and storage temperatures (5ºC and 20ºC) were evaluated for a duration of 6 days by bioassay.

## 2.3. Microbiological analysis of the samples

All experiments were carried out in duplicates.
The microbiological testing to isolate and identify aerobic spore formers, anaerobic spore formers, *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* on the leafy vegetables was carried out according to International Standard Organization protocols as described by IJABADENIYI *et al.* (2011).

### 2.3.1. Bacterial inoculum preparation

A 24 h old culture of *L. monocytogenes* ATCC 7644 and *E. coli* O157:H7 ATCC 4388 were aseptically transferred into 10 ml Brain Heart Infusion broth and Tryptic soy broth respectively. The broths were incubated at 37ºC for 24 h and washed by centrifugation (4629x*g* for 15 min) at 4ºC. Serial dilutions of the washed inocula were performed to obtain the desired dilution using absorbance at 600 nm.

### 2.3.2 Assay with Essential oil and organic acid treatment suspension preparation

Suspensions were made by dispersing the treatments into sterile distilled water according to AKBAS and OLMEZ (2007). The combination treatment was made by mixing the most effective concentrations. Fresh lettuce samples that had negative results for the presence of *E. coli* O157:H7 and *L. monocytogenes* ATCC 7644 were selected for simulative study. Lettuce leaves were washed with cold sterile deionized water at 21°C for 2 min. Thereafter the leaves were left to dry under a safety hood bio-cabinet and cut into appropriate sizes. Leaves were artificially contaminated according to SAMARA *et al.* (2009). Ten grams of the treated samples were immersed into 200 ml of each treatment solution for 2 min and 5 min each, with gentle agitation at room temperature and different concentrations (acetic acid- 0.1 and 0.3%; oregano EO- 0.05 and 0.1%). Thereafter, the lettuce was removed from solutions and then placed in 10g samples into polyethylene bags and stored at 5ºC and 22ºC for 6 days. Samples were taken for enumeration every 2 days' interval for a period of 6 days.

## 2.4. Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and t-test using statistical analysis system to determine the significant difference in treatment methods. Colony counts were converted into logarithmic values (CFU/g), means and standard deviations were calculated and significance was expressed at $P \leq 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Microbiological quality analysis of lettuce and spinach

The microbiological quality of leafy vegetables is of great concern as most are usually consumed raw with minimal processing during production. Consumer demands for microbiologically safe, fresh vegetables with no chemical preservation have enhanced the need for routine quality analysis of these commodities (CARELLA, 2014). As observed, the total mean log CFU/g of lettuce in open markets (OM) were higher and significantly different ($p<0.05$) than retail markets (RM) while there was no significant difference in spinach samples. All lettuce and spinach samples were positive for *S. aureus*, aerobic and anaerobic spore formers (Table 1).

**Table 1.** Microbiological quality of spinach and lettuce collected at different retail markets.

| Type of leafy vegetable | Microorganisms | Retail Market (Supermarket) | Open Market |
|---|---|---|---|
| Lettuce | TPC | $5.06\pm0.81^{c}$ | $6.02\pm0.54^{b}$ |
| | *S. aureus* | $0.37\pm0.99^{h}$ | $3.28\pm0.50^{e}$ |
| | ASF | $1.72\pm0.95^{g}$ | $3.70\pm0.21^{e}$ |
| | AASF | $1.46\pm0.71^{g}$ | $2.90\pm0.73^{f}$ |
| Spinach | TPC | $7.38\pm0.08^{a}$ | $7.76\pm0.39^{a}$ |
| | *S. aureus* | $4.57\pm0.19^{d}$ | $4.91\pm0.17^{d}$ |
| | ASF | $2.33\pm0.28^{f}$ | $2.61\pm0.28^{f}$ |
| | AASF | $1.94\pm0.29^{g}$ | $1.83\pm0.55^{g}$ |

Results represented as means±Standard deviation. Means with same superscript letters in the same row are not significantly different ($p > 0.05$). n= 30 (lettuce), n=30 (Spinach). TPC –Total Plate Count, ASF- Aerobic Spore Formers, AASF- Anaerobic spore formers.

Total plate counts had higher values in both samples and markets (Lettuce: RM-5.06 log CFU/g; OM- 6.02 log CFU/g; Spinach:RM-7.38 log CFU/g; OM- 7.76 log CFU/g) while *S. aureus* had the least in lettuce (RM-0.37 log CFU/g; OM- 3.28 log CFU/g) and anaerobic spore formers had the least in spinach (RM-1.94 log CFU/g; OM- 1.83 log CFU/g). The high levels of aerobic bacteria found on lettuce and spinach could be due to the large surface area, which allows for easy and fastidious attachment of microorganisms (KORIR *et al.*, 2016). The difference in *S. aureus* in the lettuce in both markets could be due to the different environmental conditions handling and cross contamination. Improper handling, abuse of temperature, unhygienic practices, un-sanitized contact surfaces that products are exposed to in the open market serve as good sources for the contamination of fresh produce (WIEDERODER *et al.*, 2012). Furthermore, reports have shown that the quality of water used for irrigation during growing seasons, age of leaves and water used for cleaning the leaves before display influences the incidence of bacteria in final produce (MERLINI *et al.*, 2018). The presence of spore formers may suggest pathogenic bacteria, which may exhibit strong resistance towards chemical and physical sanitizers. Contamination of fresh produce by these microorganisms can lead to serious diseases and harm to human health. Similar results have been reported by KORIR *et al.* (2016) with

bacterial counts of 8.02 and 7.49 log CFU/g for spinach and lettuce, respectively. (PINGULKAR, 2001) also reported aerobic bacterial growth range of 4.3 to 8.9 log CFU/g for fresh-cut vegetables. According to USDA regulations, *S. aureus* observed in open market samples are at unacceptable levels.

As observed earlier, the incidence of other pathogens was higher in samples from OM than RM. Out of the 60 samples, *Salmonella* spp. was detected on 43 - [RM: Lettuce 6(10.00%), Spinach 11(18.33%); OM: Lettuce 14(23.33%), Spinach 12(20.00%)], *E. coli* was detected on 15 (25.00%)- [RM: Lettuce 0(0.00%), Spinach 2(3.33%); OM: Lettuce 5(8.33%), Spinach 8(13.33%)], while *L. monocytogenes* was detected on 42 (70.00%) [RM: Lettuce 4(6.67%), Spinach 10 (16.67%); OM: Lettuce 12(20.00%), Spinach 16(26.67%)]. *E. coli*, *Salmonella* and *Listeria* all have mechanisms for adherence onto surfaces and all adhere differently onto leaf surfaces (TOPALIĆ-TRIVUNOVIĆ *et al.*, 2014). Similarly, KORIR *et al.* (2016) in their analysis 144 fresh produce samples from retail stores, only four samples were positive for pathogens. *E. coli, Listeria* and *Salmonella* are pathogenic microorganisms that are prominently associated with the diseases/infections caused by the consumption of poor quality contaminated leafy vegetables (SINGH *et al.*, 2002). Environmental sources such as water, soil, air, insects, animals and human activity can cause contamination of leafy vegetables by *L. monocytogenes* (MERLINI *et al.*, 2018). The South African guidelines stipulate that these pathogens should not be present in ready-to-eat foods (DEPARTMENT OF HEALTH, 2002; BEHARIELAL *et al.*, 2018), therefore, this could represent a public health threat. The low rate of detection of pathogens in samples purchased at retail could be proper handling and storage of the samples. Storage temperature and storage period of fresh produce can influence the growth of bacteria (KORIR *et al.*, 2016).

### 3.2. Antimicrobial effects of AA and OEO on *E. coli* O157:H7 ATCC 4388 at 5 and 22°C

Generally, storage at 5ºC was more effective than 22 ºC and EOE showed higher log reductions at both storage temperatures than AA (Table 2). However, there was no significant difference in log-reductions with an increase in exposure time from 2 to 5 min. Furthermore, there was a complete inhibition of *E. coli* with 0.1% EOE at day 4. Furthermore, the log-reduction increased with increase in storage days. Similar result was observed by (POIMENIDOU *et al.*, 2016) who reported a 2.0-2.4 log CFU/g reduction of *E. coli* O157:H7 on lettuce samples rinsed with acetic acid.

### 3.3. Antimicrobial effects of AA and OEO on *L. monocytogenes* ATCC 7644 at 5 and 22°C

Table 3 shows the antimicrobial effects of AA and OEO on *L. monocytogenes*. Similar to the results observed for *E. coli* reductions, Storage at 5ºC was seen to be more effective as compared to storage at 22ºC and a 2 min dip treatment at 0.1% AA yielded 1.54 log CFU/g but an increase in exposure time to 5 min was not significant (1.63 log CFU/g). However, an increase in treatment concentration showed 1.96 log CFU/g reduction at 2 min while 2.17 log CFU/g) was observed at 5 min. (Table 4). In addition, there was a progressive log-reduction as storage days increased. Contrary to the behavior of *E. coli*, *L. monocytogenes* showed higher resistance towards treatment with acetic acid, particularly on samples stored at 22ºC. The difference of the pathogens susceptibility could be due to inherent properties of each organism, differences in outer layer (Gram positive and negative) and nature of attachment of the pathogen to the lettuce leaf tissues. Organic acids generally

have a low pH that prevents or inhibit bacterial growth. CARELLA (2014) reported that Gram negative bacteria are more susceptible to low pH treatments while essential oils more effective against Gram positive bacteria. Also, microbial inhibition depends on the concentration of treatment, contact time, mode of application and storage temperature. The limiting factors of effectiveness can be due to the total number and type of microorganisms they are introduced to and how these microbes interact with the acid (SAMARA and KOUTSOUMANIS 2009).
Organic acids have been successfully used for the preservation of fresh fruits and vegetables during pre-harvest and post-harvest operations in the fresh produce industry (HIRSHFIELD *et al.*, 2003). They are generally regarded as safe (GRAS) for use in food production and preservation. Antimicrobial activity of organic acids (lactic, citric, acetic, and ascorbic acid) against *E. coli* and *L. monocytogenes* was compared on iceberg lettuce and the combination effect of lactic and acetic acid with chlorine to reduce *L. monocytogenes* on shredded lettuce has been evaluated (PARK *et al.*, 2011). The individual efficacy of antimicrobials against *E.coli* O157:H7 and *Listeria monocytogenes* has been reported in numerous studies (AKBAS and OLMEZ 2007; SAMARA and KOUTSOUMANIS 2009; HUANG and CHEN 2011; SOLGI and GHORBANPOUR 2014; de MEDEIROS BARBOSA *et al.*, 2016) but higher doses have very strong odours that may negatively affect sensory qualities of food (MIYAGUE *et al.*, 2015), hence, the combination of preservatives serves a promising method to be able to achieve optimum pathogen inhibition without affecting the quality of food (NAZER *et al.*, 2005; MIYAGUE *et al.*, 2015).

### 3.4. synergistic Effect of OEO and AA on *E. coli* O157:H7 ATCC 4388 and *L. monocytogenes* ATCC 7644

Table 4 shows the antimicrobial effects of artificially contaminated lettuce in a combined solution of oregano EO and acetic acid (0.1% oregano essential oil + 0.3% acetic acid). At 2 min dip treatment, reductions of 4.86 log CFU/g and 4.95 log CFU/g were observed in *E. coli* O157:H7 and *L. monocytogenes* ATCC 7644 respectively. At 5 min dip treatment the reductions were not significantly different in *E. coli* and *L. monocytogenes* (5.04 log CFU/g, 5.12 log CFU/g). Generally, higher log-reduction was observed at 5 ºC than 22 ºC. However, pathogens were not detected on day 4 and 6 storage period. The combined treatment reduced and inhibited the growth of both pathogens. At day 2, storage at temperatures 5 and 22 ºC (Table 4), resulted in further reduction of *E. coli* O157:H7 and *L. monocytogenes*.
The use of AA and OEO lead to a significant reduction of *E. coli* O157:H7 and *L. monocytogenes* on lettuce when used individually, more so, the combination of AA and OEO resulted in a further significant reduction of *E. coli* and O157:H7 *L. monocytogenes*. Combined treatment with OEO and AA completely inhibited the growth of *E. coli* O157:H7 and *L. monocytogenes* at day 4.
The combination of technologies with antimicrobial/preservative effects is called 'hurdle technology' and it has been used in the food industry to maintain food safety (NAZER *et al.*, 2005). Some studies have reported that the use of essential oils in combination with each other as well as combination with other natural antimicrobials results in enhanced antimicrobial effectiveness as opposed to being used individually (DIMITRIJEVIĆ *et al.*, 2007).

**Table 2.** Comparative antimicrobial effect of OEO and AA on inactivation of *E. coli* O157:H7 on iceberg lettuce (log CFU/g) over a period of 6 days at 5ºC and 22°C.

| Type of treatment | Day 0 | | Day 2 | | Day 4 | | Day 6 | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 5 min | 2 min | 5 min | 2min | 5min | 2 min | 5 min |
| **Treatment at 5 ºC** | | | | | | | | |
| Control | 8.13±0.021$^{a}$ | | 7.27±0.044$^{b}$ | | 7.04±0.024$^{b}$ | | 7.25±0.023$^{b}$ | |
| AA @ 0.1% | 5.39±0.031$^{b}$ | 5.20±0.008$^{b}$ | 5.11±0.028$^{c}$ | 5.01±0.021$^{c}$ | 4.36±0.015$^{d}$ | 4.07±0.031$^{d}$ | 3.98±0.025$^{e}$ | 3.78±0.067$^{e}$ |
| AA @ 0.3% | 5.07±0.018$^{b}$ | 5.02±0.020$^{b}$ | 4.42±0.012$^{d}$ | 4.19±0.014$^{d}$ | 3.98±0.019$^{e}$ | 3.71±0.030$^{e}$ | 3.64±0.048$^{e}$ | 3.40±0.084$^{e}$ |
| OEO @ 0.05% | 5.97±0.036$^{b}$ | 5.37±0.012$^{b}$ | 5.02±0.015$^{c}$ | 4.37±0.012$^{d}$ | 4.82±0.023$^{d}$ | 4.07±0.018$^{d}$ | 4.33±0.005$^{d}$ | 3.83±0.022 |
| OEO @ 0.1% | 3.50±0.057$^{c}$ | 3.22±0.056$^{c}$ | 3.15±0.044$^{e}$ | 2.98±0.082$^{e}$ | ND | ND | ND | ND |
| **Treatment at 22 ºC** | | | | | | | | |
| Control | 8.13±0.020$^{a}$ | | 8.26±0.013$^{a}$ | | 8.46±0.012$^{a}$ | | 8.91±0.041$^{a}$ | |
| AA @ 0.1% | 5.39±0.031$^{b}$ | 5.20±0.008$^{b}$ | 5.46±0.015$^{c}$ | 5.41±0.015$^{c}$ | 5.60±0.046$^{c}$ | 5.51±0.028$^{c}$ | 5.63±0.052$^{c}$ | 5.47±0.021$^{c}$ |
| AA @ 0.3% | 5.07±0.018$^{b}$ | 5.02±0.020$^{b}$ | 5.04±0024$^{c}$ | 4.77±0.047$^{d}$ | 4.81±0.03$^{d}$ | 4.50±0.057$^{d}$ | 4.95±0.041$^{d}$ | 4.63±0.057$^{d}$ |
| OEO @ 0.05% | 5.97±0.036$^{b}$ | 5.37±0.012$^{b}$ | 5.45±0.013$^{c}$ | 5.09±0.021$^{c}$ | 5.39±0.01$^{c}$ | 5.06±0.019$^{c}$ | 5.47±0.009$^{c}$ | 5.23±0.013$^{c}$ |
| OEO @ 0.1% | 3.50±0.057$^{c}$ | 3.22±0.056$^{c}$ | 3.30±0.031$^{e}$ | 3.09±0.025$^{e}$ | ND | ND | ND | ND |

Means with same superscript letters in the same row are not significantly different ($p > 0.05$).
Results represented as means±Standard deviation; Means (n= 2); ND- Not Detected, OEO- Oregano Essential Oil, AA- Acetic Acid.

**Table 3.** Comparative antimicrobial effect of OEO and AA on inactivation of *L. monocytogenes* on iceberg lettuce (log CFU/g) over a period of 6 days at 5ºC and 22ºC.

| Treatment | Day 0 | | Day 2 | | Day 4 | | Day 6 | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 5 min | 2 min | 5 min | 2min | 5min | 2 min | 5 min |
| **Treatment at 5 ºC** | | | | | | | | |
| Control | 8.01±0.033$^{a}$ | | 7.24±0.021$^{b}$ | | 6.93±0.05$^{b}$ | | 7.07±0.018$^{b}$ | |
| AA @ 0.1% | 6.47±0.013$^{b}$ | 6.38±0.027$^{b}$ | 6.22±0.022$^{c}$ | 6.13±0.014$^{c}$ | 6.06±0.037$^{b}$ | 6.02±0.029$^{b}$ | 6.33±0.011$^{b}$ | 6.26±0.023$^{b}$ |
| AA @ 0.3% | 6.05±0.008$^{b}$ | 5.84±0.031$^{c}$ | 5.89±0.016$^{d}$ | 5.76±0.022$^{d}$ | 5.31±0.006$^{c}$ | 5.19±0.01$^{c}$ | 5.21±0.021$^{c}$ | 4.99±0.031$^{c}$ |
| OEO @ 0.05% | 3.63±0.022$^{d}$ | 3.25±0.034$^{d}$ | 2.84±0.083 | ND | ND | ND | ND | ND |
| OEO @ 0.1% | 3.06±0.027$^{d}$ | 2.69±0.124$^{e}$ | ND | ND | ND | ND | ND | ND |
| **Treatment at 22 ºC** | | | | | | | | |
| Control | 8.01±0.033$^{a}$ | | 8.349±0.021$^{a}$ | | 8.46±0.014$^{a}$ | | 9.23±0.032$^{a}$ | |
| AA @ 0.1% | 6.47±0.013$^{b}$ | 6.38±0.027$^{b}$ | 6.41±0.015$^{c}$ | 6.35±0.011$^{c}$ | 6.45±0.016$^{b}$ | 6.31±0.019$^{b}$ | 6.45±0.029$^{b}$ | 6.37±0.034$^{b}$ |
| AA @ 0.3% | 6.05±0.008$^{b}$ | 5.84±0.031$^{c}$ | 6.37±0.016$^{c}$ | 6.25±0.008$^{c}$ | 6.33±0.009$^{b}$ | 6.16±0.015$^{b}$ | 6.46±0.011$^{b}$ | 6.26±0.016$^{b}$ |
| OEO @ 0.05% | 3.63±0.022$^{d}$ | 3.25±0.034$^{d}$ | 3.08±0.051$^{d}$ | 2.59±0.15$^{d}$ | ND | ND | ND | ND |
| OEO @ 0.1% | 3.06±0.027$^{d}$ | 2.69±0.124$^{e}$ | ND | ND | ND | ND | ND | ND |

Means with same superscript letters in the same row are not significantly different ($p > 0.05$).
Results represented as means±Standard deviation; Means (n= 2); ND- Not Detected, OEO- Oregano Essential Oil, AA- Acetic Acid

The mechanism of action of both essential oils and organic acid involves the penetration of bacterial cell membranes and disrupting cytoplasmic functions which eventually lead to cell death, hence the combination of these antimicrobials would results in rapid and increased cell death at low concentrations and contact times (NAZER *et al.,* 2005). The addition of small amounts of different natural antimicrobials can play a role in balancing sensory attributes and antimicrobial efficacy of these compounds (ZHOU *et al.,* 2007).

**Table 4.** Antimicrobial effect of combined OEO and AA on inactivation of *E. coli* O157:H7 and *L. monocytogenes* on lettuce (log CFU/g) over a period of 6 days at 5 and 22ºC.

| Storage | Day 0 | | Day 2 | | Day 4 | | Day 6 | |
|---|---|---|---|---|---|---|---|---|
| | 2 min | 5 min | 2 min | 5 min | 2min | 5min | 2 min | 5 min |
| *E. coli* O157:H7 | | | | | | | | |
| Control at 5 ºC | 8.13±0.021$^{a}$ | | 7.28±0.044$^{b}$ | | 7.00±0.024$^{b}$ | | 7.25±0.023$^{c}$ | |
| Sample at 5 ºC | 3.27±0.049$^{b}$ | 3.09±0.074$^{b}$ | 2.54±0.088$^{c}$ | 1.00±1.411$^{d}$ | ND | ND | ND | ND |
| Control at 22 ºC | 8.13±0.021$^{a}$ | | 8.26±0.013$^{a}$ | | 8.46±0.012$^{a}$ | | 8.91±0.042$^{b}$ | |
| Sample at 22 ºC | 3.27±0.049$^{b}$ | 3.09±0.074$^{b}$ | 2.50±0.281$^{c}$ | 2.151±0.213$^{c}$ | ND | ND | ND | ND |
| *L. monocytogenes* | | | | | | | | |
| Control at 5 ºC | 8.01±0.021$^{a}$ | | 7.24±0.021$^{b}$ | | 6.93±0.05$^{c}$ | | 7.07±0.02$^{c}$ | |
| Sample at 5 ºC | 3.13±0.023$^{b}$ | 2.89±0.077$^{b}$ | 2.00±0.001$^{c}$ | ND | ND | ND | ND | ND |
| Control at 22 ºC | 8.01±0.021$^{a}$ | | 8.36±0.021$^{a}$ | | 8.46±0.014$^{a}$ | | 9.23±0.032$^{a}$ | |
| Sample at 22 ºC | 3.13±0.023$^{b}$ | 2.89±0.077$^{b}$ | 2.38±0.124$^{c}$ | 2.00±0.001$^{c}$ | ND | ND | ND | ND |

Results represented as means±Standard deviation; Means (n= 2); ND - Not Detected.

## 4. CONCLUSION

Oregano essential oil and acetic acid, used singly or combined, were effective against *E. coli* O157:H7 and *L. monocytogenes* on the fresh leafy vegetable. However, the efficacy of these antimicrobial agents vary with treatment concentration, exposure time and storage temperature. The combined application of 0.1% EOE and 0.3%AA was most effective and achieved a complete inhibition at 4$^{th}$ day at 5ºC storage. This result could serve as preliminary investigation in order to determine the best experimental conditions. Research should be done towards evaluating the survival of other pathogens, potential virulence, compatibility to human diet and ready application as sanitizing solutions.

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PAPER

# PREVALENCE AND CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATES FROM RETAIL MEAT IN SOUTH ITALY

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## ABSTRACT

This study aimed to estimate the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) from 500 retail meat products in South Italy from June 2016 to June 2018, including 150 raw bovine, 120 pork, 150 chicken and 80 horse meat.

After bacteriological analysis, 12 (2.4%) samples tested positive for MRSA. Isolates were characterized by antimicrobial susceptibility, *spa* typing and MLST. MRSA were also investigated by PCR for the presence of enterotoxins, *lukF-PV-lukS-PV* and *icaA-icaD* genes. Antimicrobial susceptibility testing showed that MRSA isolates were multidrug resistant. One strain harboured PVL-encoding genes (8.3%). Seven MRSA isolates of 12 (58.3%) carried *seh* enterotoxin encoding gene. The *icaA* and *icaD* genes were both present in 10 isolates (83.3%).

MRSA isolates in retail meat may serve as a potential source of exposure to MRSA for humans and monitoring of food-producing animals and hygiene standards should be strictly and carefully considered throughout the entire meat chain to ensure food safety.

*Keywords*: biofilm, food safety, Methicillin-resistant *Staphylococcus aureus*, Panton Valentine Leukocidin, retail meat

## 1. INTRODUCTION

*Staphylococcus aureus* is considered as one of the major foodborne pathogens and is responsible for a wide spectrum of infections worldwide (Wu *et al*., 2018). Methicillin-resistant *S. aureus* (MRSA) poses a public health issue because of its multiple antimicrobial resistance and data on the occurrence of MRSA in food-producing animals and food is underestimated as the report is currently voluntary (EFSA and ECDC, 2019).
Traditionally, MRSA strains are distinguished into two distinct epidemiological groups, hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) (Tang *et al*., 2017). CA-MRSA frequently harbour staphylococcal cassette chromosome *mec* (SCC*mec*) types IV or V and the genes *lukS-PV-lukF-PV* encoding the subunits of the Panton-Valentine leucocidin, a cytotoxin that causes leukocyte lysis or apoptosis via pore formation (BOYLE-VAVRA and DAUM, 2007). HA-MRSA typically possess larger-size SCC*mec* types I, II or III and often exhibit resistance to multiple classes of antimicrobial agents strains (Shore *et al*., 2014). A third group of MRSA, known as livestock-associated MRSA (LA-MRSA), has recently been identified and it infects livestock and companion animals, as well as some other farm animal species and wild animals. LA-MRSA have mainly SCC*mec* types IVa or V, although, non-typeable cassettes and SCC*mec* type XI have also been found (BUTAYE *et al*., 2016).
Methicillin resistance is primarily attributed to the altered penicillin binding protein (PBP2a), encoded in the *mecA* gene, which has a reduced affinity for β-lactam antibiotics. Recently, a homolog of the *mecA*, *mecC* ($mecA_{LGA251}$) was identified in MRSA strains from humans and livestock that were phenotypically resistant to methicillin, but tested negative for the *mecA* gene. The *mecC* gene shares about 70% nucleotide homology with *mecA* and is located in SCC*mec* XI (Velasco *et al*., 2015).
The *nuc* gene is considered a marker for the detection of *S. aureus* and encodes for a thermostable nuclease (COSTA *et al*., 2005).
*S. aureus* has the ability to form biofilms on various materials and surfaces. Biofilms in the food industry can cause serious hygienic problems as the bacteria could adhere to the food contact surfaces and contaminate foodstuffs (RODE *et al*., 2007).
The mechanism of biofilm formation is promoted by *ica* locus containing four genes, namely *icaA*, *icaB*, *icaC*, *icaD*. The product of *ica* locus is the polysaccharide intracellular adhesin (PIA), that mediates intercellular aggregation of bacterial cells. PIA was found to be the main exopolysaccharide component of the staphylococcal biofilm (ARCIOLA *et al*., 2015). The *icaA* gene encodes for a transmembrane enzyme, N-acetylglucosaminyltransferase that contribute to the synthesis of the poly-N-acetylglucosamine polymer and requires *icaD* for full functioning (CIFTCI *et al*., 2009).
In recent years, MRSA isolation from fresh retail meat has been reported in U.S.A., Saudi Arabia, Korea, Denmark, Finland, Germany, Spain and Switzerland (EFSA and ECDC, 2019; GE *et al*., 2017; KIM *et al*., 2015; TANG *et al*., 2017), suggesting that these products may pose a potential risk for MRSA transmission to humans (BUYUKCANGAZ *et al*., 2013).
To the best of our knowledge, there is little data available on the prevalence of MRSA contamination in fresh meats sold at retail prices in Italy.
Genotyping of *S. aureus* isolated from retail meat is an important tool in epidemiological studies of infection and contributes to better understanding of the pathogen's dissemination. Several molecular methods have been developed for typing *S. aureus* isolates, such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing

(MLST) and *spa* typing (STROMMENGER *et al*., 2006; ENRIGHT *et al*., 2000; BANNERMAN *et al*., 1995).
The aims of this study were to evaluate the prevalence of MRSA in fresh meat samples sold at retail prices in southern Italy and investigate the molecular characteristics of MRSA isolates as regards some virulence-associated genes, and antimicrobial resistance profiling for epidemiological studies and risk assessment purposes in the "One Health" perspective.

## 2. MATERIALS AND METHODS

### 2.1. Isolation and identification of MRSA

A total of 500 fresh meat samples, over a two-year period (June 2016-June 2018), were collected at retail markets by local health officials, and transported to the laboratories of the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (IZS PB) and analysed for the detection of *S. aureus*. These samples comprised 150 raw bovine, 120 pork, 150 chicken, and 80 horse meat samples.
Isolation and identification of *S. aureus* were performed according to EN ISO 6888 1-2 1999. Presumptive *S. aureus* colonies (black colonies with a zone of clearing of the medium) were identified by conventional biochemical methods and plated onto blood agar. After 18-24 h of incubation at 37°C, *S. aureus* isolates were subcultured on CHROMagar™ MRSA (CHROMagar, Paris, France). DNA was extracted from an isolated bacterial colony using the InstaGene Matrix™ (Bio-Rad, Segrate (MI), Italy), following the manufacturer's instructions. All *S. aureus* isolates were screened by multiplex PCR for 16S rRNA (MONDAY and BOHACH, 1999), *nuc* (COSTA *et al*., 2005) and *mecA*/*mecC* (GARCÍA-ÁLVAREZ *et al*., 2011) genes in order to confirm *S. aureus* species and to detect methicillin resistance. Confirmed MRSA isolates (one strain per sample) were further characterized and tested for antimicrobial susceptibility.

### 2.2. In vitro antimicrobial susceptibility

Antimicrobial susceptibility of MRSA isolates was determined by disc diffusion method according to the guidelines of Clinical Laboratory Standards Institute (2013). A total of eleven antibiotics were included: penicillin (10 units), oxacillin (1 μg), cefoxitin (30 μg), cephalothin (30 μg), gentamicin (10 μg), kanamycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (25 μg), chloramphenicol (30 μg), enrofloxacin (5 μg). The MIC of teicoplanin of the MRSA isolates was determined by Etest® (bioMérieux Italia Spa, Bagno a Ripoli (FI) Italy), following CLSI interpretative breakpoints (2017). *S. aureus* ATCC 25923 was included for quality control.

### 2.3. Genotyping

The polymorphic X region of the protein A gene (*spa* typing) was amplified according to a published protocol (STROMMENGER *et al*., 2006). Amplification of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi*L) by Multilocus sequence typing (MLST) was performed as described by ENRIGHT (2000). The DNA sequences were submitted to the *Staphylococcus* MLST database (http://saureus.mlst.net/) to obtain the allelic profiles of MRSA strains.

### 2.4. SCC*mec* typing and detection of enterotoxins, *lukF-PV-lukS-PV* and *icaA-icaD* genes

SCC*mec* elements were typed by multiplex PCR as previously described (GARCÍA-ÁLVAREZ *et al*., 2011; KONDO *et al*., 2007). The SCC*mec* type IV was subtyped according to ZHANG (2012).
MRSA isolates were screened by three multiplex PCR for the detection of 12 genes encoding staphylococcal enterotoxins (for *sea* to *see*, for *seg* to *sej*, for *sem* to *seo*), using twelve specific primer sets as previously described (BOEREMA *et al*., 2006; JARRAUD *et al*., 2002; LØVSETH *et al*., 2004; MONDAY and BOHACH, 1999; ROSEC and GIGAUD, 2002). All MRSA strains were subjected to a PCR assay to test the presence of *lukF-PV-lukS-PV* and *icaA-icaD* genes encoding respectively, Panton-Valentine Leukocidin (PVL) and polysaccharide intercellular adhesin (PIA), as described elsewhere (HESJE *et al*., 2011; ZMANTAR *et al*., 2008). Furthermore, the ability of the MRSA strains to form biofilm was tested using a semi-quantitative adherence assay by microtiter plate (MTP) according to ZMANTAR (2010). The assay recorded the optical density (OD) at 570nm of adherent biofilm after incubation for 24 h at 37°C. Biofilm formation was classified as highly positive ($OD_{570} \geq 1$), low grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$).

## 3. RESULTS AND DISCUSSION

Methicillin-resistant *Staphylococcus aureus* is a public health concern and food contaminated by MRSA may serve as a potential vehicle for transmission to humans (EFSA, 2009). This study reports on the prevalence of MRSA isolated from fresh meat and sold at retail prices as well as the characterization of some virulence-associated genes and antimicrobial resistance profiling. In this survey, among 500 retail fresh meat samples subjected to bacteriological analysis, 72 (14.4%; 72/500) tested positive for *S. aureus* and 12 (2.4%; 12/500) for MRSA. Total *S. aureus* counts performed using standard microbiological procedures were below $10^3$ colony forming units per gram (CFU/g) in all tested samples. All MRSA isolates carried the *mecA* gene and none carried the *mecC* gene. Several surveys have estimated the prevalence of MRSA in retail meat worldwide. In a study from U.S.A., GE *et al*. (2017) found that 27.9% of the retail meats examined was contaminated by *S. aureus* and 1.9% tested positive for MRSA. Another study from U.K. recovered MRSA from 7.3% of retail meat samples (FOX *et al*., 2016). Previous studies conducted in Italy reported a contamination rate of meat product samples of 10% and 0.5%, but no MRSA strain was found among the isolates (NORMANNO *et al*., 2007a; NORMANNO *et al*., 2007b; TRAVERSA *et al*., 2015). Probably, these differences in the prevalence of MRSA in retail meats could be due to the different geographical area, sampling and collection period.
In this report, most MRSA isolates (5/12; 41.7%) were t127/ST1/SCC*mec* type IVa, *seh* positive and PVL-negative. The 16.7% (2/12) of the isolates was t174/ST1/SCC*mec* type IVa, SEs and negative PVL genes. Other recovered MRSA strains were: t386/ST1/SCC*mec* type IVa (1/12; 8.3%) and t599/ST1/SCC*mec* type IVa (1/12; 8.3%), both *seh* positive and PVL-negative, t044/ST80/SCC*mec*type IVc (1/12; 8.3%), SEs negative and PVL positive, ST97 t1236/ST97 (1/12; 8.3%) and t899/ST398 SCC*mec* type V, both SEs and PVL-negative (1/12; 8.3%) (Table 1). ST1 is a clone frequently implicated in human infections and spa-type 127 is the prevalent clone involved in cases of invasive MRSA infections in Europe (MONACO *et al*., 2013). MRSA t127/ST1 was also found in cows, sheep, goats and pigs in

Italy and other European countries (AGERSØ *et al*., 2012; ALBA *et al*., 2015; PAPADOPOULOS *et al*., 2018). MRSA t127/ST1 clone is often detected in Italian pig industry and the presence of a pig reservoir from this lineage has been hypothesized (ALBA *et al*., 2015; FRANCO *et al*., 2011). Moreover, *seh* gene is considered to be constitutive of ST1, independently from the host origin (MONECKE *et al*., 2011). MRSA with genotype t044/ST80/SCC*mec*type IVc PVL-positive belongs to CC80 and lacks enterotoxin genes. First recognized in Denmark in 1993, now it is widely spread throughout Europe, North Africa, sub-Saharan Africa and the Middle East (MONECKE *et al*., 2011). It is mainly associated with skin infections in the community, but rarely causes invasive infections (DAVID *et al*., 2010). The presence of this clone in retail horse meat underlies the spread of this MRSA-ST80 clone. Indeed, there are studies from various countries about nosocomial infections in horses due to MRSA causing a variety of infections (CUNY *et al*., 2017; ISLAM *et al*., 2017; STEINMAN *et al*., 2015). In this survey, only one isolate (1/12; 8.3%) harboured PVL-encoding genes. The finding of MRSA ST80/t044 PVL-positive suggests human handlers as potential source of contamination of meat, with PVL being a marker of CA-MRSA. Another genotype recovered in this study from bovine retail meat was MRSA t1236/ST97. In this isolate, SCC*mec* was not detected. Other studies reported this genotype to be associated with sheep, goats and cows as methicillin-susceptible *S. aureus* (FELTRIN *et al*., 2016; PORRERO *et al*., 2012). ST97 (CC97) is generally responsible for bovine mastitis. Less commonly, it was found in small ruminants, pigs, and humans. This clonal complex is the second most prevalent MRSA lineage in pig finishing holdings in Italy and one of the *S. aureus* lineages associated with cattle, particularly with bovine mastitis (FELTRIN *et al*., 2016). The finding of MRSA strains in raw fresh meat should be considered carefully since meat may expose humans to this microorganism. It would be desirable monitoring health status of animals and implement control measures for breeding and slaughtering to avoid contaminations of their meat by *S. aureus*. MRSA t899/ST398 SCC*mec* type V PVL-negative is considered an important livestock-associated (LA)-MRSA present in pigs, poultry, calves, companion animals, horses and other farm animal species in many countries. This clone was found in retail chicken meat in England (FOX *et al*., 2016), in Germany (KRAUSHAAR *et al*., 2017) and China (WANG *et al*., 2014). LA-MRSA may also pose an occupational risk for those people in close contact with livestock and their derived carcasses, especially pig farmers, cattle farmers, poultry farmers, slaughterhouse workers and veterinarians (HADJIRIN *et al*., 2015).

These persons are more likely to be colonized with MRSA and spread the microorganism in the community. Hence, retail meat may be a route for transmission of CA-MRSA (e.g. MRSA ST1-t127) and LA-MRSA to humans.

Several foods are implicated in Staphylococcal food poisoning (SFP) such as raw meat, sausages, raw milk and raw milk cheese, in which contamination could be due to animal carriage or to infections of animal origin. Colonized food handlers, rather than animals, are likely sources of contamination after heat treatment of the food (BASANISI *et al*., 2017). The emetic activity of enterotoxins has been demonstrated only for SEA, SEB, SEC, SED, and SEE (JOHLER *et al*., 2015). The 58.3% (7/12) of the MRSA isolated in this study harboured *seh* gene, but in literature, there is little data available on the prevalence of MRSA in SFP (Table 1) (JØRGENSEN *et al*. 2005; and OSTYN *et al*. 2012). Nevertheless, the risk of human infection cannot be ignored. Consequently, more attention should be paid during food handling and storage in order to reduce the potential role of food in the dissemination of successful MRSA lineages.

Biofilm matrix is considered to be a significant virulence factor because when growing in this mode of life, microorganisms become more tolerant to antimicrobial agents and extremely difficult to eradicate. In this survey, the 83.3% (10/12) of MRSA isolates carried both the *icaA* and *icaD* genes; the16.7% (2/12) of the isolates harboured only *icaD* gene (Table 1). As regard the ability to form biofilm, the MRSA strains were biofilm producers with MTP method, although, production level varied. Among these, 83.3% of isolates (10/12) were low grade biofilm positive and 16.7% (2/12) were strongly biofilm producers (Table 1). These strains could be of concern for the meat industry since bacteria in biofilms can be resistant to the normal disinfection and prophylaxis methods. Special attention should be paid to hygiene procedures in farms and food facility.

**Table 1.** Antimicrobial resistance profiles, genotypic characteristics and virulence-associated genes of the 12 MRSA isolates analyzed in this study.

| N° | Source of meat | Resistance to*: | SCC*mec* | *spa* type | MLST | *SEs* | *lukF-PV/lukS-PV* | *icaA* | *icaD* | #$OD_{570}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Horse | P, OX, FOX, KF, K, E, TE | Iva | t174 | ST1 | - | - | - | + | ++ |
| 2 | Horse | P, OX, FOX, KF, K, E, TE | Iva | t174 | ST1 | - | - | - | + | ++ |
| 3 | Horse | P, OX, FOX, KF, CN, K, E, ENR | IVc | t044 | ST80 | - | + | + | + | ++++ |
| 4 | Bovine | P, OX, FOX, KF, E, TE | ND | t1236 | ST97 | - | - | + | + | ++++ |
| 5 | Bovine | P, OX, FOX, K, TE, KF, STX | Iva | t127 | ST1 | *seh* | - | + | + | ++ |
| 6 | Bovine | P, OX, FOX, K, TE, KF, STX | Iva | t386 | ST1 | *seh* | - | + | + | ++ |
| 7 | Pork | P, OX, FOX, CN, K, E, KF, TE | Iva | t127 | ST1 | *seh* | - | + | + | ++ |
| 8 | Pork | P, OX, FOX, CN, K, E, KF, TE | Iva | t127 | ST1 | *seh* | - | + | + | ++ |
| 9 | Pork | P, OX, FOX, CN, K, E, KF, TE | Iva | t127 | ST1 | *seh* | - | + | + | ++ |
| 10 | Pork | P, OX, FOX, CN, K, E, KF, TE | Iva | t127 | ST1 | *seh* | - | + | + | ++ |
| 11 | Pork | P, OX, FOX, K, E, TE, KF | Iva | t599 | ST1 | *seh* | - | + | + | ++ |
| 12 | Chicken | P, OX, FOX, TE, KF, STX | V | t899 | ST398 | - | - | + | + | ++ |

*Antibiotic abbreviations: P, penicillin; OX, oxacillin; FOX, cefoxitin; KF cephalothin; CN, gentamicin; K, kanamycin; E, erythromycin; TE, tetracicline; ENR, enrofloxacin; STX, trimethoprim-sulfamethoxazole. #Strongly biofilm positive (++++), low grade biofilm positive (++).

Global consumption of antimicrobials has increased worldwide causing the development of resistance to several antimicrobial agents in bacteria and this might result to serious problems. In this study, MRSA isolates were resistant to penicillin, oxacillin, cefoxitin, cephalothin followed by tetracycline (11/12; 91.7%), kanamycin (10/12; 83.3%), erythromycin (8/12; 66.7%), gentamicin (5/12; 41.7%), trimethoprim-sulfamethoxazole (3/12; 25%), enrofloxacin (1/12; 8.3%). The MIC value of teicoplanin was ≤ 1.5 $\mu$g/ml for all MRSA isolates (Table 1). Multidrug resistance in retail meat was also observed in other studies (JACKSON *et al.*, 2013; TANG *et al.*, 2017). Aminoglicosides, macrolides,

penicillins, and tetracyclines are some of the classes of antimicrobial agents extremely important for veterinary medicine considering the wide range of applications and diseases to be treated (WENDLANDT *et al*., 2015). Therefore, controlling the use of antibiotics in farming could limit the risk of transmission of multidrug resistant pathogens among animals and potentially to humans through the food chain.

## 4. CONCLUSIONS

In this study, raw bovine, pork, chicken and horse meat samples were positive for MRSA, although, the level of prevalence was low and varied between meats of different origin. The data obtained from this survey suggest that the presence of MRSA in fresh retail meats could be the result of human contamination due to colonized food handlers or cross-contamination of carcasses during food processing. Furthermore, the most MRSA clonal complexes found in the present survey are responsible for community infections, suggesting that food may contribute to the spread of MRSA in the environment. Further studies should be designed to collect more exhaustive data on the prevalence and evolution of these pathogens.
In conclusion, monitoring of food-producing animals and strict hygienic standards should be carefully considered throughout the entire meat chain, from primary production to retail in order to prevent or reduce the transmission of multidrug resistant pathogens to consumers from the "One Health" perspective.

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PAPER

# EFFECTS OF PECTIN-BASED EDIBLE COATINGS CONTAINING A BACTERIOCIN OF *BACILLUS METHYLOTROPHICUS* BM47 ON THE QUALITY AND STORAGE LIFE OF FRESH BLACKBERRIES

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## ABSTRACT

The aim of the current research is to investigate the effects of edible coatings based on celery pectin singly and in combination with a bacteriocin of *Bacillus methylotrophicus* BM47 on the quality and storage life of fresh blackberries under refrigeration conditions. In this study three experimental groups were prepared: uncoated blackberries as a control, blackberries with 1% pectin coatings and blackberries with 1% pectin coatings+bacteriocin of *B. methylotrophicus* BM47. During the storage at 4°C and 75% RH for 16 days, the weight loss, decay percentage, total soluble solids (TSS), titratable acidity (TA), pH, organic acids, sugars, total phenolic content, total anthocyanins and antioxidant activity were analyzed.

The results showed that the application of pectin and pectin+bacteriocin coatings led to a reduction in weight loss with 6.3% and 6.7% compared to the control fruit on the 16-th day of storage. A decrease in decay percentage was also noticed, which was most pronounced in the pectin+bacteriocin coated fruit compared to the pectin coatings and control. The pectin and pectin+bacteriocin coatings reduced TSS levels with 0.4% and 0.6%, respectively compared to the control on the 16-th day of the storage, but did not affect TA and pH values. The pectin and pectin+bacteriocin coatings had no effect on decreasing total phenolic and anthocyanin contents or the concentration of sugars (glucose and fructose) in both treatments and the control fruit. The pectin and pectin+bacteriocin edible coatings exhibited a protective effect on the ascorbic acid content, maintaining concentrations of 57.5 mg/100 g of fw and 58.8 mg/100 g of fw (day 16), which were close to the initial values. The pectin and pectin+bacteriocin treatments had also a positive impact on antioxidant activity in the coated blackberries. Both edible coatings effectively inhibited its decrease with the prolongation of the storage time and kept antioxidant levels of 231.8 TE/100 g of fw and 232.4 TE/100 g of fw (day 16) that were close to the initial values.

*Keywords*: *Bacillus methylotrophicus*, bacteriocin, biopreservation, blackberry, edible coatings

## 1. INTRODUCTION

Blackberry (*Rubus fruticosus*) is a perennial plant in the family *Rosaceae* whose native populations grow primarily in the Mediterranean region of Europe. The cultivated blackberry is grown all over the world, and in recent years, worldwide production and consumption of blackberries continue to increase (STRIK *et al.*, 2007). The blackberry fruit is an excellent source of antioxidants, including various phenolic compounds (phenolic acids, tannins, stilbenes, flavonoids and anthocyanins) and ascorbic acid as well as large amounts of other compounds such as vitamins, minerals, fibers and volatiles (DE SOUZA *et al.*, 2014). The rich phytochemical composition and nutritional characteristics of blackberry fruit have been shown to exert a positive impact on human health. For example, the high levels of antioxidants in blackberries have been found to exhibit anti-inflammatory, anticarcinogenic, antimutagenic and antimicrobial properties (GYAWALI and IBRAHIM, 2014). The bioactive components in blackberries also reduce cholesterol levels, cause an analgesic effect and help to strengthen blood vessels (NILE and PARK, 2014). Some recent studies have shown that blackberry polyphenols exhibit a neuroprotective effect by delaying degenerative brain processes (TAVARES *et al.*, 2012).

On the other hand, blackberry is a highly perishable fruit with a very short market life. The storage time of blackberries after harvesting is limited due to their high susceptibility to physical injuries, desiccation and fungal spoilage caused by *Botrytis cinerea*, *Rhizopus* sp. and *Mucor* sp. which results in a rapid loss of commercially acceptable appearance. These post-harvest changes can be suppressed by storage at low temperatures or by creating modified atmospheres (lower oxygen and increased carbon dioxide levels) that delay tissue senescence. Advanced technologies such as ozone treatment and gamma radiation can also be used to decrease microbial decay and prolong the storage life of blackberries (MAFTOONAZAD *et al.*, 2007; OLIVEIRA *et al.*, 2013).

The conservation, distribution and marketing of blackberries can also be significantly improved through some non-conventional methods for extending the storage time such as the application of thin layers of biopolymers known as edible coatings, which protect the fruit from physical injuries, chemical and microbiological activities (FALGUERA *et al.*, 2011). Edible coatings and films act as barriers against moisture loss, oxygen, carbon dioxide and lipid transfer, thus preventing dehydration, desiccation and deterioration of the fruit quality (CUI *et al.*, 2016). Edible coatings provide additional benefits in perishable fruits by reducing respiration rate, improving textural quality, and helping to retain natural color and volatiles, thereby protecting the fruit's nutritional value (CORBO *et al.*, 2015).

The nature of the edible coating material is essential for the coating's effectiveness. Existing data in the literature show that hydrocolloids (polysaccharides and proteins) and lipids are among the most suitable structural matrices for edible coatings production. Most edible coatings include cellulose and its derivatives, starch, alginate, chitosan and pectin. Numerous studies have described and evaluated the protective effects of cellulose derivatives, starch, alginate and chitosan, but research on the application of pectin-based edible coatings is very limited (MAHAJAN *et al.*, 2018).

Pectins are biopolymers comprised of (1→4) $\alpha$-D-galactopyranosyluronic acid units naturally esterified with methanol. Based on the content of methyl esters or the degree of esterification (DE) that has a decisive effect on solubility and gel formation properties, pectins are divided into two groups – high-methoxylated (DE>50%) and low-methoxylated (DE<50%) pectins (CORBO *et al.*, 2015). Pectins are also polysaccharides that are commonly used as gelling agents in the food industry. As such, pectins are of great

interest as edible coating agents in fruit biopreservation due to their unique colloidal characteristics, strong gel formation properties and ability to provide an excellent oxygen barrier and aroma preservation. However, the primary disadvantage of all polysaccharide-based coatings is that they do not ensure a good moisture barrier due to their hydrophilic nature (OMS-OLIU *et al.*, 2008; VALDÉS *et al.*, 2015).
Berries contain high levels of sugars, other nutrients and water that make them an excellent substrate for microbial growth. In addition, the low pH of berry fruits is a prerequisite for fungal spoilage, which affects product quality, storage and distribution, resulting in significant economic losses (TOURNAS and KATSOUDAS, 2005). The resistance of some fungi to conventional fungicides and the proven negative effects of chemical treatments on human health have led to many limitations in the use of such chemicals. Consequently, research efforts have become more focused on the development of safer preservation methods that employ non-toxic biologically active compounds. Thus, the biological control of spoilage by antimicrobial substances such as bacteriocins synthesized by *Bacillus* sp. and lactic acid bacteria (LAB) are a promising alternative to the traditional fungicide application. Bacteriocins are safe; they do not alter the organoleptic characteristics of food and can be applied directly or indirectly by *in situ* production. Some LAB bacteriocins have been tested and have already shown promising potential against microbial spoilage of fruits and fruit products (BARBOSA *et al.*, 2017). Other bacteriocins with strong antifungal activity such as those produced by *Bacillus methylotrophicus* BM47 might also be prospective candidates for solving the problems associated with fungal decay (TUMBARSKI *et al.*, 2018).
The current study thus aimed to examine the application of edible coatings based on celery pectin alone and in combination with a bacteriocin synthesized by *Bacillus methylotrophicus* BM47 on the quality and extension of the storage life of fresh blackberry fruit.

## 2. MATERIALS AND METHODS

### 2.1. Materials

#### 2.1.1 Fruit

Fresh blackberries (*Rubus fruticosus*) were purchased from the local fruit market in Plovdiv, Bulgaria. The fruit was selected based on size, shape, color and absence of physical injuries. The blackberries were placed in brown paper bags and then immediately transferred into a fridge bag (7°C) to the laboratory to conduct the experiments.

#### 2.1.2 Pectin

Low-methoxylated pectin was extracted from celery tubers (*Apium graveolens* var. *rapaceum* D.C.) purchased from the local fruit market in Plovdiv, Bulgaria. The tubers were washed with tap water, checked for impurities, and then peeled, coarsely chopped and dried at 40°C. Before extraction, the tubers were finely ground in a laboratory homogenizer and pre-washed with 70% ethanol acidified with 2% hydrochloric acid in order to obtain alcohol insoluble solids. Pectin was extracted by ultrasound-assisted extraction with 2% aqueous ammonium oxalate using the method of PETROVA *et al.* (2017). The degree of esterification (DE) and anhydrouronic acid content (AUAC) were determined by titration

method (PETROVA *et al.*, 2017). The degree of acetylation (DA) was measured by the hydroxamic acid reaction method with β-D-glucose pentaacetate as a standard. All chemicals were purchased from Sigma-Aldrich, Merck (St. Louis, MO, USA). The molecular mass (Mm) was assessed by high-performance size-exclusion chromatography (HPSEC) analysis. Separation was conducted using an Elite LaChrom HPLC system (VWR™ Hitachi, Tokyo, Japan) coupled with a column Shodex OH-pack 806M (8 mm × 300 mm) and a refractive index detector Chromaster 5450 with an aqueous 0.1 M sodium nitrate solution at a flow rate of 0.8 mL/min (OGNYANOV *et al.*, 2018). The obtained celery pectin was characterized as low-esterified pectin with DE 46%, DA 2%, AUAC 70% and average Mm of 912694 g/mol.

2.1.3 Bacteriocin

A bacteriocin synthesized by the strain *Bacillus methylotrophicus* BM47 (previously isolated from a natural thermal spring in the Haskovo region of Bulgaria) was used in the experiment. The bacteriocin, purified by fast protein liquid chromatography (FPLC), contained an antimicrobial peptide with Mm of 19578 Da as characterized in an earlier research (TUMBARSKI *et al.*, 2018).

**2.2. Methods**

2.2.1 Experiment design

The edible coating solution (1%) was prepared by dissolving 4 g of celery pectin in 400 mL of distilled water at 45°C in a magnetic stirrer IKA® RCT classic (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 800 rpm for 30 min. Next, 0.5% glycerol monostearate Cutina® GMS (Henkel, Düsseldorf, Germany) was added as a plasticizer, and the solution was stirred under identical conditions as outlined above for 15 min. The pectin solution was then divided into two equal parts of 200 mL each. After cooling to room temperature, 100 AU/mL (0.15 mg/mL) of the purified bacteriocin of *B. methylotrophicus* BM47 was added to the second part and stirred without heating for 15 min (TUMBARSKI *et al.*, 2019).
The blackberries were disinfected by being dipped in 1% sodium hypochlorite (Sigma-Aldrich, Merck, Germany) for 3 min, then washed three times with tap water and dried at 25°C for 2 h in a forced air MLW drying chamber (Labexchange, Burladingen, Germany). After drying, the blackberries were separated into three experimental groups of 30 berries each as follows: uncoated blackberries as a control (group 1), blackberries with pectin coatings (group 2) and blackberries with pectin coatings + bacteriocin (group 3). The blackberries from group 2 and group 3 were immersed in the relevant coating solutions for 2-3 min. The treated fruits were then dried at 25°C for 2 h in a drying chamber with forced air MLW (Labexchange), after which all groups were placed in plastic boxes and stored under refrigeration conditions (4°C and 75% RH) for 16 days (TUMBARSKI *et al.*, 2019).

2.2.2 Visual observation

All groups were examined at the beginning of the experiment (i.e. 0 day) and on the 4-th, 8-th, 12-th and 16-th day of storage. During the storage period, an observation of the morphological changes and fungal growth was made, and samples for analyses were taken.

*2.2.2.1 Weight loss percentage*

For determination of weight loss (WL) and decay percentage, three separate groups of 10 blackberries each with the same treatments - control, pectin and pectin + bacteriocin were prepared and stored under identical conditions. To measure WL, each group was weighed at the beginning of the experiment (i.e. 0 day) and on the 4-th, 8-th, 12-th and 16-th day of storage. WL was defined as the difference between the initial weight of each experimental group and the weight of the same group determined on the relevant monitoring day. The results were calculated as a percentage loss of the initial weight (TUMBARSKI *et al.*, 2019).

*2.2.2.2 Decay percentage*

The decay percentage was determined as follows: the number of berries with visible decay or morphological changes was expressed as a percentage of the initial number of all berries in the relevant experimental group (TUMBARSKI *et al.*, 2019).

2.2.3 Physico-chemical parameters

*2.2.3.1 Total soluble solids, titratable acidity and pH*

The total soluble solids (TSS) content was determined by a portable Abbe refractrometer (Officine Galileo, Campi Bisenzio, Italy). The samples were preliminary homogenized with a special device Polytron (Kinematica AG, Luzern, Switzerland), a few drops of blackberry juice were put on the prism glass, and the TSS value was immediately read and recorded. The titratable acidity (TA) was measured by titration of 2 mL of blackberry juice with 0.1 N NaOH (Sigma-Aldrich, Merck) using phenolphthalein (Sigma-Aldrich, Merck) as an indicator until the appearance of a pale pink color persisted for over 1 min. The results were calculated as the mean value of three successive experiments and expressed as the percent of malic acid. The pH values for each experimental group were measured by a pH-meter WTW pH 7110 (WTW, Weilheim, Germany) at 23°C (TUMBARSKI *et al.*, 2019).

*2.2.3.2 Total phenolic content*

The total phenolic content (TPC) was measured using a Folin-Ciocalteu reagent (Sigma-Aldrich, Merck) by the method of STINTZING *et al.* (2005). To conduct the analysis, 1 mL of Folin-Ciocalteu reagent was mixed with 0.2 mL blackberry juice and 0.8 mL 7.5% $Na_2CO_3$ (Sigma-Aldrich, Merck). The reaction was performed in darkness at room temperature for 20 min. The absorbance was measured by UV/Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, UK) against a blank at $\lambda$=765 nm, and the results were expressed as mg equivalent of gallic acid (GAE)/100 g of fresh weight (fw) according to a calibration curve (IVANOV *et al.*, 2014).

*2.2.3.3 Total anthocyanins content*

The total anthocyanins content (TAC) was estimated using the pH differential method (LEE *et al.*, 2005). The samples of blackberry juice (0.2 mL) were mixed with buffers (Sigma-Aldrich, Merck) at pH 1.0 and pH 4.5 (1.8 mL), and the absorbance was measured by UV/Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd.) against a blank

at λ=510 nm and λ=700 nm. The results were expressed as mg cyanidin-3-glycoside equivalents/100 g of fw.

*2.2.3.4 Antioxidant activity*

The antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging method. To perform this assay, 0.15 mL of blackberry juice was mixed with 2.85 mL of a freshly prepared 0.1 mM methanol solution of DPPH (Sigma-Aldrich, Merck). The samples were incubated in darkness for 15 min at 37°C. The reduction of absorbance was measured at λ=517 nm by UV/Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd.) against a blank prepared with methanol (Sigma-Aldrich, Merck), and the percentage of inhibition was calculated (IVANOV *et al.*, 2014).

*2.2.3.5 High pressure liquid chromatographic (HPLC) analysis of organic acids*

The content of organic acids was determined by an Elite LaChrom HPLC-DAD system (VWR™ Hitachi) according to IVANOV *et al.* (2017). The separation was conducted on a Discovery® HS C18 column (5 μm, 25 cm × 4.6 mm) at 30°C. The isocratic elution was conducted with 25 mM $KH_2PO_4$ (pH 2.4) as a mobile phase at a flow rate of 0.5 mL/min. L-(+)-ascorbic acid was detected at λ=244 nm and L-malic acid at λ=210 nm. The obtained results were expressed as mg/100 g of fw.

*2.2.3.6 HPLC analysis of mono- and disaccharides*

Chromatographic separation and measurement of sugars were performed on an Elite LaChrom HPLC system coupled with a refractive index detector Chromaster 5450 (VWR™ Hitachi). The separation was performed on a Shodex® Sugar SP0810 column (300 mm × 8.0 mm) with $Pb^{2+}$ and a guard column Shodex SP-G (5 μm, 6 mm × 50 mm) operating at 85°C mobile phase d.$H_2O$ with a flow rate of 1.0 mL/min and injection volume of 20 μl. The results were expressed as g/100 g of fw (PETKOVA *et al.*, 2014).

2.2.4 Statistical analysis

Each analysis was independently replicated three times, the data were presented as mean value, and the standard deviation (±SD) was calculated (TUMBARSKI *et al.*, 2019).

## 3. RESULTS AND DISCUSSION

### 3.1. Visual observation

During the first four days of storage at 4°C and 75% RH, a slight increase in weight loss (WL) in all experimental groups was observed, which was greater in the control blackberries than in the pectin and pectin+bacteriocin coated fruit. During this time no morphological changes in any experimental group were observed (Figs. 1 and 2). On the 8-th day of the refrigerated storage, the first visible signs of dehydration in the control blackberries appeared, while the coated fruit remained unaffected (Fig. 3). The WL of the control blackberries was greater with 1.8% compared to that of the treated fruit. On the 12-

th day of the storage period, the first visible signs of fungal growth in the control and pectin-coated fruit appeared, while the bacteriocin-containing coatings prevented effectively the blackberries from decay and no spoilage changes were observed (Fig. 4). The WL of the control blackberries was greater with 2.1% (pectin) to 2.4% (pectin+bacteriocin) compared to the coated fruit. At the end of the observation period (day 16), the dehydration in the control group continued to increase, and the difference in the WL between the uncoated blackberries and the coated fruit reached 6.3% (pectin) and 6.7% (pectin+bacteriocin), respectively. The fungal spoilage process was most pronounced in the control and pectin-containing coatings, while the blackberries whose coatings contained a bacteriocin remained unaffected (Fig. 5).

As seen from the results summarized in Table 1, the pectin-based edible coatings singly or in combination with a bacteriocin of *B. methylotrophicus* BM47 effectively protected the treated fruit from moisture/weight loss and desiccation during the entire storage period, which helped to extend their storage life and to improve their commercial appearance. The protective effect of the bacteriocin of *B. methylotrophicus* BM47 and the attendant decrease in the decay percentage were related to the bacteriocin's strong antifungal properties as described in our previous research (TUMBARSKI *et al.*, 2018).

A similar tendency for weight loss during storage at low temperature was reported by GUERREIRO *et al.* (2015), who used citrus pectin and alginate as edible coatings for red raspberry fruit. In addition, it was demonstrated that pectin active coatings improved the physico-chemical, microbiological and sensory quality of strawberries from 6 (control) to 15 days at 4°C and 90% RH (VALDÉS *et al.*, 2015).


**Figure 1.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the appearance and morphology of fresh blackberries at the beginning of the experiment (day 0).
C - control; P - pectin (1%); P+B - pectin (1%) + bacteriocin.

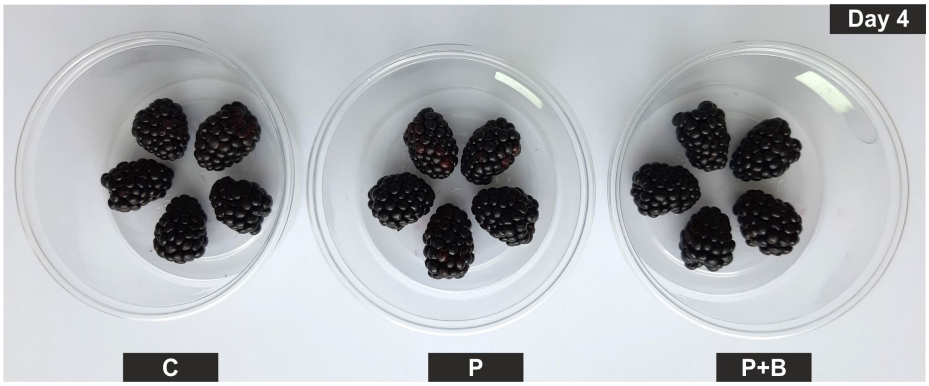


**Figure 2.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the appearance and morphology of fresh blackberries at day 4 during refrigerated storage (4°C and 75% RH). C - control; P - pectin (1%); P+B - pectin (1%) + bacteriocin.

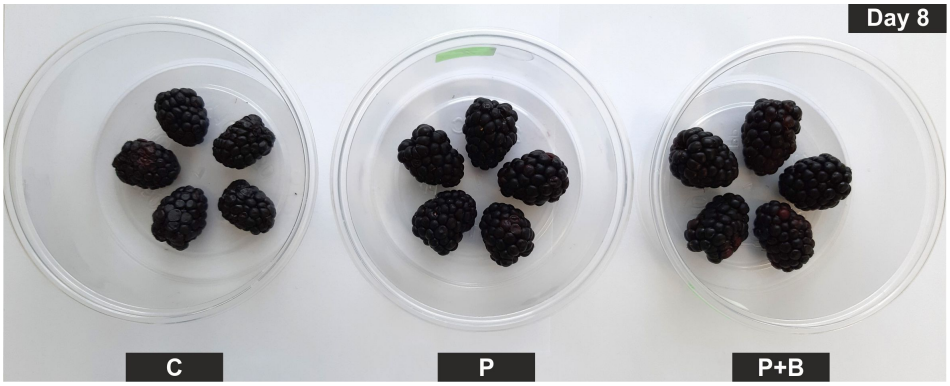


**Figure 3.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the appearance and morphology of fresh blackberries at day 8 during refrigerated storage (4°C and 75% RH). C – control; P - pectin (1%); P+B – pectin (1%) + bacteriocin.


**Figure 4.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the appearance and morphology of fresh blackberries at day 12 during refrigerated storage (4°C and 75% RH). C - control; P - pectin (1%); P+B - pectin (1%) + bacteriocin.

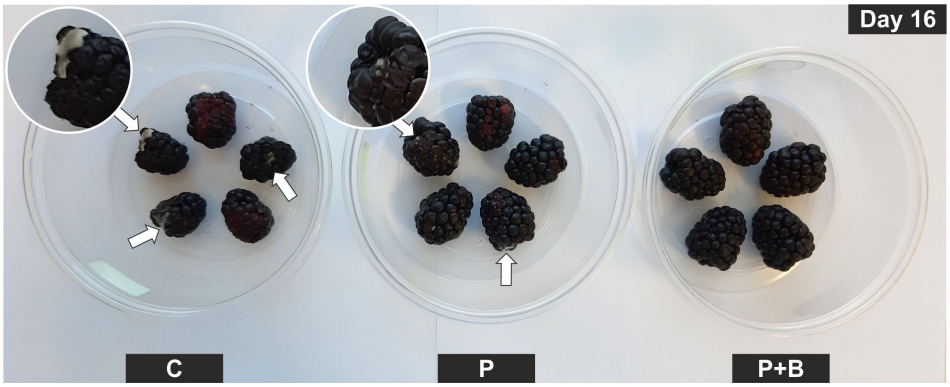


**Figure 5.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the appearance and morphology of fresh blackberries at day 16 during refrigerated storage (4°C and 75% RH). C - control; P - pectin (1%); P+B - pectin (1%) + bacteriocin.

### 3.2. Changes in total soluble solids, titratable acidity and pH

The total soluble solids maintained a constant level of 14.1% in all experimental groups during the first four days of storage. On the 8-th day, a slight increase in TSS in the control group and pectin-coated blackberries was detected, while pectin+bacteriocin coatings maintained TSS levels equal to the initial (14.1%). The TSS levels continued to rise gradually over the storage period due to water migration in the environment and fruit desiccation. By the end of the observation period (days 12 and 16) the treated blackberries had lower TSS values (with 0.2% to 0.4% for pectin-coated fruit and 0.4% to 0.6% for pectin+bacteriocin-coated fruit) compared to the uncoated blackberries (Table 1). These results demonstrated that pectin-based edible coatings, especially those with the addition of bacteriocin, provided a protective barrier against moisture loss and consequently a reduced attenuation of the fruit quality.

A slight increase in TA and a decrease in pH values in all groups were observed, both of which are normally associated with post-harvest changes of fruits. The results presented in Table 1 demonstrate that pectin and pectin+bacteriocin coatings did not consistently influence these two parameters, which remained similar to those observed in the uncoated blackberries until the end of the storage period. TOSUN *et al.* (2008), who studied the behaviour of pH during blackberry ripening, observed average values of 3.20 in unripe fruits, 2.64 in red fruits and 3.14 in mature fruits. According to ANTUNES *et al.* (2003), the initial values of pH 3.59 and 3.39 reached 3.94 and 4.09 after 12 days of storage, respectively; there was an increase in pH of 0.40 and 0.70. However, in our case, a decrease in pH values of 0.30 was observed at the end of 16 days.

In our study, an increase in titrable acidity was observed during the storage (Table 1) from 1.09 to 1.70 %. Our values were higher than reported from MENEGHEL *et al.* (2008), who showed constant values for acidity during 18 days of storage, with average values of 0.86, 0.89 and 0.85 g of citric acid per 100 g for coated blackberry cv. Comanche. The similar observation of increase in titrable acidity was reported by OLIVEIRA *et al.* (2012) in blackberry (*Rubus* spp.) conservation with edible coating.

**Table 1.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the physicochemical characteristics of fresh blackberries during refrigerated storage.

| Day | Coating | Parameter | | | | |
|---|---|---|---|---|---|---|
| | | WL(%) | Decay(%) | TSS(%) | TA(%) | pH |
| 0 | C | n.a. | n.a. | 14.1 | 1.09±0.06* | 3.72±0.03 |
| | P | n.a. | n.a. | 14.1 | 1.08±0.03 | 3.71±0.06 |
| | P+B | n.a. | n.a. | 14.1 | 1.09±0.07 | 3.72±0.08 |
| 4 | C | 7.6 | 0 | 14.1 | 1.16±0.02 | 3.63±0.04 |
| | P | 6.7 | 0 | 14.1 | 1.19±0.04 | 3.62±0.02 |
| | P+B | 6.7 | 0 | 14.1 | 1.20±0.03 | 3.54±0.06 |
| 8 | C | 12.0 | 0 | 14.5 | 1.30±0.05 | 3.60±0.04 |
| | P | 10.2 | 0 | 14.3 | 1.41±0.01 | 3.56±0.03 |
| | P+B | 10.2 | 0 | 14.1 | 1.55±0.06 | 3.52±0.01 |
| 12 | C | 17.7 | 20.0 | 14.7 | 1.57±0.03 | 3.57±0.02 |
| | P | 15.6 | 10.0 | 14.5 | 1.61±0.01 | 3.45±0.04 |
| | P+B | 15.3 | 0 | 14.3 | 1.67±0.04 | 3.42±0.05 |
| 16 | C | 23.7 | 40.0 | 15.2 | 1.70±0.06 | 3.42±0.01 |
| | P | 17.4 | 20.0 | 14.8 | 1.72±0.02 | 3.46±0.05 |
| | P+B | 17.0 | 0 | 14.6 | 1.74±0.01 | 3.44±0.03 |

C - control; P - pectin (1%); B - bacteriocin; WL - weight loss; TSS - total soluble solids; TA - titratable acidity; n.a. - not analyzed; * - ±standard deviation (±SD).

### 3.3. Changes in organic acid content

The results showed that the content of ascorbic acid in blackberries declined in all treatments during the first 4 days of storage (Table 2). This finding was most likely associated with a stress reaction resulting from the effects of low temperatures on fruit metabolism. Thereafter, ascorbic acid concentrations gradually increased in all treatments relative to the storage period. The application of pectin-based coatings singly and in combination with bacteriocin of *B. methylotrophicus* BM47 effectively maintained higher levels of ascorbic acid in the treated fruit compared to the control fruit during the entire monitoring period. By the end of the storage period (16-th day), ascorbic acid levels had reached 56.8 mg/100 g of fw (control), 57.5 mg/100 g of fw (pectin) and 58.8 mg/100 g of fw (pectin+bacteriocin). These levels were close to the value noted at the beginning of the experiment (62.3 mg/100 g of fw). Our values for ascorbic acid content in fresh and coated blackberries were higher than reported values for different fresh blackberry cultivars (12.3–16.4 mg/100 g of fw) (DEIGHTON *et al*. 2000). The detected levels of ascorbic acid content were near to Cornelaian cherry cultivars (48.4-73.1 mg/100 g of fw) and higher than its level in other berries as raspberry (21.2- 31.1 mg/100 g of fw) and strawberries (46 mg/100 g of fw) (PANTELIDIS *et al*. 2007).

The concentration of malic acid in both the control and coated blackberries progressively increased, with storage time, reaching levels of 56.9 mg/100 g of fw (control), 58.3 mg/100 g of fw (pectin) and 61.3 mg/100 g of fw (pectin+bacteriocin) by the 16-th day of storage as compared to the initial level of 31.3 mg/100 g of fw. Our values for malic acid were more than twenty times higher than reported content in raspberry cultivars (ZORENC *et al*.

2017) and wild growing blackberries (MIKULIC-PETKOVSEK *et al.* 2012). Neither type of coating had an inhibitory effect on the increasing concentration of malic acid that is normally associated with post-harvest changes in fruits (Table 2). These results correlated with the increasing levels of titratable acidity and decreasing pH values in all experimental groups during the storage period (Table 1). This could be explained with by increased metabolism of fruits after harvesting, in which a greater consumption of organic acids is needed as substrates for the respiratory process. As a result of this, there is a greater conversion into simple sugars during maturation (OLIVEIRA *et al*. 2012).

**Table 2.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on organic acids content and sugar content of fresh blackberries during refrigerated storage.

| Day | Coating | Organic acid (mg/100 g) | | Sugar (g/100 g) | |
|---|---|---|---|---|---|
| | | Ascorbic | Malic | Glucose | Fructose |
| 0 | C | 62.3±0.14* | 31.3±0.22 | 3.0±0.21 | 3.4±0.22 |
| | P | 62.2±0.15 | 31.1±0.21 | 3.0±0.24 | 3.4±0.19 |
| | P+B | 62.1±0.13 | 31.2±0.17 | 3.0±0.18 | 3.4±0.23 |
| 4 | C | 47.5±0.16 | 31.3±0.14 | n.a. | n.a. |
| | P | 49.8±0.23 | 33.1±0.15 | n.a. | n.a. |
| | P+B | 51.8±0.21 | 31.3±0.23 | n.a. | n.a. |
| 8 | C | 53.4±0.19 | 33.2±0.24 | 3.0±0.29 | 3.4±0.27 |
| | P | 54.9±0.22 | 45.6±0.16 | 2.8±0.23 | 3.3±0.21 |
| | P+B | 55.2±0.18 | 46.2±0.13 | 2.7±0.25 | 3.4±0.28 |
| 12 | C | 56.0±0.15 | 47.7±0.15 | n.a. | n.a. |
| | P | 56.4±0.17 | 56.1±0.18 | n.a. | n.a. |
| | P+B | 58.2±0.21 | 56.2±0.22 | n.a. | n.a. |
| 16 | C | 56.8±0.24 | 56.9±0.21 | 2.8±0.25 | 3.2±0.28 |
| | P | 57.5±0.15 | 58.3±0.19 | 2.5±0.22 | 3.1±0.24 |
| | P+B | 58.8±0.21 | 61.3±0.15 | 2.3±0.29 | 3.2±0.23 |

C - control; P - pectin (1%); B – bacteriocin; n.a. - not analyzed; * - ±standard deviation (±SD).

### 3.4. Changes in sugar content

The values of glucose and fructose in blackberries were measured at the beginning (day 0), middle (day 8) and end (day 16) of the storage period (Table 2). The detected sugars in all samples were only glucose and fructose as their content was closer to reported values for wild grown blackberry 35g/kg of fw (MIKULIC-PETKOVSEK *et al*. 2012). However, in our study sucrose was not found in blackberry samples. The results showed that on the 8-th day of the experiment, the concentration of glucose in the control fruit matched the initial level of 3.0 g/100 g of fw, while the glucose level in the treated fruit decreased slightly to 2.8 g/100 g of fw (pectin) and 2.7 g/100 g of fw (pectin+bacteriocin). Thereafter, the concentration of glucose decreased in all treatments and reached levels of 2.8 g/100 g of fw (control), 2.5 g/100 g of fw (pectin) and 2.3 g/100 g of fw (pectin+bacteriocin) by the 16-th day of storage. Fructose levels remained almost constant despite prolongation of the storage time. During the first 8 days of storage, all treatments maintained their initial level

of 3.4 g/100 g of fw, except the pectin-coated fruits, which showed a concentration of 3.3 g/100 g of fw. At the end of the storage period (day 16), the concentration of fructose had decreased slightly in all experimental groups, reaching levels of 3.2 g/100 g of fw (control), 3.1 g/100 g of fw (pectin) and 3.2 g/100 g of fw (pectin+bacteriocin) (Table 2). These results demonstrated that pectin-based edible coatings singly or in combination with bacteriocin did not protect the sugar content in coated blackberries under refrigerated storage conditions.

### 3.5. Changes in total anthocyanins content

As seen in Fig. 6, the initial level of total anthocyanins of 109.2 mg/100 g of fw was in accordance with that reported in the literature (104 – 198 mg/100 g of fw) (PANTELIDIS *et al.*, 2007), but gradually decreased in all treatments. This trend was recorded through the end of the observation period. By the 16-th day of storage, total anthocyanins reached concentrations of 100 mg/100 g of fw (control), 94.2 mg/100 g of fw (pectin) and 92.3 mg/100 g of fw (pectin+bacteriocin), indicating that the application of both coatings failed to delay the reduction of anthocyanins in blackberries during refrigerated storage. The negative effect of low temperatures on anthocyanin concentration during the storage of fresh berries was previously reported by KALT *et al*. (1999). Lowered anthocyanins and ascorbic acid content during cold storage of strawberries was also confirmed by CORDENUNSI *et al.* (2005). However, the authors observed a positive impact on other parameters such as sugars, while levels of flavonols, ellagic acid, TPC and antioxidant activity remained almost the same or even decreased at all tested temperatures (6, 16 and 25°C).

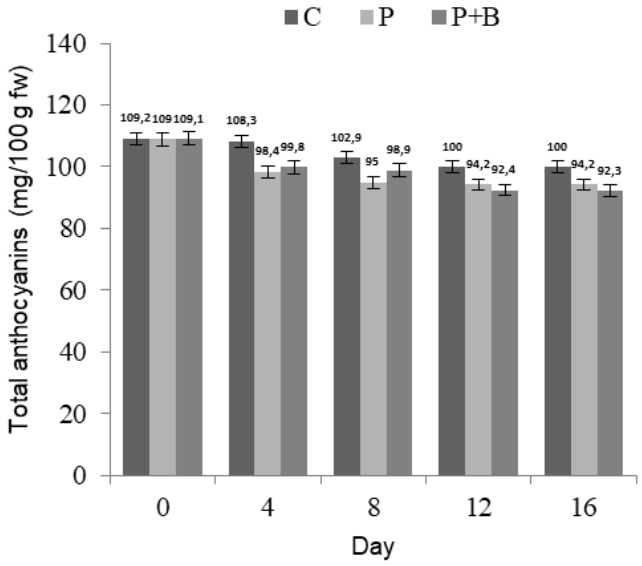


**Figure 6.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the total anthocyanins content of fresh blackberries during refrigerated storage. C - control; P - pectin (1%); P+B - pectin (1%) + bacteriocin.

### 3.6. Changes in total polyphenols content

During the first four days of refrigerated storage, the TPC in both the control and treated blackberries maintained relatively high levels that were close to the initial concentration of polyphenols of 186 mg GAE/100 g of fw (0 day) (Fig. 7). However, by the end of the observation period (16-th day), the TPC declined for all experimental groups to concentrations of 174.2 mg GAE/100 g of fw (control), 173.8 mg GAE/100 g of fw (pectin) and 173.1 mg GAE/100 g of fw (pectin+bacteriocin). These results demonstrated that the coatings did not effectively protect the polyphenolic content, and this progressive decrease was most likely associated with the breakdown of cell structures that is caused by natural senescence processes in the fruits (TUMBARSKI *et al.*, 2019).

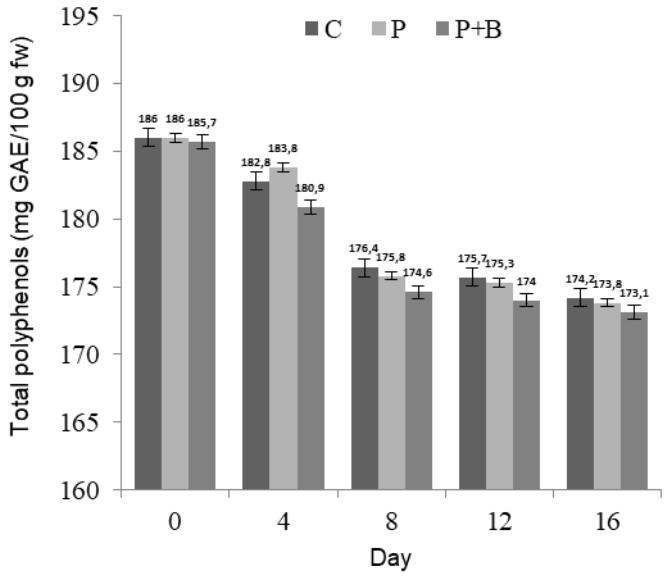


**Figure 7.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the total phenolic content of fresh blackberries during refrigerated storage. C - control; P - pectin (1%); P+B - pectin (1%) + bacteriocin.

### 3.7. Changes in antioxidant activity

The results obtained by the DPPH method showed that the antioxidant activity of control blackberries gradually decreased throughout the monitoring period, reaching its lowest value of 222.2 mmol TE/100 g of fw on the 16-th day. As seen from the results presented in Fig. 8, the pectin and pectin+bacteriocin coatings effectively inhibited this decrease and maintained antioxidant levels in the treated blackberries that were higher than those in the uncoated ones, keeping the antioxidant levels close to the initial value of 233.9 mmol TE/100 g of fw (0 day). The positive impact of pectin and pectin+bacteriocin coatings on antioxidant activity could be associated with a reduction in the respiration rates and the resultant protective effect on other biologically active compounds that possess strong antioxidant activity such as phenolic acids, hydrolysable tannins, vitamin E, carotenoids, minerals and enzymes. Further exploration of these antioxidant compounds is warranted in a future study.

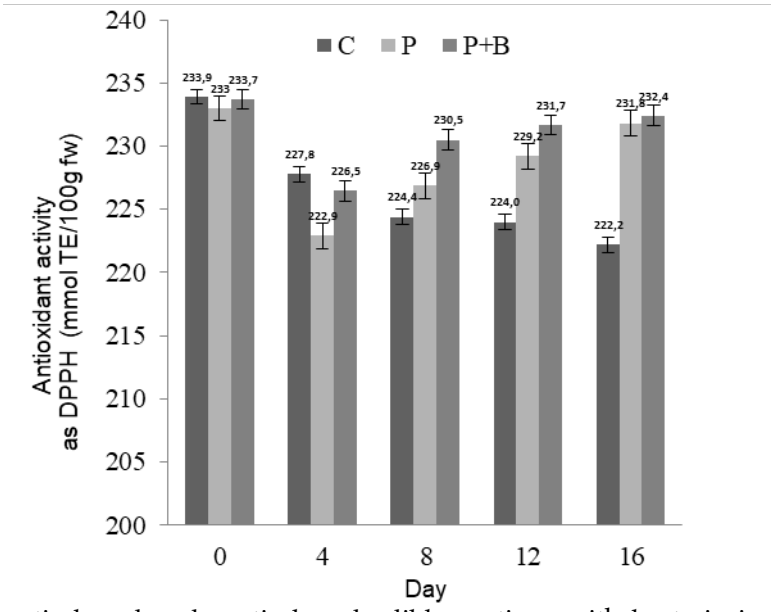


**Figure 8.** Effects of pectin-based and pectin-based edible coatings with bacteriocin of *B. methylotrophicus* BM47 on the antioxidant activity of fresh blackberries during refrigerated storage. C - control; P - pectin (1%); P+B – pectin (1%) + bacteriocin.

The biopreservation of blackberry fruit using different edible coatings has also been investigated by other authors. For example, OLIVEIRA *et al.* (2012) reported the effects of three types of edible coatings - chitosan (1.5%), cassava starch (2.5%) and kefir grains in water (20%) on the storage life of the blackberry cultivar *Tupy* stored at temperatures of 0°C and 10°C. The authors stated that during the 18 days of storage, all coatings effectively reduced the loss of weight at 0°C, but cassava starch and kefir grains-based coatings were not efficient at 10°C. During storage, a significant increase in pH was observed in all treatments at both 0°C and 10°C, with the control fruit showing the lowest pH values. The authors also reported that at 10°C, the anthocyanins levels in all treatments progressively decreased until the 12-th day but thereafter increased significantly in the control fruit and in blackberries with chitosan coatings. At 0°C, the same trend of lowered anthocyanins concentrations in the control and chitosan coatings was observed, while cassava starch and kefir grains-based edible coatings maintained elevated and relatively stable anthocyanins levels.

PÉREZ-GALLARDO *et al.* (2014) developed more hydrophobic edible coatings based on modified tapioca starch added to 0.5 and 1.0% beeswax microparticles and then examined the quality of freshly harvested blackberries during storage at 4°C for 16 days. This study showed that the coated fruit exhibited greater weight loss than uncoated fruit, but the increase in concentration of beeswax particles from 0.5 to 1% led to a decrease in weight loss of 11.55±0.71 and 9.72±0.42%, respectively, compared to the control fruit (7.6±0.13%) at the end of the experiment. Uncoated blackberries showed higher anthocyanins content than coated ones. The coated blackberries with 0.5% beeswax microparticles exhibited low but similar TAC, while those coated with 1% beeswax microparticles revealed higher values and a slight, but significant decrease at the end of the storage period. By the end of the storage period, the TPC was significantly higher in uncoated blackberries than in coated fruit.

GOL *et al.* (2015) evaluated the effects of edible coatings comprised of chitosan, alginate and carboxymethyl cellulose on the improvement in the quality and storage life of Indian blackberry or Jamun fruit (*Syzygium cumini* L.). The results from this study showed that fruit treated with these three coatings at concentrations of 1% and 1.5% exhibited a significant delay in WL along with a reduction in decay percentage as well as positive effects on TSS, pH, TA and sugars in comparison to the uncoated fruit. These coatings also had a positive impact on maintaining a higher concentration of antioxidants during the 16 days of storage and had a positive effect on the inhibition of cell wall-degrading enzyme activities.

## 4. CONCLUSIONS

The present study demonstrated that the application of celery pectin-based edible coatings singly and in combination with a bacteriocin of *Bacillus methylotrophicus* BM47 represents a promising approach for extending storage life in processed blackberries. The bacteriocin-containing edible coatings effectively inhibited the fungal growth, significantly reduced the decay incidence, delayed the increase in TSS and helped to preserve some of the health benefitial properties of the fresh fruits, specifically the ascorbic acid content and antioxidant activity. Our results suggested that the bacteriocin synthesized by *B. methylotrophicus* BM47 in the composition of edible coatings could find a successful application as a biopreservative for improving product quality, storage life and the safety of blackberry fruit.

## ACKNOWLEDGEMENTS

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PAPER

# IMPACT OF HYDROCOLLOIDS ON THE PHYSICO-CHEMICAL AND SENSORY PROPERTIES OF GLUTEN-FREE INSTANT NOODLES FROM RICE FLOUR AND MUNG BEAN STARCH

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## ABSTRACT

The physico-chemical properties of gluten-free (GF) instant noodles prepared from rice flour and mung bean starch containing hydrocolloids that were carboxymethyl cellulose (CMC), hydroxypropylmethyl cellulose (HPMC), guar gum (GG) and xanthan gum (XG) had been investigated. The results were found that the sample that contained CMC had the least fat uptake and cooking time. Different hydrocolloids had no effect on the gelatinization parameters of the noodle dough. The addition of GG improved the textural properties and cooking yields of the GF instant noodles. The samples that were enriched with HPMC showed a significantly lower cooking loss than the others did. An undesirable characteristic was found in the GF noodles that incorporated XG. Microstructural image of sample containing XG revealed a non-continuous matrix. The addition of either GG or HPMC improved the sensory properties of the GF instant noodles.

*Keywords*: gluten-free, guar gum, hydrocolloid, instant noodles, sensory evaluation, thermal properties

## 1. INTRODUCTION

Instant noodles are a popular food consumed in Asian countries due to their convenience, taste, prolonged shelf life, nutrition and low cost. They are an internationally recognized food (GULIA *et al.*, 2014). Moreover, 100 million servings of instant noodles were consumed worldwide in 2017 (WINA, 2018). The main ingredients of instant noodles are wheat flour, water, salt and alkaline salt. However, consuming wheat-containing foods, which contains gluten, may cause an allergenic response, especially in those who have celiac disease. In addition, there is a rising demand for gluten-free (GF) products over the past decade (CHRISTOPH *et al.*, 2018; HOUBEN *et al.*, 2012). One way to develop such a product is by using non-gluten flour, such as rice flour, as a raw material to substitute wheat flour. Rice is hypoallergenic, mild and colorless (GARCIA *et al.*, 2016; WU *et al.*, 2019). It is also a staple food and the most important crop in Thailand.

Rice flour, however, cannot form a cohesive dough structure since rice protein lacks the functionality of wheat gluten (elasticity) (KAWAMURA-KONISHI *et al.*, 2013; SOZER, 2009). Therefore, the application of heat moisture treated rice flour (CHAICHAW *et al.*, 2011), guar gum (GG) (SABBATINI *et al.*, 2014), xanthan gum (XG) (SABBATINI *et al.*, 2014; YALCIN and BASMAN, 2008), locust bean gum (YALCIN and BASMAN, 2008) and hydroxypropylmethyl cellulose (HPMC) (SABBATINI *et al.*, 2014) are added to obtain a viscoelastic dough when preparing the GF noodles.

Hydrocolloids provide a broad range of functional properties that make them suitable for each application (ANTON and ARTFIELD, 2008). Carboxymethyl cellulose (CMC), GG, locust bean gum and alginates are hydrocolloids that widely used in instant wheat noodle processing (GULIA *et al.*, 2014). Hydrocolloids mimic the viscoelastic properties of gluten (ANTON and ARTFIELD, 2008; SUWANNAPORN and WIWATTANAWANICH, 2011) and improve cooking quality and textural properties of gluten-free pasta (MARTI and PAGANI, 2013; PADALINO *et al.*, 2013; SUSANNA and PRABHASANKAR, 2013). The improvement of cooking and textural properties of GF noodles that contains GG has been shown (KUNYANEE *et al.*, 2015; SABBATINI *et al.*, 2014). The incorporation of XG and gelatinized rice flour improved the dough forming ability and the cooking and sensory properties of the rice noodles (YALCIN and BASMAN, 2008). The HPMC and CMC can form thermo-gelling films (MELLEMA, 2003), which result in a reduction of oil absorption in instant fried wheat noodles (REKAS and MARCINIAK-LUKASIAK, 2015) and deep-fried legume snack foods (PRIYA *et al.*, 1996). However, CMC has a small effect on the reduction of fat uptake in instant fried wheat noodles (CHOY *et al.*, 2012). Wheat-rice noodles that contain CMC have an improved textural quality and they possess sensory qualities that are comparable to wheat noodles (SUWANNAPORN and WIWATTANAWANICH, 2011). In addition, CMC and HPMC may have quite different properties i.e. solubility, thermal gelation, thickening capacity that make them adequate as food additives (CORREA *et al.*, 2010).

The ingredients, formulation and processing parameters have an influence on the quality of instant noodles (GULIA *et al.*, 2014). This study, therefore, aims to investigate the effect of hydrocolloids including GG, HPMC, CMC and XG on the qualities of GF instant noodles.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Rice flour was purchased from Varavoot Industry Co. Ltd. in Angthong, Thailand. Mung bean starch was purchased from a local market in Nakhon Pathom, Thailand (Tonson, Sitthinan Co. Ltd). The hydrocolloids used in this study included GG (Union Chemical 1986 Co. Ltd., Bangkok, Thailand), HPMC (Methocel K4M, Dow Chemical Co. Ltd., Samut Prakan, Thailand), CMC (Chemipan Corporation Co., Ltd., Bangkok, Thailand) and XG (Thai Food and Chemical Co. Ltd., Bangkok, Thailand).

### 2.2. Preparation of instant fried noodles

The dough was formulated by mixing 55.6% rice flour and 2.8% mung bean starch, 0.5% salt (NaCl), 0.5% alkaline salt (Sodium carbonate), 5.6% pasteurized liquid whole eggs, 1.7% hydrocolloid and 33.3% water. Rice flour, mung bean starch and hydrocolloid were mixed in a mixer (Kitchen Aid K5SS, USA). The pasteurized liquid whole eggs and water containing dissolved salts and alkaline salt were then added, respectively. The mixer was operated at speed 2 until the resultant dough became crumbly (10 minutes) and then it was manually kneaded to form a dough ball. It was then sheeted using a pasta machine (Marcato ATLAS 150, Italy) to obtain a final thickness of 1.0 mm, and it was cut into strips 15 cm in length and 0.18 cm in width. The noodle strands were steamed for 10 minutes, cooled at room temperature and fried in palm oil (Morakot Industries PCL., Thailand) at 150°C for 45 seconds. The fried noodles were cooled at room temperature and the excess oil was drained. Finally, the instant fried noodles were stored in resealable plastic bags for analysis.

### 2.3. Chemical analysis

The proximate composition (moisture, protein and ash) of the instant noodles was analyzed using the Association of Official Analytical Chemists method no. 925.10, 920.87 and 923.03, respectively (AOAC, 2000) and each sample was carried out in triplicate. An analysis of fat content was performed using automated Soxhlet extraction according to a modified method that is described by REKAS and MARCINIAK-LUKASIAK (2015). The total carbohydrate content was determined by calculation using the difference method (100 - (weight in grams [protein + fat + ash] in 100 g of food (dry solid))).

### 2.4. Scanning electron microscopy (SEM)

The cross-section images of the instant fried noodles prior to cooking in boiling water were observed using a Field Emission Scanning Electron Microscopy (FE-SEM, Tescan Mira3, Kohoutovice, Czech Republic) at the operating voltage of 5.0 kV. The instant noodle samples were fractured into pieces of an approximately 1 cm in length and defatted using the automated Soxhlet extraction. The defatted instant fried noodles were attached to a circular specimen stub with double-sided adhesive tape and coated with gold using a sputter coater.

## 2.5. Thermal properties

An analysis of the thermal properties of the fresh noodles before they were steamed and fried was conducted using a Differential Scanning Calorimeter *(*DSC8000, Perkin Elmer, Shelton, CT, USA*)*. An empty stainless steel pan was used as a reference and each noodle dough sample was weighed in a stainless steel pan. Distilled water was added into the sample pan to bring the water content to 70%. The sample pan was sealed hermetically and equilibrated for 1 hour at room temperature before the DSC measurement. The sample pan was heated from 25°C to 130°C at a ramp rate of 10°C/min to obtain the characterization of gelatinization. The onset temperature ($T_o$), peak temperature ($T_{p1}$ and $T_{p2}$), final temperature ($T_f$), and enthalpy of gelatinization ($\Delta H$) were determined.

## 2.6. Cooking properties

The optimum cooking time, cooking loss and cooking yield (or water absorption) of the instant noodles were determined according to the American Association of Cereal Chemists Official Methods (AACC, 2000) with slight modification. The noodle samples, which was approximately 10g were broken into pieces with an approximate lengths of 5 cm, they were placed in a beaker that contained 120ml of boiling distilled water and then the timing started. The noodle strands were removed from the cooking water at 10 seconds time intervals and they were squeezed between two pieces of glass plates. The time required to the sample as having an "optimum cooking time" was when the opaque central core of the sample disappeared. Samples that were 15g were cooked at optimum cooking time in 180 ml of boiling distilled water. After cooking, the samples were rinsed with 50 ml of distilled water, placed in the water at room temperature for one minute and drained before the weight was recorded. The results were calculated for percentage of "cooking yield." The cooking loss was the amount of solid loss in the cooking water. The cooking water was added to a pre-weighed beaker and evaporated over a steamed bath, and then it was put into hot air oven at a temperature of 105°C until a constant weight was obtained and reported as a percentage of "cooking loss." The analysis was performed in triplicate for each sample.

## 2.7. Texture profile analysis

The texture measurements of the cooked instant noodles were evaluated using a TA-XT2 texture analyzer *(*Stable Micro System, London, England*)* and the procedure described by CHOY *et al.* (2012) with modifications. The samples were cooked for the optimum cooking time as described above and cooled in tap water *(*~17°C*)* for 1 minute. Then, the cooked noodles were retained at room temperature in a covered plastic container. The noodles were compressed using a cylinder probe (P/50) at 2.00 mm/s speed *(*pre-test, test and post-test*)* and 75% strain. The parameters that were obtained from the force-time curve of the texture profiles analysis (TPA) were hardness, adhesiveness, springiness, cohesiveness and chewiness.

## 2.8. Sensory evaluation

The instant noodles were served after cooking and evaluated by fifty untrained panelists who like eating instant noodles (48 females and 2 males). The sensory evaluations were

performed using a 9-point hedonic scale. The panelists scored each sample and assigned scores on a scale of 1 (extremely disliked) to 9 (extremely liked) for appearance, color, firmness, springiness and overall acceptability.

### 2.9. Statistical analysis

A statistical analysis was conducted on the experimental data using a one-way analysis of variance (ANOVA), and a comparison of the means was completed by Tukey's test with a significance level of $p<0.05$. Analysis of variance (ANOVA) was carried out using the SPSS 10.0 (SPSS Inc., USA).

## 3. RESULTS AND DISCUSSION

### 3.1. Proximate composition of instant noodles

The chemical composition of the GF instant noodles are shown in Table 1. Moisture, fat, ash and carbohydrate contents of all samples were 5.01-7.80%, 14.09-18.09%, 1.56-1.94% and 73.98-77.83%, respectively. The moisture content of the noodles was reduced from around 40% to 5-8% when they were fried in oil. The fat content of the fried noodles was generally in a range of between 15-20% (GULIA *et al.*, 2014). All the samples met the standard requirements as specified by the Notification of Ministry of Industry, Thailand. Hydrocolloid type had an influence on moisture and fat contents of the final products. The GF instant noodles that contained GG had the lowest moisture content ($p<0.05$). The lowest fat content was obtained in the sample containing CMC. Fat uptake are related to two main mechanisms: condensation and capillary mechanisms; in both, oil penetrates through the pores inside the product (MELLEMA, 2003). The differences in fat content of instant fried noodles come from the microporous structure of the product and from the amount of water absorbed in the evaporation process (MARCINIAK-LUKASIAK *et al.*, 2019; MELLEMA, 2003). The ability of CMC to reduce oil absorption was linked to its hydrophilic character (ANG and MILLER, 1991) and the final product porosity (PINTHUS *et al.*, 1995).

**Table 1.** Proximate composition of the GF instant fried noodles containing different hydrocolloids.

| Samples | Content (g/100 g of dry basis) | | | | |
|---|---|---|---|---|---|
| | Moisture | Protein$^{NS}$ | Fat | Ash | Carbohydrate |
| GG | 5.01±0.09$^{c}$ | 6.16±0.02 | 18.09±0.19$^{a}$ | 1.56±0.02$^{b}$ | 74.19±0.16$^{b}$ |
| HPMC | 7.80±0.02$^{a}$ | 6.29±0.01 | 18.09±0.08$^{a}$ | 1.67±0.02$^{b}$ | 73.96±0.07$^{b}$ |
| CMC | 7.72±0.07$^{a}$ | 6.15±0.04 | 14.09±0.81$^{b}$ | 1.94±0.03$^{a}$ | 77.83±0.80$^{a}$ |
| XG | 5.97±0.08$^{b}$ | 6.20±0.09 | 16.74±0.65$^{a}$ | 1.86±0.04$^{a}$ | 75.21±0.51$^{b}$ |

Guar gum (GG), hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC) and xanthan gum (XG) were used in this study.
Results are expressed as mean values ± standard deviations.
Means with same superscripts in a row are not significantly different ($p\geq0.05$) as assessed by Tukey's test.
$^{NS}$ = values in the same column are not significantly different (*probability value*, $p\geq0.05$).

In addition, CMC had greater hydrophilicity than HPMC (ADEDEJI A.A. and MO, 2011). The thermal gelation of this hydrocolloid also created an oil-resistant film around the fried product and resulted in an increased in its water holding capacity because it entrapped the food moisture inside and lowered the fat absorption (ANG and MILLER, 1991; SAKHALE *et al.*, 2011; YAZDANSETA *et al.*, 2015).

### 3.2. SEM

The microstructure in the cross section of the GF instant noodle strands (after frying) is shown in Fig. 1. Generally, during the frying process, many microporous were created as the water was quickly removed, which left empty spaces in the hole structures of the noodles that were replaced by oil (HOU, 2001; ZIAIIFAR *et al.*, 2008). The cross-section structure of the GF instant fried noodles that were incorporated with different hydrocolloids had many pores with various sizes and thicknesses. The structure of the instant fried noodles with CMC (Fig. 1c) presented small pore sizes and a fewer number of voids and hollows compared to the others. The sample with XG (Fig. 1d) showed a non-continuous matrix noodle structure, a more open area and a large voids and hollows.

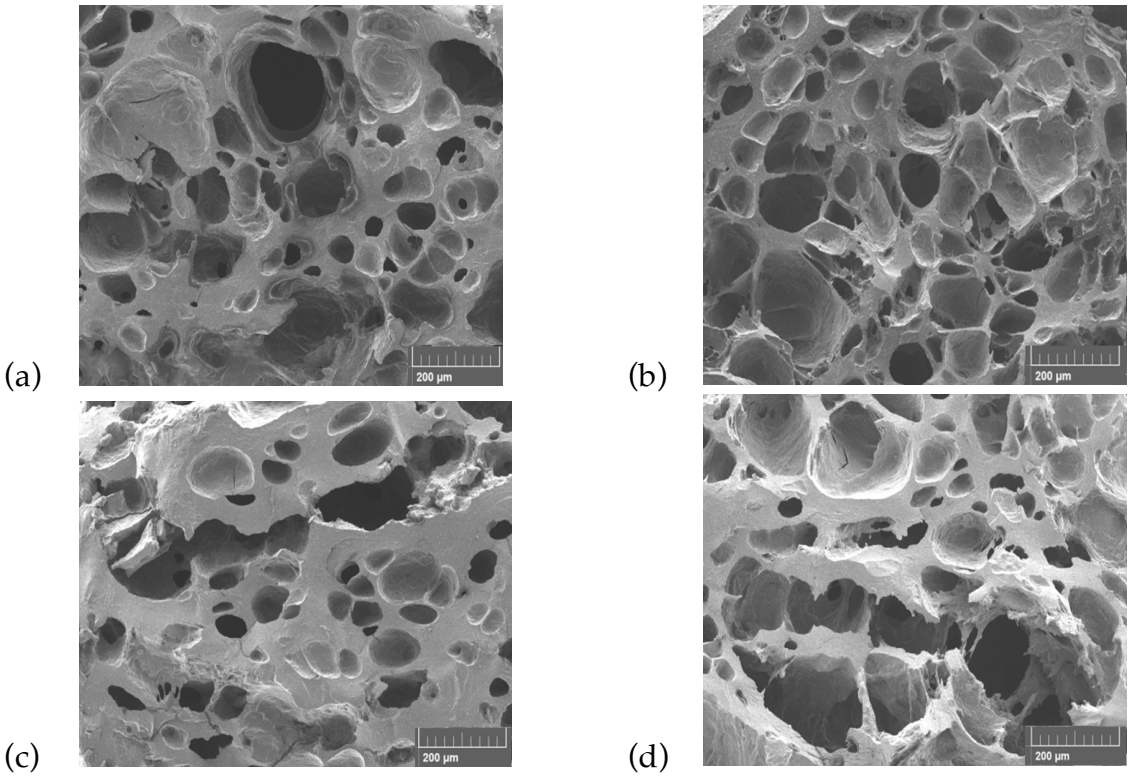


**Figure 1.** Scanning electron microscopy (SEM) of the GF instant fried noodles that contained **(**a**)** guar gum (GG), **(**b**)** hydroxypropylmethyl cellulose (HPMC), **(**c**)** carboxymethyl cellulose (CMC) and **(**d**)** xanthan gum (XG).

### 3.3. Thermal properties

The gelatinization temperature and enthalpy changes of the dough are important for an understanding of these phenomena during the thermal process (SABANIS and TZIA, 2011). All the GF instant fried noodles did not observe an endothermic peak of gelatinization, which indicates that the starches in those samples were fully gelatinized after frying (data not shown). DSC thermograms of the fresh noodles (before steaming and frying) with different hydrocolloids showed biphasic endotherm referred to as G and M1 endotherms, which is in accordance with the finding of PRAKAYWATCHARA *et al.* (2018). The first peak ($Tp_1$ or G endothermic peak) is attributed to the swelling of partially degraded starch chains and the second peak ($Tp_2$ or M1 endothermic peak) reflects the ''melting'' of the remaining crystallites (KIM *et al.*, 2014; XING *et al.*, 2017). The samples with different hydrocolloids were not significantly changed in all thermal properties ($p \geq 0.05$) (Table 2). It indicated that the hydrocolloids used in this study had no effect on the thermal properties of the noodle dough.

**Table 2.** Thermal properties of fresh GF noodles that contained different hydrocolloids.

| Samples | $T_o$ (°C)$^{NS}$ | $T_{p1}$ (°C)$^{NS}$ | $T_{p2}$ (°C)$^{NS}$ | $T_f$ (°C)$^{NS}$ | ΔH (J/g)$^{NS}$ |
|---|---|---|---|---|---|
| **GG** | 68.57±0.15 | 74.10±0.01 | 82.96±0.04 | 90.43±0.10 | 9.31±0.41 |
| **HPMC** | 68.55±0.10 | 74.39±0.09 | 82.86±0.29 | 90.35±0.03 | 8.08±0.64 |
| **CMC** | 68.66±0.13 | 74.33±0.01 | 83.29±0.08 | 90.49±0.08 | 8.60±0.05 |
| **XG** | 68.54±0.06 | 74.10±0.16 | 82.72±0.23 | 90.43±0.07 | 8.26±0.23 |

Guar gum (GG), hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC) and xanthan gum (XG) were used in this study.
Results are expressed as mean values ± standard deviations.
$^{NS}$ = values in the same column are not significantly different (*probability value*, $p \geq 0.05$).

### 3.4. Cooking qualities of the instant noodles

The cooking quality characteristics of the GF instant fried noodles, including optimum cooking time, cooking loss and cooking yield, are presented in Table 3. The optimum cooking times of all the samples ranged from 2.50 to 3.10 minutes. The samples that contained XG had the longest optimum cooking time. The longer optimum cooking times may be attributed to the limited availability of water to the starch granules, which resulted in the retardation of starch swelling (KAUR *et al.*, 2015). On the other hand, the noodles that were incorporated with CMC had the shortest optimum cooking time. This may be due to the carboxyl and hydroxyl groups in the structure of the gum, which allowed them to bind with the readily available water and caused an increment in the swelling index and water absorption (GULL *et al.*, 2016).
Cooking loss was related to the structural strength of the noodles, and a higher value indicated a lower structural strength. Because the soluble starch and other soluble components, including non-starch polysaccharides, leached out into the water during cooking (GULL *et al.*, 2016). The cooking loss for a good quality noodle should be lower than 12% (UGARCIC-HARDI *et al.*, 2007). In this study, the cooking loss of the GF instant noodles was between 8.50 and 14.46% (Table 3). The sample that contained HPMC was the

most effective for reducing the cooking loss because the hydrated HPMC network may have been surrounded in the starch-protein matrix and confined the excessive swelling and diffusion of the amylose content (PURNIMA *et al.*, 2012). Finally, the noodles that contained XG had the highest cooking loss, perhaps because the XG interfered with the gel compactness (HAN *et al.*, 2011) as observed in Fig. 1(d).
The cooking yield explains the ability of the noodles to absorb water during the cooking process (TAN *et al.*, 2016). The highest cooking yield was observed in the samples in which GG was added, followed by CMC, HPMC and XG, respectively. This result may be due to the ability of the GG to absorb water in its interrelated network and interact with starch granules (RODGE *et al.*, 2012).

**Table 3.** The cooking qualities of the GF instant fried noodles incorporating different hydrocolloids.

| Samples | Optimum cooking time (min) | Cooking loss (%) | Cooking yield (%) |
|---|---|---|---|
| GG | 3.00 | $10.84\pm0.86^{b}$ | $297.26\pm6.13^{a}$ |
| HPMC | 3.00 | $8.50\pm0.79^{c}$ | $270.32\pm5.43^{c}$ |
| CMC | 2.50 | $10.34\pm0.59^{bc}$ | $281.11\pm5.29^{b}$ |
| XG | 3.10 | $14.46\pm1.70^{a}$ | $228.79\pm7.49^{d}$ |

Guar gum (GG), hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC) and xanthan gum (XG) were used in this study.
Results are expressed as mean values ± standard deviations.
Means with same superscripts in a row are not significantly different (*probability value,* $p \geq 0.05$) as assessed by Tukey's test.

### 3.5. Textural properties of the instant noodles

Textural characteristics are an important parameter that determines the acceptance of the noodles **(**WU *et al.*, **2015)**. Hardness, springiness, cohesiveness and chewiness of the instant GF noodles with GG addition presented the highest values (Table 4). These results indicated that a strong network could be formed by an interaction between amylose in the molecules of starch and GG, resulting in a three-dimensional structure and an increase in gel hardness (KUNYANEE *et al.*, 2015). The springiness referred to the elasticity of the noodles and assessed the ability of the noodle to regain its original shape after compression (EPSTEIN *et al.*, 2002). The addition of XG presented the lowest amount of springiness in the instant noodles. Similar results have been reported by SUWANNAPORN and WIWATTANAWANICH (2011). XG did not enhance the elasticity of wheat-rice noodles (SUWANNAPORN and WIWATTANAWANICH, 2011) and increase the dough resistance during extension (COLLAR *et al.*, 1999). HPMC was effective for reducing the cooking loss of the instant GF noodles, but it showed a relatively high adhesiveness in the cooked samples. Similarly, HAN *et al.* (2011) also observed the same trend in noodles that contained locust bean gum and they reported that the soluble starch that resided on the noodle surface directly affected their adhesiveness. Finally, they found that this hydrocolloid was also effective for the promotion of a gel matrix formation (HAN *et al.*, 2011).

**Table 4.** The textural characteristics of the GF instant noodles containing different hydrocolloids.

| Samples | Hardness (N) | Adhesiveness (N.sec) | Springiness | Cohesiveness | Chewiness |
|---|---|---|---|---|---|
| GG | 34.12±2.19$^{a}$ | 0.78±0.13$^{b}$ | 0.88±0.02$^{a}$ | 0.71±0.02$^{a}$ | 21.17±1.94$^{a}$ |
| HPMC | 30.18±2.63$^{b}$ | 1.02±0.16$^{a}$ | 0.85±0.05$^{ab}$ | 0.64±0.02$^{b}$ | 16.52±2.13$^{b}$ |
| CMC | 25.86±2.73$^{c}$ | 1.07±0.10$^{a}$ | 0.84±0.05$^{ab}$ | 0.69±0.04$^{a}$ | 15.02±2.23$^{bc}$ |
| XG | 27.70±2.60$^{c}$ | 0.67±0.13$^{c}$ | 0.82±0.05$^{b}$ | 0.61±0.03$^{b}$ | 13.86±2.17$^{c}$ |

Guar gum (GG), hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC) and xanthan gum (XG) were used in this study.
Results are expressed as mean values ± standard deviations.
Means with same superscripts in a row are not significantly different (*probability value, p*≥0.05) as assessed by Tukey's test.

### 3.6. Sensory evaluation

The results of the sensory evaluation of the cooked GF instant noodles with mixed hydrocolloids are presented in Table 5. The instant noodle that contained GG and HPMC had significantly higher ($p$<0.05) scores for color, firmness, springiness and overall acceptability. The scores for springiness of the cooked instant noodles were improved with the addition of GG and HPMC. They have medium correlation with hardness (r = 0.621, p<0.05), springiness (r = 0.588, p<0.05) and chewiness (r = 0.631, p<0.05) evaluated by instrument (Table 3). The samples that contained GG and HPMC exhibited the highest overall acceptability scores. On the other hand, the samples that contained CMC and XG showed lower overall qualities, namely that they possessed less elasticity and softer texture than the others.

**Table 5.** The sensory evaluation of the GF instant noodles containing different hydrocolloids.

| Samples | Appearance$^{NS}$ | Color$^{NS}$ | Firmness | Springiness | Overall acceptability |
|---|---|---|---|---|---|
| GG | 6.58±1.18 | 6.92±1.07 | 6.34±1.38$^{ab}$ | 6.18±1.41$^{a}$ | 6.58±1.11$^{ab}$ |
| HPMC | 6.90±1.22 | 7.00±1.07 | 6.58±1.07$^{a}$ | 6.18±1.55$^{a}$ | 6.70±1.22$^{a}$ |
| CMC | 6.64±1.10 | 6.68±1.08 | 5.92±1.61$^{b}$ | 5.56±1.77$^{ab}$ | 6.14±1.37$^{bc}$ |
| XG | 6.52±1.30 | 6.78±1.15 | 6.08±1.58$^{ab}$ | 5.20±1.71$^{b}$ | 5.98±1.44$^{c}$ |

Guar gum (GG), hydroxypropylmethyl cellulose (HPMC), carboxymethyl cellulose (CMC) and xanthan gum (XG) were used in this study.
Results are expressed as mean values ± standard deviations.
Means with same superscripts in a row are not significantly different (*probability value, p*≥0.05) as assessed by Tukey's test.
$^{NS}$ = values in the same column are not significantly different (*probability value, p*≥0.05).

## 4. CONCLUSIONS

The GF instant noodles that consisted of GG and HPMC provided a comparable sensory score. Compared with HPMC, the sample with GG had a significantly higher cooking

yield, cooking loss, hardness, cohesiveness and chewiness, and it also presented a significantly lower adhesiveness. CMC was effective for the reduction of fat uptake and required the least cooking time. Thermograms of the fresh noodles with different hydrocolloids before they were steamed and fried showed no significant difference.

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PAPER

# COLD AND HOT SMOKED NILE TILAPIA FILLETS: QUALITY AND YIELD OF PIGMENTED AND UNPIGMENTED FILLETS

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## ABSTRACT

Aiming evaluate the effects of smoking techniques on the quality of tilapia fillets, a 2x2 factorial scheme experiment was conducted comprising two smoking techniques (hot and cold) and two pigmentation (with and without). Cold smoked fillets and fillets with pigmentation demonstrated a greater yield. Hot smoked fillets were tenderer and presented lower moisture and greater ash and protein contents. The pigmentation did not influence the smoked fillet composition, but ash content was greater in fillets without pigmentation. The sensory acceptance of hot smoked fillets was better. The pigmentation influenced the color and appearance; however, fillets without pigmentation gave better flavor.

*Keywords*: benzo(a)pyrene, chemical composition, *Oreochromis niloticus*, organoleptic aspects, smoked yield

## 1. INTRODUCTION

Smoking is a technique that has been used since antiquity in order to preserve food from the effects of natural degradation and oxidation (VARLET *et al*., 2007). The degree of conservation of the fish depends on the synergistic actions between the addition of salt, the preservative effects from smoke compounds (phenols, aldehydes, and organic acids), and the dehydration that occurs during the smoking process (Fuentes *et al*., 2010). However, smoking is currently more utilized for its organoleptic qualities, as it is a process that provides fewer preservative benefits but more sensory qualities such as aroma, flavor, color, and it also adds value to the product (CARDINAL *et al*., 2006). Smoked fish is a highly nutritional food that contains polyunsaturated fatty acids, fat-soluble vitamins, essential minerals, and essential amino acids for humans (BILGIN *et al*., 2008).

Traditionally, there are two methods of smoking: hot and cold; these differences are obtained by temperature changes in the smoking chamber. Cold smoking is done at 33°C so that the intense thermal treatment is avoided and the nutrient structure is preserved (ARVANITOYANNIS and KOTSANOPOULOS, 2012). As a result, cold smoking does not offer adequate protection against harmful microorganisms, thus decreasing the shelf-life of the cold smoked fish. In the hot smoking process, the temperature ranges from 70 to 80°C, which results in baking of the meat (ARVANITOYANNIS and KOTSANOPOULOS, 2012). The heat and dehydration reduce the water activity of the fish, thus limiting the growth of microorganisms and increasing the shelf-life (ABOLAGBA and OSIFO, 2004).

The smoking process occurs in three steps: salting, heating, and smoking. Salt is used to preserve and to enhance the flavor of the smoked fish (GUIZANI *et al*., 2014), to help in the dehydration process, inhibit microorganism growth, and extract the salt soluble protein (CHENG *et al*., 2007).

The appearance is the first factor that influences the consumer who is buying smoked products. To get a better color in smoked fish, artificial coloring can be added, or the smoking time can be extended, the latter of which would lead to more weight loss in the fish and possible economic losses (BERAQUET and MORI, 1984). In the smoking process, color can be added to either intensify or subdue the golden red color. Natural dyes in foods have been utilized to give or to intensify color as well as to restore the color in products after the smoking process. The artificial pigmentation can ensure greater color uniformity, and the product becomes more attractive, which can significantly influence its acceptability with consumers.

Nile tilapia (*Oreochromis niloticus*) is the fourth most farmed fish worldwide (FAO, 2018) and the most widely farmed in Brazil (PEIXEBR, 2019). Its meat has a high nutritional value, featuring good taste and texture, and its fillet provides a good acceptance (SOUZA *et al*., 2015).

Therefore, an experiment was conducted to evaluate the effects of hot and cold smoking techniques on the quality, yield, and organoleptic characteristics in fillets of *in natura* Nile tilapia (*Oreochromis niloticus*).

## 2. MATERIALS AND METHODS

### 2.1. Animals and experimental procedures

Method was carried out in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).
We used 250 Nile tilapia (*Oreochromis niloticus*) and submitted them to depuration for 48 hours in tanks with running water and without feed. After the depuration, animals were euthanized by severing the spinal cord followed by hand filleting. The fillets were vacuum-packed and kept cooled for 12 hours until the smoking time. From the 500 fillets that were obtained, half were assigned to the cold smoking treatment while the other half was assigned to hot smoking. The two fillets from each fish were randomly distributed to be pigmented or not.
The smoking of the fillets was achieved by using an industrial smoker (model Arprojet, Arprotec, Valinhos, Brazil), with smoke produced via wood friction (wooden rafter from pink eucalyptus).
For the smoking process, the fillets (with and without pigmentation) were immersed in a 20% brine solution at a 2:1 ratio of brine solution (weight/volume) for 30 minutes. After this period, the fillets were washed in running water and were placed in the screen of the smoking cart.
For the pigmentation process, the fillets were submerged in a water solution (1 kg fillets/1 liter of solution) with annatto extract (6 mL/L) for 15 minutes. The pigmented and unpigmented fillets were taken to the cold chamber (0 to 1°C), where they remained for 7 hours to remove superficial water (drainage). Afterwards, the fillets were placed in the smoking chamber to achieve partial drying (cold smoking = 30ºC and hot smoking = 50ºC) for 60 minutes. After, smoke was added to the process; the temperatures in the hot smoking treatment ranged from 50ºC to 80ºC for 3 hours, while in the cold smoking treatment the temperatures ranged from 30ºC to 40ºC for 5 hours.
At the end of the smoking process, the fillets were taken to a cold chamber (0° to 1ºC), and after the cooling process, they were vacuum packed and individually labeled.

### 2.2. Scanning electron microscopy

Three samples from the dorsal part of the smoked fillets were removed for scanning electron microscopy analysis; they were fixed in 2.5% buffered glutaraldehyde and then received 1% osmium tetroxide for 2 hours. Afterwards, the samples were washed in phosphate buffer, dehydrated in ethanol, and dried at a critical point with $CO_2$. The specimens were metallized with gold-palladium ions and electron-micrographed with JEOL JSM-5410.

### 2.3. Fillet yields and areas

In order to determine the yield, 90 fillets per treatment were utilized, and each weight was multiplied by 2 (number of fillets per fish). All yield data was calculated in relation to the total animal weight. Fillet yields were analyzed for *in natura* and smoked treatments, and calculations were made to determine the losses that occurred during the smoking process (*in natura* fillet yield minus smoked fillet yield).
To determine the fillet area, the fillets were placed on parchment paper and were circumscribed by using a pencil. After, a geographic information system was utilized with

the Geocoded Information Processing System – Spring program (INPE, 1999), which was developed by the National Institute of Spatial Research – INPE in order to complete the calculation of the fillet areas.

**2.4. Quality parameters in smoked fillets compared with in natura fillets**

2.4.1 Chemical composition analysis

Four fillets per treatment collected before and after the smoking process were used for the chemical composition analysis. These samples were packed in plastic bags, identified, and stored at -18ºC until the analyses.
The samples of *in natura* and smoked fillets were ground using a multiprocessor to obtain a homogeneous sample. Aliquots from this sample were used for the chemical composition determinations (4 replications; moisture, ash, and crude protein) according to the official methodology of AOAC (2005), while total lipid determination was achieved based on the methodology described by BLIGH and DYER (1959).

**2.5 Color, texture, and pH**

Ten smoked fillets per treatment and three *in natura* fillets (control) were used for the evaluation of the color. The fillet color was determined using a portable colorimeter (MiniScan XE, HunterLab, Reston, VA, USA) with a D65 light source (6500ºKelvin), observation angle of 10º, and a 30 mm opening measurement cell while using the scale L*, a*, and b* of the CIELab system that was developed by Judd and Hunter (HUNTER, 1975).
The shear force was determined in 10 smoked fillets per treatment and 3 *in natura* fillets (control). The fillets were sheared with a Warner Bratzler catheter at 500 mm/min by using a texturometer (TA.XT2i, SMS, Surrey, England). The force as a function of the deformation was calculated using the average of 10 measures in different positions per fillet sample, which was expressed in kg using the program Texture Expert V.1.15 (SMS, Surrey, England).
The pH of the smoked fillets was determined using a portable pH meter (DM2, Digimed, São Paulo, Brazil) in 10 fillets per treatment after the end of the smoking process.

**2.6. Organoleptic characteristics**

For the sensory analysis, the method utilized to evaluate the acceptability of the products was the preference test, which represents the sum of all sensory perceptions and considers the opinions of the consumers. This test measures consumer preference in order to predict the acceptability of a product. Thus, the acceptance attributes test was conducted by using a 9-point hedonic scale varying from "extremely disliked" (1 point) to "extremely liked" (9 points), as according to DUTCOSKY (2007).
Forty non trained tasters were used for the sensory evaluation. Within 36 hours after the smoking process, the fillets were cut and the samples were standardized in terms of weight and portion of the fillet (25 g). Then, the samples were packed in aluminum papers and identified. The tasters randomly received the samples on plates coded with three random numbers, and a sheet for sensory analysis was provided to evaluate flavor, internal color, aroma, texture, salt content, and general acceptance. Also, entire fillets per treatment were evaluated in terms of appearance attributes and the superficial fillet color.

### 2.7. Benzo(a)pyrene production

Aliquots (n = 4) from samples of *in natura* and smoked fillets that were utilized in the chemical composition were also used to determine the benzo(a)pyrene content. The samples were subjected to saponification with methanol KOH, liquid-liquid extraction with cyclohexane and dimethylformamide water (9:1, v/v), and cleaning via column chromatography of silica gel. The determination of benzo(a)pyrene was performed using high performance liquid chromatography with fluorescence detection (HPLC).

### 2.8. Experimental design

The experiment was completely randomized in a 2 × 2 factorial design consisting of two smoking techniques (HS = hot smoking and CC = cold smoking) and two pigmentation treatments (FWP = fillet with pigmentation and FOP = fillet without pigmentation) with 90 replications per treatment to determine yield analysis and losses during processing. For the chemical composition (n = 4), color determination (n = 10) and texture (n = 10) characteristics of smoked fillets, *in natura* fillets (control) were added in the design. Three *in natura* replications were used for color and texture, while for the other measurements we used the same number of replications as the smoked fillets (n = 4). For the determination of fillet area (n = 20) the type of process was included in the analysis (*in natura* and smoked). For the determination of benzo(a)pyrene (n = 3), we compared the HS and CC fillets with the *in natura* fillets (control). The fillet was considered the experimental unit.

The results of the analyzed variables were submitted to variance analysis by GLM procedure from the statistical computer program Statistical Analysis System (SAS, 2005). and the means were compared using the Tukey test with a 5% probability level. For the sensory analysis, the Friedman test (Chi-square test) was utilized with the non-parameterized Tukey test ($\alpha = 0.05$).

## 3. RESULTS

### 3.1. Yield, area, and area losses due to processing

*In natura* fillets presented with a similar weight, while smoked fillets presented with an average weight of 73.77 g/fillet. The smoking and the pigmentation processes affected the smoked fillet weight (Table 1). The hot smoked fillets presented with a lower weight ($p<0.01$) than cold smoked fillets, while the pigmented fillets were heavier ($p<0.01$) than those without pigmentation. A significant difference was not observed in the fillet yields.

A significant interaction was observed ($p<0.01$) in the smoked fillet yield and the smoked fillet losses (Table 1, Fig. 1). We observed that the cold smoking technique demonstrated a greater yield independent of fillet pigmentation (fillets with pigmentation = 24.90%) and lack of pigmentation (fillets without pigmentation = 25.03%) (Fig. 1A). Considering hot smoked fillets, the fillets with pigmentation presented with a greater yield (23.85%) than fillets without pigmentation (22.20%).

Regarding the losses that occurred during the smoking process, an interaction was observed between smoking techniques and pigmentation (Table 1, Fig. 1B). Cold smoked fillets (fillets with pigmentation = 11.54% and fillets without pigmentation = 11.52%)

presented with lower losses during the smoking process than hot smoked fillets (fillets with pigmentation = 12.51% and fillets without pigmentation = 14.43%).

**Table 1.** Means of weight, yield, losses and areas that occurred during the processing of Nile tilapia fillets (*Oreochromis niloticus*) that were submitted to cold and hot smoking techniques, with and without pigmentation.

| Factors of variation | Fillet weight (g) | | Yield (%) | | Smoked losses (%) |
|---|---|---|---|---|---|
| | *in natura* | Smoked | Filleting | Smoked | |
| Smoking technique (T) | | | | | |
| Cold (CC) | 112.32±17.25 a | 76.94±13.35 a | 36.51±1.12 a | 24.97±1.68 | 11.53±1.52 |
| Hot (HS) | 112.43±16.10 a | 70.81±11.56 b | 36.52±0.90 a | 22.98±1.96 | 13.51±1.97 |
| Pigmentation (P) | | | | | |
| With (FWP) | 113.37±16.87 a | 75.82±12.67 a | 36.40±0.95 a | 24.37±2.19 | 12.03±2.08 |
| Without (FOP) | 111.43±16.42 a | 71.82±12.68 b | 36.59±1.05 a | 23.54±1.89 | 13.05±1.84 |
| F test | | | | | |
| Technique (T) | 0.01ns | 14.67** | 0.00 ns | 77.42** | 87.04** |
| Pigmentation (P) | 0.83ns | 5.84* | 2.25 ns | 11.71** | 20.81** |
| Interaction T × P | 0.02ns | 3.55ns | 0.36 ns | 16.20** | 21.63** |

Means in the same column that are followed by different letters differed by Tukey test (P<0.05) ** Significant ($p$<0.01); ns- non-significant ($p$>0.05). (*) Means with the same lowercase letter for smoking technique within the kind of pigmentation, while uppercase letters for pigmentation within smoking technique did not differ by Tukey test (P>0.05). Data expressed as mean ± standard deviation.

Smoking technique significantly affected (P<0.01) the fillet area. The hot smoked fillets had a greater superficial area (41.92 $cm^2$) than cold smoked fillets (37.17 $cm^2$). The use of pigments did not affect the fillets' area, but a significant reduction ($p$<0.01) in area was observed when the fillet processing technique was evaluated (*in natura* = 41.99 $cm^2$ and smoked = 37.11 $cm^2$). The smoking process decreased the fillet area by 11.62% (4.88 $cm^2$) when compared to *in natura* fillets. The pigmentation did not interfere ($p$>0.05) in the loss of fillet area.

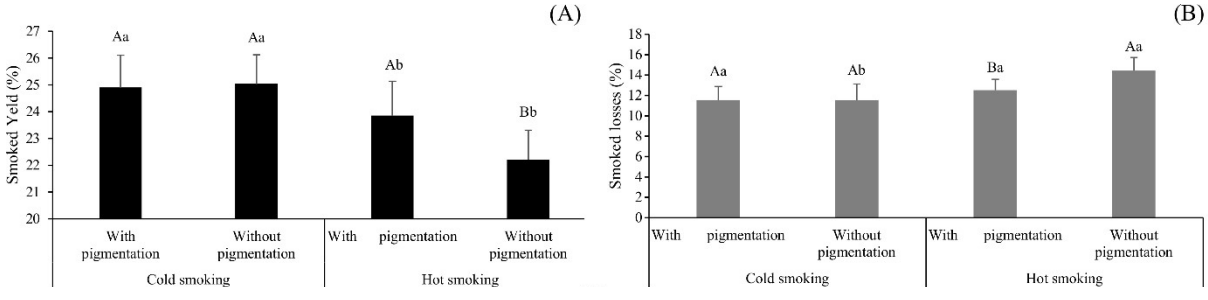


**Figure 1**. (A) Smoked yeld (%); (B) Smoked losses (%), unfolding of the interaction between Smoking Technique *x* Pigmentation in fillets of Nile tilapia (*Oreochromis niloticus*). (*) Means followed by the same lowercase to the factor Smoking Technique and uppercase to Pigmentation does not differ by Tukey test ($p$>0.05). Vertical bars represent the standard deviation of the mean.

### 3.2. Chemical composition

Variation occurred in the chemical composition of *in natura* fillets when compared to the final product (Table 2). Hot smoked fillets had a lower moisture content (68.90%) than cold smoked fillets (72.24%), while crude protein and ash contents were significantly greater (25.20 and 3.85% respectively) in hot smoking.
The smoking technique did not influence total lipid content (Table 2). It was observed that fillet pigmentation affected the ash and energy content. The pigmented fillets presented with lower ash contents (2.84%) than those without pigmentation (4.37%).

**Table 2.** Means of chemical composition[*] of Nile tilapia fillets (*Oreochromis niloticus*) submitted to cold and hot smoking techniques with and without pigmentation.

| Factors of variation | Moisture (%) | Protein (%) | Lipids (%) | Ash (%) |
|---|---|---|---|---|
| Smoking technique (T) | | | | |
| Cold (CC) | 72.24±2.37 a[(1)] | 22.50±2.30 b | 2.10±0.25 a | 3.19±0.87 b |
| Hot (HS) | 68.90±2.29 b | 25.20±1.71 a | 2.08±0.99 a | 3.85±0.91 a |
| Pigmentation (P) | | | | |
| With (FWP) | 71.45±2.60 a | 23.60±1.98 a | 2.19±0.98 a | 2.84±0.43 b |
| Without (FOP) | 69.67±2,92 a | 24.07±2.87 a | 2.04±0.26 a | 4.37±0.58 a |
| Control = *in natura* | 80.83±0.58 | 17.48±0.13 | 1.18±0.07 | 1.00±0.01 |
| F test | | | | |
| Control vs Fatorial | 102.62** | 45.36** | 5.74* | 227.61** |
| Smoking technique (T) | 13.46** | 10.31** | 0.004 ns | 23.59** |
| Pigmentation (P) | 3.74 ns | 0.45 ns | 0.26 ns | 103.60** |
| Interaction T x P | 0.09 ns | 0.31 ns | 0.47 ns | 0.32 ns |

[(1)]For each factor, means of the same factor in a column that are followed by the same letter did not differ by Tukey test ($p > 0.05$). ns - Non-significant ($p > 0.05$) *Significant ($p < 0.05$) **Significant ($p < 0.01$)
(*) 2 replications per sample were used for protein, lipids, and ash content, while 3 replications were used for moisture content. Moisture content is showed in Franco *et al*. (2013). Data expressed as mean ± standard deviation.

### 3.3. Color, texture, and pH of the fillets

When analyzing the fillet color (Figure 2), we found that unpigmented *in natura* fillets had an average chroma value of a* and b* of -1.5 and 9.54, respectively. The values of a* and b* increased in function of the smoking technique and pigmentation. Pigmented fillets had values for a* and b* that were significantly greater than those without pigmentation, while the hot smoking process yielded greater chroma values than cold smoked fillets.
The unpigmented hot smoked fillets presented with a lightness (66.04) that was significantly greater ($p < 0.05$) than pigmented fillets (62.45). Cold smoked fillets with pigmentation (48.74) did not differ from those cold smoked fillets with pigmentation (49.46) in terms of lightness.

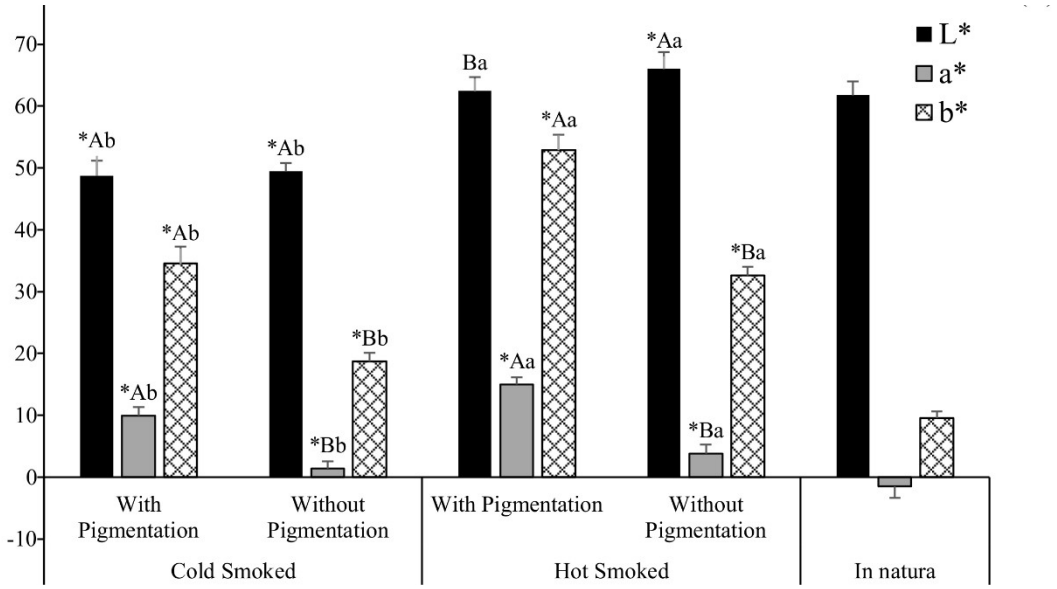


**Figure 2.** Means of color system (CIELab) for smoked and *in natura* fillets with interactions between smoking technique and fillet pigmentation. Means following the same uppercase letter (lowercase) for the factor pigmentation within each smoked technique (smoked technique within each pigmentation) did not differ by Tukey test at 5% of probability level. *indicates that for each smoking technique and pigmentation treatment, the mean differs from control (*in natura*). Vertical bars represent the standard deviation of the mean.

There was an interaction between smoking techniques and pigmentation for shear force of the fillets (Fig. 3A). Cold smoked fillet shear force values (with pigmentation = 6.40 kg and without pigmentation = 6.11 kg) were greater ($p$ <0.05) than hot smoked fillets values (with pigmentation = 2.71 kg and without pigmentation = 3.09 kg). There was a significant difference between smoking techniques and pigmentation in fillets in comparison to *in natura* fillets.

Through scanning electron microscopy, we can observe the film surrounding the fillets (Fig. 3B). In cold smoked fillets, collagen fibers were observed (Fig. 3C).

Cold smoked fillets presented with a lower ($p$ <0.01) pH value (6.43) than HS fillets (6.94). The fillets without pigmentation had a lower pH (6.59) in relation to fillets with pigmentation (6.78) due to dehydration in fillets without pigmentation. The pH changes that occurred in cold versus hot techniques are due to water loss during the process, with more water loss occurring in hot smoked fillets (pH = 6.94). The greatest smoking time decreased the pH values in cold smoked fillets (pH = 6.43).

### 3.4. Benzo(a)pyrene production

When analyzing the benzo(a)pyrene content in cold smoked fillets (0.45 $\mu$g/kg) and hot smoked fillets (0.49 $\mu$g/kg) and comparing them to *in natura* fillets (0.26 $\mu$g/kg), we observed that the smoking process affected the benzo(a)pyrene content, wich was significantly higher ($p$ <0,05) in the fillets subjected to smoking (regardless of type, hot or cold) compared to fillets *in natura*.

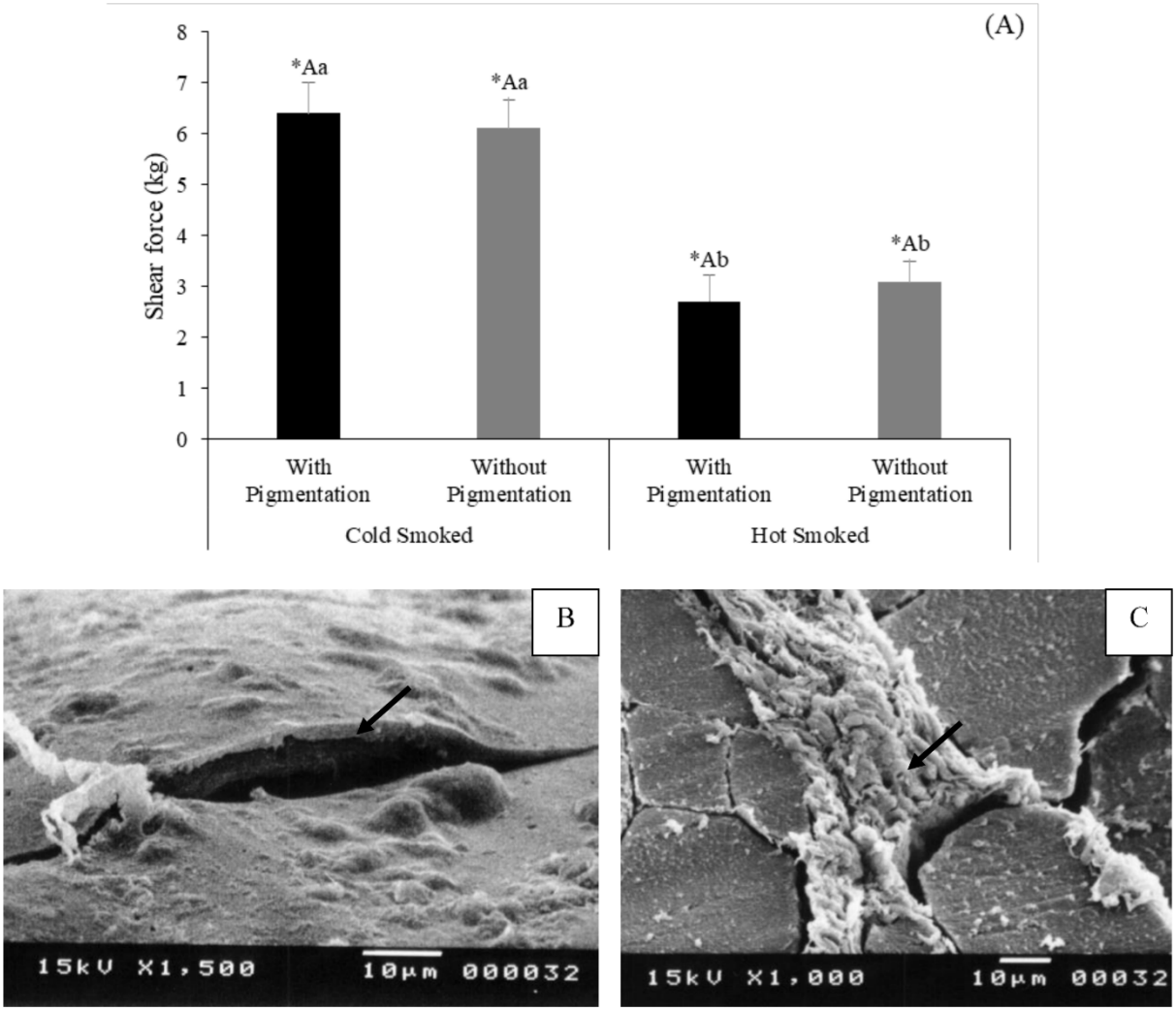


**Figure 3.** (A) Means of shear force (kg) of interaction when compared between smoking technique (cold and hot) and pigmentation (with = FWP and without = FOP). Means following the same uppercase (lowercase) letter for the factor pigmentation within of each smoking technique (smoking technique within each pigmentation) did not differ by Tukey test at a 5% probability level. * indicates that for each smoking technique and pigmentation treatment, the mean following the * differs from control (*in natura*). Vertical bars represent the standard deviation of the mean. (B) Electron-micrograph of smoked fillets depicting the film that is formed by protein denaturation and leaching of fat, which is associated with the compound action generated by organic matter pyrolysis. (C) Presence of collagen fiber bundles among muscle tissue of cold smoked fillets.

### 3.5. Organoleptic characteristics

There was a significant effect ($p$ <0.05) of smoking technique on flavor, aroma, texture, salt content, superficial and internal color, and general acceptance. Hot smoked fillets were the best evaluated by tasters. The pigmentation had a significant effect on attributes such as appearance, flavor, superficial color, and salt content in smoked fillets. Fillets with pigmentation received greater scores than fillets without pigmentation for appearance, superficial and internal color, and flavor (Table 3).

There was a significant interaction ($p$<0.05) between smoking technique and pigmentation on salt content. Hot smoked fillets presented with greater scores by tasters when compared to pigmented and unpigmented cold smoked fillets in terms of salt content.

**Table 3.** Probability value for the Friedman Chi-square test, means and non-parameterized Tukey multiple comparison test values ($\alpha$ = 0.05) based on the scores assigned by tasters for Nile tilapia smoked fillets.

| | Entire fillet | | | Portion fillet | | | | |
|---|---|---|---|---|---|---|---|---|
| | Appearance[(1)] | Color | Internal color | Aroma | Flavor | Texture | Salt content | General acceptance |
| Smoking technique (T) | | | | | | | | |
| Cold (CC) | 6 a | 6 b | 5 b | 5 b | 4 b | 4 b | 5,5 b | 4 b |
| Hot (HS) | 6 a | 7 a | 8 a | 7 a | 8 a | 8 a | 8 a | 7 a |
| Pigmentation (P) | | | | | | | | |
| With (FWP) | 7 a | 7 a | 6 a | 6 a | 6 b | 6 a | 6 b | 6 a |
| Without (FOP) | 5 b | 6 b | 6 a | 6 a | 7 a | 6 a | 7 a | 7 a |
| F test | | | | | | | | |
| Smoking technique (T) | 0.6985 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Pigmentation (P) | 0.0001 | 0.0001 | 0.3897 | 0.3481 | 0.0445 | 0.4320 | 0.0183 | 0.2893 |
| Interaction T×P | 0.8575 | 0.4652 | 0.4497 | 0.7237 | 0.1573 | 0.0947 | 0.0112 | 0.0679 |
| Coeficiente of variation (%) | 3.85 | 1.89 | 3.22 | 2.89 | 1.66 | 2.52 | 3.01 | 2.99 |

[(1)]For each factor, means in the same column that have the same letter did not differ significantly by the non-parameterized Tukey multiple comparison test ($\alpha$ = 0.05).

## 4. DISCUSSION

In the present study, the losses that occurred during the smoking process were greatest for hot smoked fillets (13.51%). According to SIGURGISLADOTTIR *et al*. (2000), fillet weight losses between 10 and 25% during the smoking process, due to dehydration and fat leaching in muscle tissues, is commonly observed.

A significant difference was not observed in the fillet yields. Thus, we can infer that fish weights were homogeneous, and that we used one filleting method and the same person to perform the filleting.

The cold smoking technique demonstrated a greater yield independent of fillet pigmentation and lack of pigmentation. Considering hot smoked fillets, the fillets with pigmentation presented with a greater yield than fillets without pigmentation. These results were also observed by FRANCO *et al*. (2010), who reported that Matrinxa fillets with skin (*Brycon cephalus*) had a lower hot smoked fillets yield (33.79%) when compared with cold smoking yields (34.46%).

Regarding the losses that occurred during the smoking process, cold smoked fillets presented with lower losses during the smoking process than hot smoked fillets. Although pigmentation did not interfere in the losses for cold smoked fillets, it did affect hot smoked fillets. This fact is due to the dehydration that occurred in the fillets, as hot

smoked fillets presented with lower moisture contents; despite the fact that there was no significant difference observed, the fillets with pigmentation had the lowest moisture content.
The smoking process decreased the fillet area by 11.62% when compared to *in natura* fillets. Thus, the losses that occurred during the smoking process are related to the weight, thickness, and area of the fillets. The hot smoked fillets demonstrated lower losses in fillet area compared to cold smoked fillets; the temperature used in the process led to greater losses in the product due to the greater reduction in moisture content of the fillet.
In the chemical composition, the greater values for crude protein and total lipids observed in smoked fillets in relation to *in natura* fillets are due to the effects of dehydration. These results were also observed by VASILIADOU *et al*. (2005) for hot smoked dourade (*Sparus aurata*); the total lipid concentration increased from 7.55% (*in natura* fish) to 12.92% (smoked fish), crude protein content ranged from 20.65% (*in natura*) to 25.67% (smoked), while moisture content decreased from 69.96 % to 57.45% after the smoking process.
The greater ash content in the *in natura* fillets compared to the smoked fillets may be due to sodium chloride absorption in the muscle tissue during the brining process of the smoked fillets. This result may also be due to the varied nutrient concentration, which is a side effect of moisture losses during dehydration, which takes place during the smoking process.
The pigmented fillets presented with lower ash contents, and this can be related to the loss of salt in the fillets due to the annatto extract solution that was used in the fillet pigmentation process.
When analyzing the fillet color, the values of a* and b* increased in function of the smoking technique and pigmentation. The processing technique (time x temperature) that was used influenced the chroma a* and b* and led to lower chroma values in cold smoked fillets. CARDINAL *et al*. (2001), when studying Atlantic salmon (*Salmo salar*), relayed that the temperature did not affect these two color parameters. BERAQUET and MORI (1984) observed that smoking time contributed intensively to color formation in smoked fishes (mackerel *Scomber japonicus*); in other words, cold smoked fishes (8 hours at temperature below 35ºC) presented with a golden yellow color that was more intense than hot smoked fishes due to the long exposure time of the fillets to smoke.
It was theorized that reactions between carbon compounds and proteins are responsible for color formation in the smoked fish surface, while absorbed phenolic compounds are deeply related to the flavor and aroma of the smoked product (HUDA *et al*., 2010).
There was an interaction between lightness (L*) and chroma a* and b*. Pigmented fillets had values for a* and b* that were significantly greater than those without pigmentation, while the hot smoking process yielded greater chroma values than cold smoked fillets.
The smoking process results in water losses of the meat, which decreases the fillet lightness (FUENTES *et al*., 2012). However, for hot smoked fillets in the present study, the increase in processing temperature led to a greater release of lipids, which resulted in brighter fillet surfaces independent of the pigmentation treatment, and consequently, caused an increase in the lightness.
Bixin, natural pigment annatto seed (*Bixa orellana* L.), is the compound responsible for food pigmentation, and it has a good thermal stability below 100ºC (GIRIDHAR *et al*., 2014). In both cold and hot smoking processes, the smoking chamber temperature did not exceed 100ºC, which therefore did not alter the fillet color. This can be demonstrated by the homogeneity of the results for chroma a* and b*.
The unpigmented hot smoked fillets presented with a lightness that was significantly greater than pigmented fillets. Cold smoked fillets with pigmentation did not differ from

those cold smoked fillets with pigmentation in terms of lightness. The lightness increased due to steam because actomyosin is denatured by heat. However, if the smoking process is independent of temperature, the lightness decreases once the smoking process deposits chemical compounds, which are produced naturally via wood pyrolysis, into the smoked product.

Hot smoked unpigmented fillets had greater values for a* and b* and yielded a golden red color; these values were greater than those of pigmented cold smoked fillets, whose red tone was also significantly less. The greater smoking time results in blackened fillets, so the dehydration and fillet color should be controlled to obtain a more acceptable final product (HASSAN, 1988).

Lysine participates with the ε-amina group in the initial steps of the Maillard reaction (Siskos *et al*., 2005), as seen in the active reaction of aldehyde (formaldehyde, glyoxal, furfural, coniferaldehyde, and sinapaldehyde) from the smoke with the amina group of the lysine. The smoking process increases the lysine content in the fish due to the Maillard reactions (AKINTOLA, 2015). Thus, this can justify the greater values (a* and b*) observed in hot smoked fillets when compared to cold smoked fillets. Despite the shorter smoking time in the hot smoking process (3h), the temperature provides greater amounts of dehydration and more reactions between lysine and smoke compounds, thereby obtaining a more intense coloration.

When we evaluated the shear force of the fillets, cold smoked fillet shear force values were greater than hot smoked fillets values. The salt that was used in the smoking process can affect the final texture of the smoked fillet, which can be seen in terms of water retention capacity, isoelectric point, and protein functionality. Thus, greater salt concentrations are responsible for a firmer texture (GALLART-JORNET *et al*., 2007), which then results in a better flavor and greater stability during storage. The production of a superficial film in smoked meat is due to the protein denaturation that occurs as a result of dehydration, which is associated with salt and heating (HASSAN, 1988). This film on the surface may prevent excessive leaching of fat or evaporation (SIGURGISLADOTTIR *et al*., 2000).

Through scanning electron microscopy, we can observe the film surrounding the fillets. In hot smoked fillets, this was very important, because they were tenderer than cold smoked fillets. Thus, they needed a more consistent structure to avoid disruption via touch or light pressure. This is important mainly for fillets or fish that are kept hanging inside of the smoking chamber in order to avoid falling during the smoking process.

Collagen is the largest component of the intramuscular connective tissue of fish, and it has an important role in maintaining fillet integrity and muscle cohesiveness (AUSSANASUWANNAKUL *et al*., 2012) since it contributes to meat stability and firmness. During the smoking process, the activity of endogenous proteases increases, and these enzymes hydrolyze muscle proteins, thereby breaking down the connective tissue (HULTMANN *et al*., 2004) and altering the texture of the smoked fillet.

Collagen contributes to the texture of *in natura* fishes, but it is not important in the texture of baked fish (HAARD, 1992). The textural resistance of baked muscle decreases with increasing moisture contents up to 79% (Lee and Toledo, 1976). Above 79% of moisture, the resistance decreases, thus reflecting the effects of shear force and compression. Morris *et al*., (2004) reported that hot smoking results in protein denaturation via heat, but there are no theoretical explanations for the phenomenon relating to the thermoviscoelastic properties of the muscle tissue. In hot smoked fillets, despite the lower moisture content (68.90%), a temperature between 50 and 80ºC was enough to alter the structure of collagen fibers and increase tenderness of the fillets, while the film on the fillet surface was responsible for keeping the surface intact until the end of the process. In cold smoked

fillets, collagen fibers were observed; therefore, the temperature utilized was not severe enough to denature the collagen fibers.

During the smoking process, polycyclic aromatic hydrocarbons (PAH) can be formed by organic matter from the wood (VISCIANO *et al*., 2008). Polycyclic aromatic hydrocarbons are a compound group that consists of three or more condensed aromatic rings, which are produced during the incomplete combustion process involving wood, coal, or oil, of which the benzo[a] pyrene is the most studied because it is highly carcinogenic (WRETLING *et al*., 2010).

Regarding the pH of the fillets, cold smoked fillets presented with a lower pH value than hot smoked fillets, and the fillets without pigmentation had a lower pH in relation to fillets with pigmentation, due to dehydration in fillets without pigmentation. The pH changes that occurred in cold versus hot techniques are due to water loss during the process, with more water loss occurring in hot smoked fillets. The greatest smoking time decreased the pH values in cold smoked fillets, and these changes are due to acid absorption from the smoke, moisture loss, as well as the reaction between phenol or polyphenol and carbonyls with proteins and amino groups, respectively (HASSAN, 1988).

When analyzing the benzo(a)pyrene content in cold smoked fillets and hot smoked fillets and comparing them to *in natura* fillets, we observed that the smoking process affected the benzo(a)pyrene content, wich was significantly higher in the fillets subjected to smoking (regardless of type, hot or cold) compared to fillets *in natura*. However, the mean values that we observed are considered low.

The temperature influences the amount of benzo(a)pyrene, as does the exposure time of the product to the smoke compound in pyrolysis. The smoked fish, in adequate conditions, normally present with a low amount of benzo(a)pyrenes, and the maximum level for benzo[a]pyrene in smoked fish and smoked fishery products is 5.0 μg/kg (WRETLING *et al*., 2010); the values in this study are within the range reported.

In sensory analysis, hot smoked fillets were the best evaluated by tasters. Fillets with pigmentation received greater scores than fillets without pigmentation for appearance, superficial and internal color, and flavor.

During the smoking process, the phenols contained in the smoke are responsible for conferring the desirable sensory properties as well as important antioxidants (STOŁYHWO and SIKORSKI, 2005). The lipids in the fish absorb the aromatic substances present in the smoke. We observed that cold smoked fillets had lower scores for flavor and aroma, which can be associated with the fat content in the fillets and the temperature used in the process. When the temperature is high (above 35ºC), the fat in the muscle moves to the surface; this improves the appearance and retention of aromatic substances in the fillets, which therefore creates a better aroma and flavor (BERAQUET and Mori, 1984).

Hot smoked fillets presented with greater scores by tasters when compared to pigmented and unpigmented cold smoked fillets in terms of salt content. This is due to the smoking process since moisture losses occurred in the hot smoked fillets. In the fillets without pigmentation, there was a consequent increase in the salt concentration in the smoked fillets, thus providing a tasty fillet in terms of salt content.

In temperatures above 30ºC, the fish acquires better flavor and coloration when compared to cold smoked products from the same time period (BERAQUET and MORI, 1984). This finding confirms the observations in the present study, where the flavor was significantly greater for hot smoked fillets than cold smoked fillets. The same phenomenon occurred in terms of superficial and internal color of the fillets, because the tasters attributed the greatest scores to hot smoked fillets when compared to cold smoked fillets.

Hot smoked Matrinxa fillets presented with the best results in terms of flavor, salt content, and acceptance, while cold smoked fillets had the best results for color and appearance (FRANCO *et al*., 2010). According to these authors, aroma and texture were not influenced by the smoking techniques.
The tasters believed that hot smoked fillets had a better texture (score 8) when compared to cold smoked fillets (score 4). This fact is due to the tenderness of the hot smoked fillets, as these fillets had a lower shear force. The tasters did not observe any difference in the texture of pigmented and unpigmented fillets, and when this characteristic was analyzed in terms of shear force, no difference was observed.

## 5. CONCLUSIONS

Time and temperature affected the fillet quality in terms of composition, sensory attributes, yield, area, salt content, color, and pH.
The pigmented hot smoked fillets presented with the best results regarding quality (chemical composition, salt content, color, texture, water activity, and pH) and organoleptic characteristics; however, they presented with the lowest yield in terms of processing, and consequently, had greater weight losses.
The smoking process altered the chemical composition of the smoked fillets by reducing moisture and providing a greater concentration of crude protein, total lipids, and ash. The hot smoked fillets presented with a lower moisture content, but had a greater crude protein and ash content. The pigmentation interfered with ash content.
The pigmentation improved the color and general acceptance of smoked fillets, and was also associated with a greater yield for hot smoked fillets. The reddish color (a*) and the yellow color (b*) produced a golden red fillet that was more pigmented in hot smoked fillets than the others; these fillets were considered to be the most adequate by tasters.
In terms of public health (or food safety), the smoking techniques utilized in this study were adequate because they produced smoked products with a benzo(a)pyrene content below the limit allowed by legislation.

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PAPER

# FOOD SERVICE OPERATORS BEHAVIOR AND KNOWLEDGE ON GLUTEN-FREE MEALS AND REQUIREMENTS OF PUBLIC CANTEENS

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## ABSTRACT

This study provides a summary of Celiac Disease (CD) epidemiology in Italy, canteens distribution and training courses for food service operators (FSOs) analyzing 2007-2017 data. Furthermore, behavior and knowledge of FSOs and organizational requirements for gluten-free (GF) meals in school and hospital canteens in central Italy were investigated. In parallel to an increased CD occurrence at national level, GF foods demand is significantly growing. Our survey in 20 inspected canteens revealed important knowledge and awareness gaps among FSOs, as well as lack of procedures and structural requirements for safely providing GF meals, underlining the need to improve education on topic.

*Keywords*: celiac people, central Italy, gluten contamination, public food supply, food preparation, training

## 1. INTRODUCTION

Celiac Disease (CD) is a chronic small intestinal immune-mediated enteropathy with a spectrum of disorders related to the trigger of gluten ingestion in susceptible individuals (GULINO *et al.*, 2016; DEGEORGE *et al.*, 2017; LEBWOHL *et al.*, 2018). Generally, CD clinical presentation encompasses classic gastrointestinal or malabsorption symptoms, such as chronic diarrhea, weight loss, bloating, flatulence, and abdominal pain (LUDVIGSSON *et al.*, 2013; DOWNEY *et al.*, 2015; ELLI *et al.*, 2015). Because of the most recent epidemiological shift, non-classical symptoms are becoming frequent either in childhood or in adulthood, and commonly include iron deficiency anemia, abnormal liver functions, bone disease, and skin disorders (RUBIO-TAPIA *et al.*, 2013; LEBWOHL *et al.*, 2018).

During the latest 10 years, CD has become a significant public health concern globally (SAPONE *et al.*, 2012; SINGH *et al.*, 2018), primarily involving the areas of the world characterized by high wheat consumption. A recent meta-analysis estimated a pooled global CD seroprevalence and biopsy-confirmed CD prevalence of 1.4% and 0.7%, respectively, with the highest prevalence in Europe (0.8%) and Oceania (0.8%), and the least in South America (0.4%) (SINGH *et al.*, 2018). Furthermore, biopsy-confirmed CD prevalence was reported to be 1.5 times more common in females than in males, and approximately twice more common in children than in adults (SINGH *et al.*, 2018).

The high CD prevalence has been mostly associated to the widespread consumption of gluten containing cereals, and to occurrence of Human Leucocyte Antigen *(HLA) DQ2* and *HLA DQ8* predisposing alleles in the global population, ranging 0-28% and 1-9%, respectively (LIONETTI and CATASSI, 2014). Although both required, these factors are not sufficient for clinical CD development, since only 30% of subjects eventually develop the disease (LIONETTI et *al.*, 2015). CD almost affects about 1% of the European population (RUBIO-TAPIA *et al.*, 2012; CHOUNG *et al.*, 2017), even though a large proportion of undiagnosed and consequently untreated subjects has been estimated (ELLI *et al.*, 2015). Furthermore, there are huge differences in CD prevalence that could be not explained only by genetic and environmental risk factors. For example, Germany has the lowest CD prevalence than other European countries, whilst Sweden and Finland the highest (MUSTALAHTI *et al.*, 2010).

In Italy, the revision of the "Essential Levels of Care" (Italian Ministerial Decree 12/01/2017) involved the shift of CD and its clinical variant of dermatitis herpetiformis in the list of chronic disabling diseases. The transition was considered as necessary because both clinical forms could not be longer included within the prevalence limits of the rare diseases established below 5/10,000 inhabitants at European level.

Furthermore, the Italian Law n. 123/2005 on "Rules for the protection of CD people" has recognized CD as a social disease and promoted adoption of measures for these patients (Legge 4 luglio 2005, n. 123; CAPUOZZO *et al.*, 2013). Indeed, the National Government assigns annually dedicated resources to the Regions, and authorizes public money spending for gluten-free (GF) meals distribution in the school, hospital and public canteens, as well as for professional training for food service operators (FSOs). In particular, according to the Law 123/2005, school (public and private) and hospital (public and private hospitals, hospices and public nursing homes, institutional care and private nursing homes) canteens, as well as those attached to public facilities (public institutes and administrations) must guarantee GF meals for CD people who request it.

At present, a balanced GF diet based on the combination of naturally and certified processed GF food represents the only effective treatment for celiac people (LA VIEILLE *et*

*al.*, 2014; GULINO *et al.*, 2016; BASCUÑÁN *et al.*, 2017), providing a rapid improvement and resolution of symptoms.
Although the effectiveness of gluten sources elimination in an absolute and permanent way is proved, numerous difficulties could affect a complete adherence to GF diet, including costs of GF food, variable quality of information regarding the status of ingredients, and potential gluten exposure when travelling or eating out (BARRATT *et al.*, 2011; RUBIO-TAPIA *et al.*, 2013). The threshold below which gluten is safe is unknown; however, the available evidences advised that exposure to less than 10 mg/day is unlikely to cause histological changes to the intestinal mucosa for CD individuals (BOLD and ROSTAMI, 2011; LA VIEILLE *et al.*, 2014, 2016). Indeed, according to the international food standards Codex Alimentarius, gluten level in GF food may not exceed 20 parts per million (ppm), which corresponds to 20 milligrams of gluten per kilogram, or per liter of product (RUBIO-TAPIA *et al.*, 2013; FARAGE *et al.*, 2017a; Standards CODEX ALIMENTARIUS FAO-WHO; EU law-EUR-Lex). Of concern, it should be considered that cross-contamination could frequently occur when GF food come in contact with gluten-containing grains and their derivatives during any stage of food preparation process, or if the same equipment/utensils handling food products or surfaces are used (KOERNER *et al.*, 2011; FARAGE *et al.*, 2017a; ROSTAMI *et al.*, 2017; VERMA *et al.*, 2017).
Therefore, avoiding cross-contamination during meals preparation, especially in restaurants and public canteens, is the key factor to ensure the quality of GF food for CD people. However, having a meal out could represent a serious risk concern when there is a lack of knowledge regarding GF food by the staff involved (FARAGE *et al.*, 2014). In order to contribute to a better quality of life for CD patients, it is important to establish effective strategies to prevent contamination and enable safe production of GF food (FARAGE *et al.*, 2017a). These perspectives clearly include the development of appropriate instruments or approaches for verification *in loco* of non-conformities related to the potential cross-contamination in the production process (FARAGE *et al.*, 2017b), and the improvement of professional training, being awareness of CD related issues among chefs and cooks highly dependent by the education level (SCHULTZ *et al.*, 2017).
This study aims to describe the state of the art and the epidemiological background regarding CD occurrence in Italy, as well as an overview of the public canteens administrating GF meals and training on this issue by examining all available data at national level. Furthermore, the results of a survey conducted among FSOs of hospital and school canteens in Terni province, Umbria region, central Italy, are reported. In particular, the investigation explored about FSOs knowledge and behaviors related to CD issues, as well as on the structural and organizational requirements for GF meals preparation and administration, and measures developed and in place established to minimize the likelihood of gluten contamination.

## 2. METHODS

### 2.1. Epidemiological data on CD, canteens mapping and training

In Italy, the General Directorate for Hygiene and Food Safety and Nutrition of the Ministry of Health releases an annual census to the Parliament on CD (www.salute.gov.it/portale/documentazione/). By searching in the thematic area of "Nutrition" and the publication years, annual reports to the Parliament on CD items were available since 2007. Indeed, data on CD prevalence, distribution of public canteens

involved in GF meals administration, and specific training for FSOs in relation to 2007-2017 reference period were pooled and analyzed (GUIDARELLI *et al.*, 2008, 2009, 2010; DE STEFANO and SILANO, 2011, 2012, 2016, 2018, 2019; DE STEFANO *et al.*, 2015, 2016).

### 2.2. Setting of the survey involving FSOs

The survey involved 20 public canteens located in Terni province, Umbria region, central Italy. Particularly, 14 nursery school, 4 primary school and 2 hospital canteens were included, being involved in the production and/or administration and/or sale of GF products to consumers at time of the study. The canteens were inspected by Technicians of the prevention in the environment and the workplace of the Food and Nutrition Hygiene Unit, Department of Prevention of Umbria region, Local Health Institution n. 2.

### 2.3. Survey tools for data collection

Data were collected during the regular inspection activities using two different approaches, an interview and a checklist, which were both administered to 20 FSOs (one for each inspected canteen) in charge of the entire food processing. Hence, FSOs were *face-to-face* interviewed by Technicians to ascertain their knowledge about CD and gluten-related aspects and their behavior for the control of gluten contamination risk. Similarly, a checklist was completed by FSOs to verify the structural and organizational requirements for GF food preparation and administration.
In details, the interview was structured in two sections concerning knowledge and behavior. The knowledge section was composed of five questions concerning general and theoretical aspects on CD, particularly referring to food permitted and forbidden (list of 20 food types) in the diet for celiacs, and specific precautions. The section referred to behavior was structured in seven questions in order to investigate about their attitudes in the management and control of the entire GF food processing. In particular, the checklist was structured into six areas, reflecting different stages of GF food production, preparation and administration to verify the compliance with procedural, structural and organizational requirements for control of gluten contamination risk. Hence, the checklist included 24 questions concerning Hazard Analysis and Critical Control Points (HACCP) plan; purchase and storage of raw food materials and available equipment (control procedures for raw ingredients; storage and equipment/utensils for food preparation and manipulation); food preparation and cooking (areas, practices, equipment); food administration; staff hygiene and procedures (clothing); personnel training (food hygiene and CD issues).

### 2.4. Data analysis

CD prevalence, public canteens involved in GF meals administration, and specific courses for FSOs obtained by reports to Italian Parliament from 2007 to 2017 were analyzed in terms of temporal trend, and through descriptive statistics (mean ± standard deviation and median encompassing all the reference period).
The overall data from the interview and checklist were collected and analyzed using Statistical Package for Social Science (SPSS) software version 25.0. To each item from both the interview and the checklist, 1-point level was assigned when a correct answer was provided, whilst 0-point if FSOs erroneously or not answered.

Therefore, results from the interview were evaluated compared to the maximum achievable score of 31, comprised of 24 plus 7-points of knowledge and behavior questionnaire sections, respectively, while the score for checklist varied between 0 and 23-points.

## 3. RESULTS

### 3.1. CD prevalence, GF meals canteens and training courses, Italy, 2007-2017

In Italy, according to the last epidemiological mapping and 2017 annual census on CD by Ministry of Health, 206,561 celiacs have been diagnosed, for a mean prevalence of 0.34% (DE STEFANO and SILANO, 2019), with a percentage increment of 4.1% compared to the previous year (Fig. 1). From 2007 to 2017, a steady increase of the number of diagnosed cases was observed (mean number of CD subjects=144,389±46,330 (SD), median=148,662), which along the whole period corresponded to +220.8% increase of diagnoses, ranging from 64,398 in 2007 to 206,561 cases in 2017 (Fig. 1). During 10 years, the highest variation occurred between 2007 and 2008 (+27.2%), and between 2008 and 2009 (+34.9%), while lowest variations were found in the most recent years (Fig. 1), although with a high number of new diagnoses. Additionally, two third of CD population were females (145,759 *vs* 60,802 males) with a 1M:2F mean proportion (DE STEFANO and SILANO, 2019), and this trend has been constantly observed in the last decade (Fig. 2). During 2007-2017 period, CD cases were on average 98,849±35,771 (median=104,334 cases; range: 35,017-145,759) among females, and 42,017±14,479 (median=44,253 cases; range: 16,239-60,802) among males, respectively.
The age group in which CD was prevalently registered corresponded to 19-40 years with 71,371 individuals (34.5%) (DE STEFANO and SILANO, 2019).

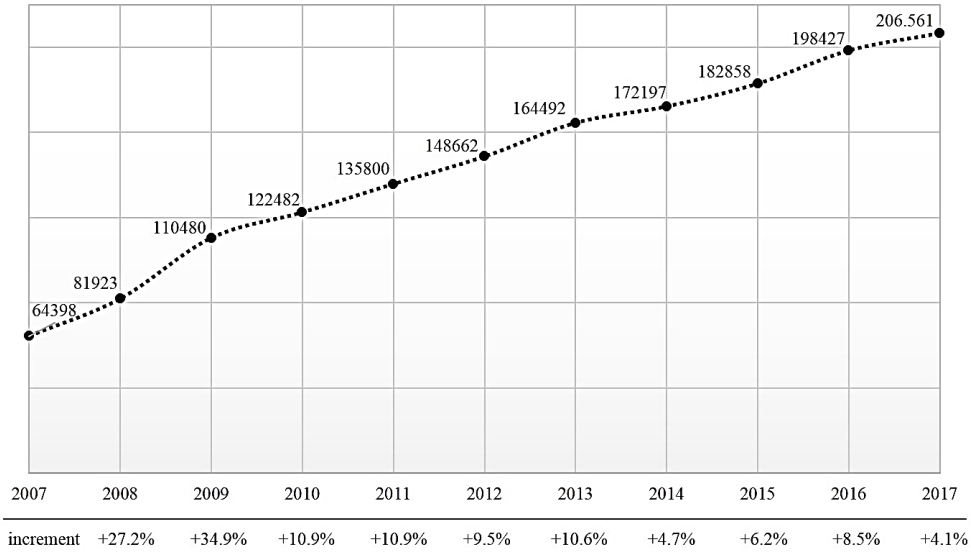


**Figure 1.** Number of CD cases, Italy, 2007-2017.

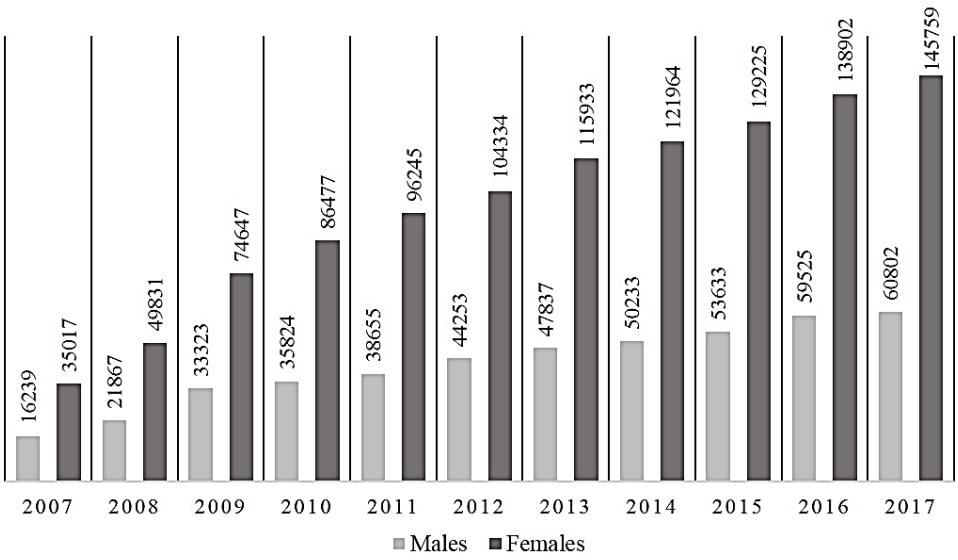


**Figure 2.** Number of CD cases stratified by gender, Italy, 2007-2017.

In 2017, the National Health Service has provided an amount of 320,111.59€ to the Italian Regions in order to guarantee GF food and 534,427.43€ for training courses to FSOs (DE STEFANO and SILANO, 2019). To support GF diet, almost 250 million euros have been spent for GF products, with an annual national average of 1,200.00€ per capita (DE STEFANO and SILANO, 2019).
Data from the regional registry offices in 2017 reported that 42,814 canteens covered the scope of Law 123/2005: 28,718 (67%) were school, 7,997 (19%) hospital and 6,099 (14%) of public organizations. During 2007-2017, a significant increase of the number of canteens was observed (mean±SD=39,761±3,040; median=39,110), with the highest prevalence of school canteens in the national territory (Fig. 3) (DE STEFANO and SILANO, 2019). In the overall period, school, public and hospital canteens were 28,103±1,704 (median=28,248; range: 24,693-30,810), 7,029±1,778 (median=6,410; range: 6,032-9,301), and 4,506±2,031 (median=3,823; range: 4,320-9,301), respectively.
From 2017 data, 755 specific training courses with an average of 5 hours for each course were activated (Fig. 4) in the national territory, involving 19,068 FSOs.
Since 2017, the number of courses was increased compared to the latest two years. In the whole period, on average, 669±362 (median=645) specific courses were managed, involving 15,120±5,403 (median=15,968) FSOs, with high participation since 2010.

### 3.2. Interview to FSOs

Among various listed food, all FSOs demonstrated to know that bread, pasta and cutlet are forbidden food in the diet for CD individuals, whilst 15% (n=3) and 20% (n=4) erroneously answered that cookies and barley coffee can be consumed, respectively (Fig. 5).
Other permitted food categories in the diet for celiacs were correctly identified, such as cooked vegetables, olive oil, tomato paste, eggs, meat, fish, sugar, honey, and coffee. Conversely, only 80% (n=16), 85% (n=17), and 55% (n=11) knew that consumption of milk, rice and strawberries are allowed in GF diet, respectively. In addition, raw ham was

improperly considered as forbidden in CD diet by 25% (n=5), as well as natural yoghurt (without cereals) and butter by 15% (n=3) and 10% (n=2), respectively (Fig. 5).

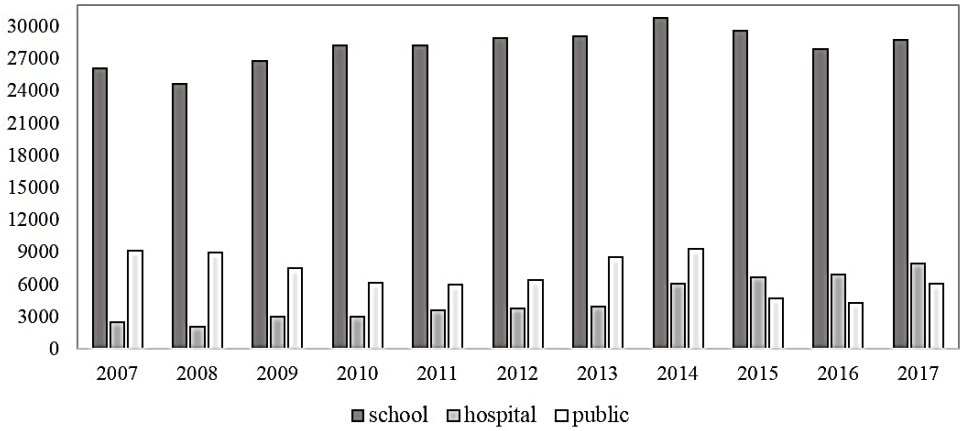


**Figure 3.** Number of school, hospital and public canteens administering GF food, Italy, 2007-2017.

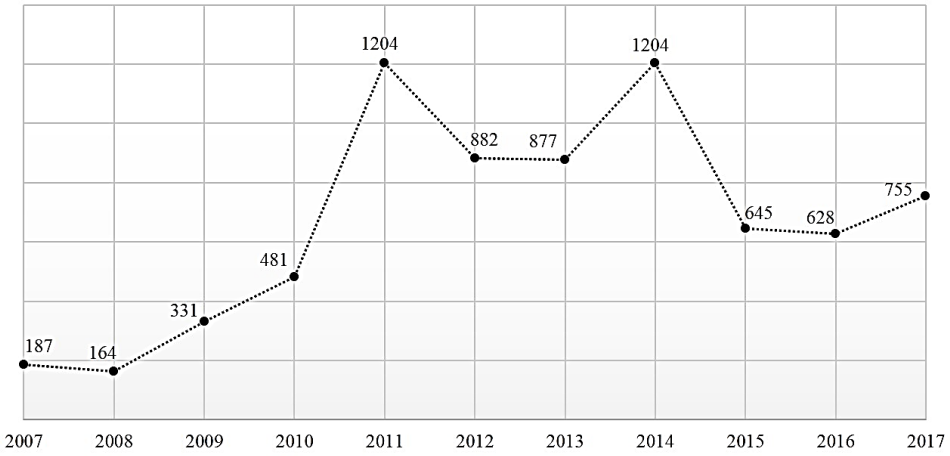


**Figure 4.** Courses on CD and GF food for FSOs, Italy, 2007-2017.

Furthermore, 30% (n=6) erroneously stated that CD patients can introduce small amounts of gluten with diet, and only 75% (n=15) proved to be aware that gluten is not removed by cooking foods (Table 1).

About management and control of gluten contamination risk in food preparation and administration, 20% (n=4) of FSOs did not know that GF food should be stored in clearly identified and separated areas.

Furthermore, GF food preparation should occur in a well-identified area and separated kitchen for 30% (n=6). Only 25% (n=5) of FSOs knew that equipment (i.e. oven, deep fryer, plates, etc.) and utensils (i.e. cookware, tableware, etc.) should be used exclusively for GF food preparation (Table 1).

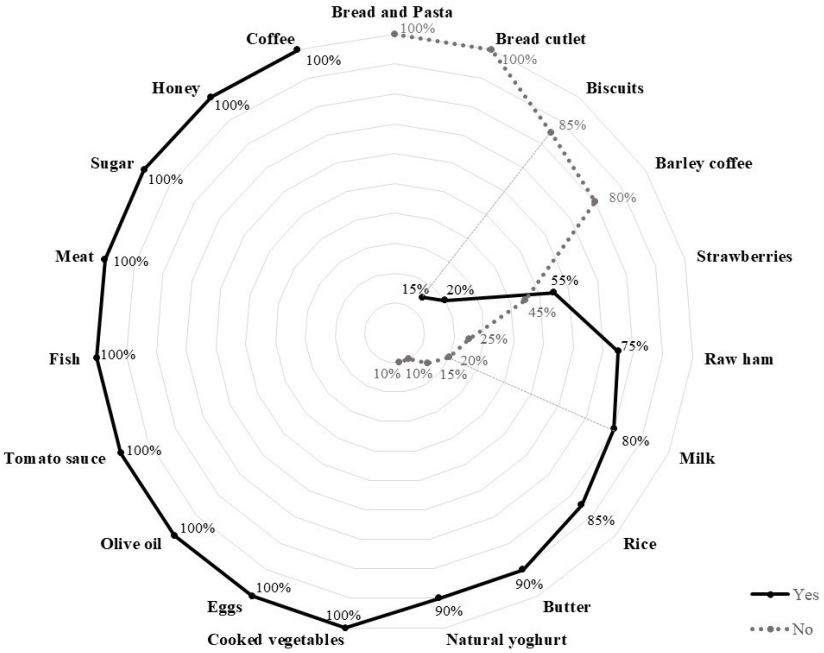


**Figure 5**. Are the following food categories permitted in the diet for CD individuals?

All FSOs demonstrated to know that, if lacking a double equipment for celiac and non-celiac people, it is a prerequisite to separate their use in time, ensuring satisfactory cleaning conditions prior to use. All FSOs proved to be aware that hand washing and control of clothing are measures highly required in the meal service, as well as the proper identification of dishes for CD consumers to avoid a promiscuous service. However, a special coat when preparing GF meals should be used by 30% (n=6) of FSOs (Table 1).
Based on the answers provided, only 6 (30%) FSOs scored 24-points being the maximum level of knowledge, while the remaining scored 18-23-points. Regarding skills related to GF meals, the maximum level was reached only for two FSOs, while the remaining ranged as 4-6, and more often (65%) with a 5-points score. Largely, the inadequate response was related to the equipment dedicated for GF food because considered unnecessary. By pooling data, none of the FSOs had the ideal level (maximum score=31) of knowledge and behavior, and only eight showed a quasi-optimal score reaching 28-30-points, while the remaining scored in the intermediate range of 23-26-points.

### 3.3. Requirements for GF meals processing resulting from checklist

With respect to HACCP plan implemented in the inspected canteens, there was a specific section on gluten and management of critical control points (CCPs) to minimize and control the risk of accidental and/or cross-contamination only for 40% (n=8) (Table 2).
Furthermore, there was no opportunity to appreciate the presence, in paper form or on-line, of the handbook of the Italian association for celiacs in 55% (n=11) of canteens. Before storage, control procedure for raw materials, semi-finished and finished food products at the reception were not available in 45% (n=9), and no specific procedure for non-conforming food was found in 60% (n=12) (Table 2).

**Table 1.** Knowledge on CD and GF food as reported by 20 FSOs through the interview.

| Items | Correct answer | Yes N (%) | No N (%) |
|---|---|---|---|
| • CD subjects could introduce small amounts of gluten with diet | No | 6 (30.0) | 14 (70.0) |
| • Gluten is removed by cooking foods | No | 5 (25.0) | 15 (75.0) |
| • GF food must be provided by the consumers | No | 4 (20.0) | 16 (80.0) |
| • GF food should be stored in a separate and well identified area | Yes | 16 (80.0) | 4 (20.0) |
| • Preparation of GF meals must take place in a separate and well identified area | Yes | 6 (30.0) | 14 (70.0) |
| • Equipment and utensils must be dedicated for GF food preparation | Yes | 5 (25.0) | 15 (75.0) |
| • It is necessary prior sanitation and time differentiation when a double equipment is not available | Yes | 20 (100) | 0 |
| • A dedicated coat should be used when preparing for CD subjects | Yes | 14 (70.0) | 6 (30.0) |
| • Hand washing and control of uniform must be regularly performed in food administration | Yes | 20 (100) | 0 |
| • GF meals must be clearly identified to avoid promiscuous service | Yes | 20 (100) | 0 |

**Table 2.** Checklist on procedural, structural and hygienic requirements in GF meals preparation and administration.

| Items | Correct answer | Yes N (%) | No N (%) |
|---|---|---|---|
| • A section for GF products is included in the HACCP plan | Yes | 8 (40.0) | 12 (60.0) |
| • In place availability of the Italian booklet of association for celiacs | Yes | 9 (45.0) | 11 (55.0) |
| • The control of any raw materials, semi-finished and finished products is performed before storage | Yes | 11 (55.0) | 9 (45.0) |
| • There is a procedure for discarding non-conforming products | Yes | 8 (40.0) | 12 (60.0) |
| • GF raw materials are stored in separate areas and in identifiable shelves | Yes | 20 (100) | 0 |
| • GF food are stored in closed and identifiable containers | Yes | 20 (100) | 0 |
| • GF food are identified and placed in separate containers in the fridge/freezer | Yes | 13 (65.0) | 7 (35.0) |
| • The storage of GF products takes place in a dedicated fridge/freezer | Yes | 4 (20.0) | 16 (80.0) |
| • There is a separate room/area for preparing GF food | Yes | 4 (20.0) | 16 (80.0) |
| • A temporal differentiation with previous sanitization is performed if a separate zone for GF food is not available | Yes | 16 (80.0) | 4 (20.0) |
| • A sanitation areas procedure is available | Yes | 5 (25.0) | 15 (75.0) |
| • The registration of the sanitization control is carried out | Yes | 5 (25.0) | 15 (75.0) |
| • There are equipment, tools and utensils dedicated for meals for CD people | Yes | 4 (20.0) | 16 (80.0) |
| • A temporal differentiation is performed with previous sanitation if utensils are used for gluten-containing foods | Yes | 4 (20.0) | 16 (80.0) |
| • There is a procedure for the sanitization of equipment and tools | Yes | 5 (25.0) | 15 (75.0) |
| • Presence of washbasins | Yes | 7 (35.0) | 13 (65.0) |
| • Hand washing and clothing control are regularly carried out | Yes | 16 (80.0) | 4 (20.0) |
| • The identification of the dishes for CD people and non-promiscuous service are always performed | Yes | 19 (95.0) | 1 (5.0) |
| • There is dedicated clothing that is placed in exclusive rooms | Yes | 2 (10.0) | 18 (90.0) |
| • A clothing and hygiene procedure for personnel is available | Yes | 12 (60.0) | 8 (40.0) |
| • A specific training on CD and related issues is regularly performed | Yes | 11 (55.0) | 9 (45.0) |

Storage activities of GF raw materials were carried out in separate areas and in special shelves, as well as GF food preserved in closed and identifiable containers. However, well-defined local or area for preparing GF food, or utensils/equipment dedicated were not available for 80% (n=16) of the canteens, although a time differentiation for their use was performed, with prior sanitization (Table 2). Similarly, a fridge or freezer exclusively dedicated to store perishable GF food was not found in 80% (n=16) of canteens, although a separation from gluten-containing products was ensured in the 65% (n=13) of cases.
When locals and equipment/tools were shared and used for both GF and non-GF foods, a formal registration of the sanitation procedure before use was not present for 75% (n=15) of the facilities. Furthermore, 20% (n=4) of FSOs declared to do not regularly practice hand washing and control of the uniform in food administration, and 5% (n=1) stated to do not identify meals/dishes intended or not intended for CD consumers (Table 2).
Assessing hygienic practices and training, 90% (n=18) did not comply with the requirements of clothes dedicated for GF food and retained in distinct cabinets. Furthermore, 60% (n=12) did not have a formalized procedure on management of staff clothing, and washbasins were present in the 35% (n=7). At time of the inspection, 45% (n=9) of FSOs have not attended a certified training course on CD and issues related to GF preparation and administration (Table 2).
Based on the responses provided for the checklist, the score ranged between 6 and 19 (ideal score=23), and the majority scored 17 (n=4, 20%), followed by 10 and 7 (each n=3, 15%).

## 4. CONCLUSION

Health protection of CD individuals is an important goal, especially for most sensitive categories, such as children, patients admitted at hospitals, and people of school age who represent a vulnerable young-adult group (FORLEO *et al.*, 2017; TAMBURRO *et al.*, 2017).
In this study, results from 20 FSOs revealed knowledge gaps on the basic and general theories about CD, as well as the lack of best practices for control risk of gluten contamination. Part of FSOs was not aware that consumption of some products could lead to high risk, while other naturally GF food were erroneously considered as forbidden. Similar findings were found in other studies (KARAJEH *et al.*, 2005; YOUNG and THAIVALAPPIL, 2018), reporting that food service personnel were less knowledgeable on CD than general population, underlining a paucity of tailored education that should be addressed through specific training. Furthermore, concerns emerged about the beliefs of some FSOs that CD subjects can introduce small amounts of gluten with diet that, however, it should not exceed 20 mg/kg (Regulation (EU) No 609/2013). Moreover, the protracted intake of products contaminated with gluten traces may cause persistent intestinal damage and symptoms in non-responsive CD patients, who fail to ever respond to GF diet, or have recurrence/relapse of symptoms despite the GF diet (HOLLON *et al*., 2013).
While FSOs were aware of the importance to use equipment and utensils in different time for GF and gluten-containing food, inappropriate behaviors were observed for special coat in GF meals preparation. In fact, although kitchen equipment and utensils used for gluten-containing foods may not pose a high risk for CD patients, cross-contamination during food preparation or cooking can occur, and it should be avoided cleaning utensils, and washing hands/surfaces regularly (STUDERUS *et al.*, 2018).

The complete removal of gluten from diet is difficult, being a pervasive nutrient that may contaminate GF products along the production chain (LEE *et al.*, 2014). Recently, a systematic review on cross-contamination from gluten higher than 20 ppm in GF food in Europe (FALCOMER et al., 2018) has revealed percentages of contamination ranging from 10%-13% in the United Kingdom and Sweden (STORSRUD *et al.*, 2003; MCINTOSH *et al.*, 2011) to 56%-70% in Finland and Spain (COLLIN *et al.*, 2004; HERNANDO *et al.*, 2008). One study conducted in Italy did not report gluten contamination among GF foods tested (MANFREDI *et al.*, 2015). Quantification of gluten is difficult, being a combination of different components (microheterogeneity) classified as gliadins, glutenins, globulins and albumins, whose measure is unpractical (VERMA *et al.*, 2017), especially in processed foods (DIAZ-AMIGO and POPPING, 2012). Nonetheless, several approaches for detecting gluten proteins are available, such as immunological tests mainly through ELISA kits, proteomic analysis with mass spectrometry, and DNA-based methods applying Polymerase Chain Reaction (HARASZI *et al.*, 2011). However, none of these methods is considered universally acceptable for a high sensitive detection, showing each technology advantages and disadvantages (SLOT *et al.*, 2016).

Our results from checklist showed a non-optimal compliance with structural and procedural requirements for warranting food safety value to CD subjects, as well as a lack of adherence to hygiene principles and in relation to a continuing education on GF diet, indicating that targeted and effective interventions are necessary. Like our survey, a retrospective study analyzing characteristics of the GF food chain in a school service in an Italian Region (BIOLETTI *et al.*, 2016) reported that 71% of sampled schools were inadequate for at least one of the production stages. Certainly, food separation for CD people has to be handled in order to reduce the chance of cross-contamination. At present, it is managed as strictly as for allergic subjects, and whether this approach is necessary remains to be tested in prospective studies. The same oven for gluten-containing and GF food can be used, as long as the two types are not baked at the same time (BIANCHI *et al.*, 2018); when specific requirements are complied, also the simultaneous cooking can be a safe procedure (VINCENTINI *et* al., 2016).

Nearly half of recruited FSOs in our survey has not attended a training course on CD and related issues, similarly to another study reporting that half of chefs had not received formal training (SCHULTZ *et al.*, 2017). Indeed, a comprehensive and specialized training has been recommended for FSOs (LEE and XU, 2015; SHAFIE and AZMAN, 2015; RADKE *et al.*, 2016), and interventions to improve knowledge and practice of food service personnel should be implemented (YOUNG and THAIVALAPPIL, 2018). Moreover, to meet GF food quality specifications, compliance with basic food safety concepts at all stages of product life cycle is decisive for ensuring harmless foods for celiacs (BIOLETTI *et al.*, 2016), as well as appropriateness of a HACCP plan (PETRUZZELLI *et al.*, 2014).

In conclusion, our study remark the need to provide effective education and proper resources for food service establishment personnel to gain knowledge and strengthen awareness on GF foods, and improve or enhance practical skills, attending courses conceived to assess their ability for safely serving celiac consumers. In addition, good manufacturing practices have a positive effect on GF foods production, and are useful to identify priority areas for improving comprehension of CD issues and practices among FSOs, to prevent accidental gluten exposure. Although additional studies are needed to better estimate the likelihood of gluten cross-contamination in food service establishments and industries, it should be underlined that risk can be controlled implementing simple rules applied on a daily basis in both meals preparation and administration.

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PAPER

# CHARACTERIZATION OF VOLATILE COMPOUNDS IN FIVE BLUEBERRY VARIETIES USING PURGE AND TRAP COUPLED TO GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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## ABSTRACT

The volatile composition of five blueberry varieties from two different regions was analysed by dynamic headspace (purge and trap, P&T) coupled to gas chromatography-mass spectrometry (GC-MS). Under the optimized conditions, the P&T method was successfully validated, showing good linearity, high accuracy, good reproducibility and a low limit of detection. A total of 80 volatiles were identified, including 19 esters, 30 alcohols, 18 aldehydes, 7 ketones and 6 other compounds. Furthermore, a spider web diagram was constructed to compare the flavour profiles of these blueberries, and the obtained results demonstrated that blueberries from different locations have different flavour profiles.

*Keywords*: aroma active compounds, blueberry, GC-MS, P&T, volatile compounds

## 1. INTRODUCTION

Blueberries have been recognized by the scientific community and consumers for their health-promoting potential (SILVA *et al.*, 2017). The history of blueberry cultivation in China is approximately 20 years old, and Chinese blueberries were mainly introduced from the United States and Japan. There are three main types of blueberries: highbush (*Vaccinium corymbosum*), lowbush (*Vaccinium angustifolium*), and rabbiteye (*Vaccinium virgatum*). Highbush blueberries can be further divided into northern highbush and southern highbush blueberries (DU and ROUSEFF, 2014). Northern highbush and lowbush blueberries are the predominant varieties in the Greater Khingan Range. Southern highbush and rabbiteye blueberries are generally grown to the south of the Yangtze River (HE and WU, 2010).

In addition to being rich in vitamins and anthocyanins, blueberries are rich in volatile compounds such as ethyl acetate, butyl acetate, and 1-nonanal. Flavour and aroma are two of the most important fruit quality characteristics and ultimately determine consumer acceptability and purchase decisions (DU and ROUSEFF, 2014). Volatile compounds are important contributors to fruit aroma, which is one of the main characteristics that determine blueberry organoleptic quality and style (SUN *et al.*, 2013). Different proportions of volatile components determine the overall aromatic properties (LV and LIN, 2015). People realized the importance of volatile compounds with regards to aroma approximately 50 years ago. However, due to equipment being less advanced, studies of blueberry aroma are still very limited. The volatile compounds of highbush blueberries were analysed by PARLIAMENT and KOLOR in 1975, and 18 individual components were identified by mass spectrometry, infrared analysis and gas chromatographic retention times (PARLIMENT and KOLOR, 1975). HALL *et al*. (1970) used gas-liquid chromatography (GLC) to examine the aromatic composition of lowbush blueberries. Acetaldehyde, methyl acetate, ethyl acetate and ethyl alcohol were reported as the major aromatic compounds. Currently, with the emergence of detection techniques with high sensitivity and accuracy, such as gas chromatography-olfactometry (GC-O), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), more volatile compounds at relatively low concentrations and thresholds are expected to be detected.

Purge and trap (P&T), also known as dynamic headspace, has been widely used for the preconcentration of volatile compounds (LARRETA *et al.*, 2008). With P&T, an inert gas is purged throughout the sample in the same way as which we breathe, making this technique suitable for correlation with organoleptic studies (AZNAR and ARROYO, 2007). It can be applied to solid or liquid matrices (MURAT *et al.*, 2012). Compared to SPME, the high recovery of very volatile compounds and the low dispersion associated with the use of a totally automated system are the main advantages of P&T-GC-MS-based methods (SORIA *et al*., 2009).

In this study, five blueberry varieties from two major blueberry production areas were identified (i) by purge and trap coupled to gas chromatography-mass spectrometry (P&T-GC-MS). To provide a representative analysis of the blueberry volatiles, we first (ii) optimized this method and evaluate its correctness and then (iii) drew a spider web diagram to compare the flavour profiles of these blueberries.

## 2. MATERIALS AND METHODS

### 2.1. Plant materials

All the samples were purchased from the Hulun Buiroroqen Pristine Production Co. Ltd. Blueberries were squeezed into juice, diluted three-fold, and filtered for analysis.
In this work, a total of five blueberry taxa were used to study volatiles. These taxa included two wild blueberries and three cultivated blueberries (Table 1).

**Table 1.** Blueberry taxa in this study*.

| Taxa | Characters | Origin | Population |
|---|---|---|---|
| Wild blueberry | around the humus | Mohe area of Greater Khingan Range | WH-M |
| Wild blueberry | around the stones | Mohe area of Greater Khingan Range | WS-M |
| Cultivated blueberry | Bluecrop | Greater Khingan Range | CB-G |
| Cultivated blueberry | Powderblue | Greater Khingan Range | CP-G |
| Cultivated blueberry | Britewell | Yangzhou | CB-Y |

*CB-G is northern highbush blueberry; CP-G and CB-Y are rabbiteye blueberries.

### 2.2. Chemicals

NaCl and n-alkanes ($C_6$-$C_{22}$) were purchased from Beijing Chemical Reagents Co. Ltd. (Beijing, China). Analytical grade 2-methylbutyraldehyde, ethyl acetate, 2-nonanone, linalool, and ethyl caprylate were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China).

### 2.3. Volatile compounds extracted by purge and trap

P&T was performed by an Eclipse 4660 purge and trap sample concentrator with a 4551A autosampler (OI Analytical Company, USA) and a #10 trap. Three millilitres of each juice sample was placed in a 5 mL purge tube. Nitrogen gas was utilized as a purge at 10 psi at 25°C.
The other analytical conditions were as follows:
Trap temperature: purge, 30°C; desorption, 190°C; transfer line, 110°C; and valve oven, 110°C.
Time: purge 11 min; desorption 1 min.

### 2.4. GC-MS conditions

Chromatographic analysis was performed in a GC-MS (QP2010 Ultra, Shimadzu Corporation, Japan) system equipped with a Rtx-5MS capillary column (0.25 mm×30 m×0.25 μm) (Restek, USA). Helium was used as the carrier gas at a linear velocity of 1.0 mL/min. The column temperature was held at 50°C for 5 min, increased to 180°C at a rate of 10°C/min, increased to 210°C for 5 min at a rate of 5°C/min, and finally increased to 280°C at a rate of 20°C/min. The mass selective was operated in the electron ionization mode at 70 eV and a scan range m/z of 45-400.

### 2.5. Identification of volatile compounds

Volatile compounds were identified by matching their mass spectra with those of the known compounds from the NIST 11/11s edition library.
The relative odour activity value (ROAV) was calculated to measure the contribution of each volatile compound towards the whole aroma profile and was calculated using the following equation (ZHUANG *et al.*, 2008; GU *et al.*, 2012). ROAVs were calculated by using Eq. (1):

$$\mathrm{ROAVi} = \frac{\mathrm{Ci\%}}{\mathrm{Cstan\%}} \times \frac{\mathrm{Tstan}}{\mathrm{Ti}} \times 100 \qquad (1)$$

where "stan" is the volatile compound that has the highest relative contents; ROAVi is the odour activity value of the compound in sample i; Ci is its content; and Ti is its odour threshold concentration. Compounds with a ROAV ≥1 significantly contribute to the aroma. (ZHUANG *et al.*,2016).

### 2.6. Statistical analysis

Significant differences in the volatile compounds of the five blueberry varieties obtained from duplicate analysis were determined by one-way ANOVA with SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Statistically significant differences were determined at $p<0.05$. The OriginPro system (v8.5 SR6, OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis.

## 3. RESULTS AND DISCUSSION

### 3.1. Optimization of the P&T-GC-MS method

This study optimized the following P&T extraction parameters: sample volume, purge temperature and purge time.
Ethyl acetate, ethyl caprylate, 2-methylbutyraldehyde, 2-nonanone and linalool were used as standard compounds for optimization of the P&T-GC-MS method. As shown in Fig. 1, varying volumes of blueberry juice (3, 4, and 5 mL) were placed in the trapping apparatus flask and purged for 11 min at 25°C. For ethyl octanoate, ethyl acetate, and linalool, there was a considerable difference between the various sample volumes ($p<0.05$). For 2-methylbutyraldehyde and 2-nonanone, the relative percentages of these standard compounds in the 3 mL groups increased compared with the high sample quality group, but there was no significant difference ($p>0.05$). This study also showed that the number of volatile substances obtained from 3, 4, and 5 mL was 52, 50, and 49, respectively. The reason for this result may be that a high liquid level is too close to the top of the purge trap, so when a large amount of $N_2$ purifies the liquid, extra water could be purged into the trap, which can shorten the trap life in the same way as a longer purge time (DENG *et al.*, 2011).

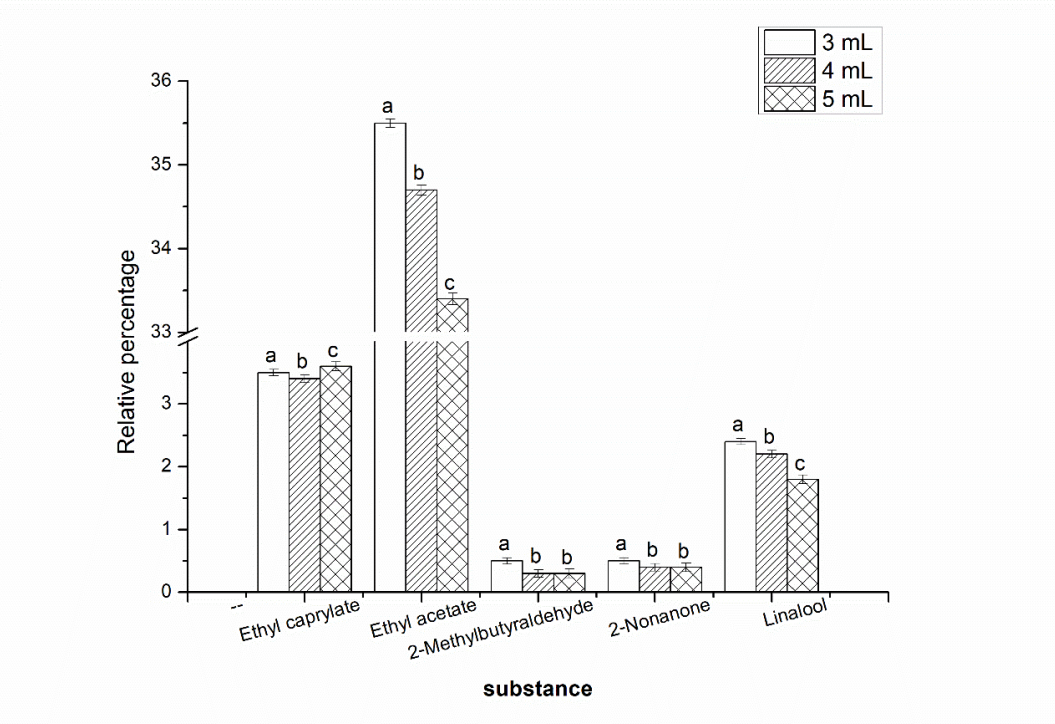


**Figure 1.** Effect of sample volume on the extraction efficiency; purge temperature = 25°C, purge time = 11 min.

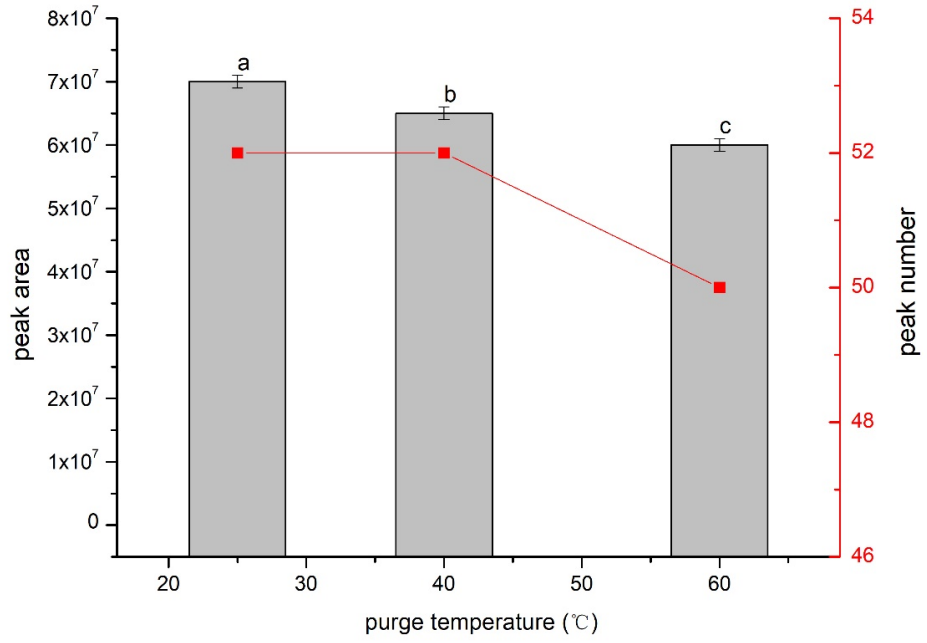


**Figure 2.** Effect of purge temperature on the extraction efficiency. Sample volume = 3 mL, purge time = 11 min.

Fig. 2 showed the effect of purge temperature on the extraction efficiency. The purge temperature varied between 25-60°C with 3 mL of sample volume for 11 min. The amount of total volatiles detected in blueberries gradually decreased as the purge temperature increased from 25°C to 60°C, probably due to the amount of water that reached the trap and decreased the sensitivity; therefore, ambient temperature was maintained in all the experiments (CAMPILLO *et al.*, 2004).
The effect of purge time on the sensitivity is shown in Fig. 3. The purge time was varied between 8 and 14 min with 3 mL of sample volume at 25°C. Finally, a value of 11 min was chosen as the optimal time, since 8 and 14 min led to a slight decrease in the peak area and total number. Eight minutes is too short mainly because the volatile substances are not fully blown out. Indeed, 14 min decreased the signals because a flow of $N_2$ that was too long could move the volatiles from the trap before desorption and reduce the final signal (CAMPILLO *et al.*, 2004).

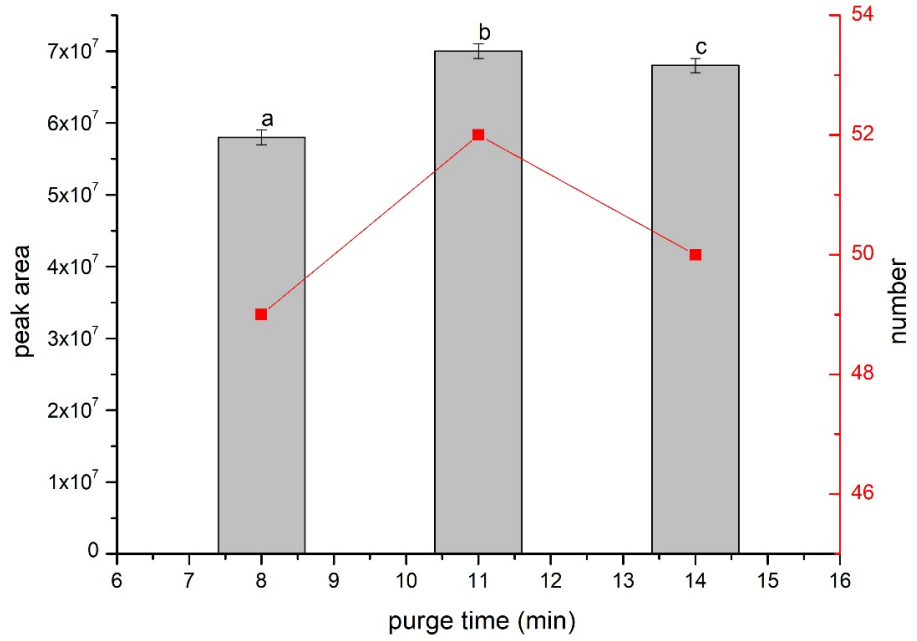


**Figure 3.** Effect of purge time on the extraction efficiency. Sample volume = 3 mL, purge temperature = 25°C.

## Verification and quantitative analysis (of P&T-GC-MS method)

Once the final purge conditions were selected, these five aroma standards were detected. Table 2 shows the results from method validation: linearity, recovery, reproducibility, LOD and LOQ.

## Linearity

The linearity of the method was evaluated by analysing a series of aromatic standards. Linearity was found in the concentration range between 5 and 160 $\mu g/L$, with high

reproducibility and accuracy. Regression analysis of the experimental data points showed a linear relationship with excellent regression coefficients ($r^2$>99%) for 2-methylbutyraldehyde, ethyl acetate, 2-nonanone, linalool, and ethyl caprylate.

**Table 2.** Performance parameters of the P&T method for the volatile compounds in blueberry.

| Compounds | Linearity ($r^2$) | Recovery (%) | C.V. (%) | LOD (µg/L) | LOQ (µg/L) |
|---|---|---|---|---|---|
| 2-Methylbutyraldehyde | 0.9982 | 117.01 | 4.3418 | 0.78 | 2.60 |
| Ethyl acetate | 0.9971 | 91.52 | 1.7276 | 0.90 | 3.00 |
| 2-Nonanone | 0.9957 | 103.47 | 2.8291 | 1.02 | 3.40 |
| Linalool | 0.9933 | 99.33 | 5.4841 | 1.06 | 3.50 |
| Ethyl caprylate | 0.9964 | 106.18 | 7.1420 | 1.29 | 4.30 |

## Recovery

Recoveries ranged between 96% and 120%, indicating that the accuracy of the method meets the experimental requirements and that the results are reliable.

## Reproducibility

Reproducibility was evaluated by using the coefficient of variation (CV%) for replicate analyses. The CV% values obtained are shown in Table 3. CV% values were found to be <8% in the case of relative proportions (HAKALA *et al.*, 2002). The smallest CV% was found for ethyl acetate (1.73%), and the largest was found for ethyl octanoate (7.14%). In the range of esters, as the carbon number increases, the coefficient of variation also increased. As shown above, the P&T-GC-MS technique was reproducible enough to allow for comparative comparison studies of the volatiles of different varieties (HAKALA *et al.*, 2002).

Determination of the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was calculated as the concentration required to obtain a signal that was three times higher than that of the baseline signal (PINO and OUERIS, 2010). Detection limits were below 1.29 $\mu$g/L for all volatiles. The LOQ can also be estimated as the concentration of analyte producing a signal that is 10 times that of the noise (S/N = 10) (PINO and OUERIS, 2010).

From the above results, good linearity, high accuracy, very good repeatability and a low limit of detection were achieved (DENG *et al.*, 2011). There were also good recoveries and reproducibility. This method can be applied for research on the volatiles in blueberries.

In conclusion, 3 mL of sample purged at 25°C for 11 min were selected as the best extraction conditions for the P&T methodology developed in this study.

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From the above results, good linearity, high accuracy, very good repeatability and a low limit of detection were achieved (DENG *et al.*, 2011). There were also good recoveries and reproducibility. This method can be applied for research on the volatiles in blueberries.

In conclusion, 3 mL of sample purged at 25°C for 11 min were selected as the best extraction conditions for the P&T methodology developed in this study.

**Table 3.** Analysis of volatile compounds from different blueberry varieties.

| No. | RI | $t_R$ (min) | Compounds | Relative content (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | WH-M | WS-M | CB-G | CP-G | CB-Y |
| | | | **Esters** | | | | | |
| 1 | 487 | 1.603 | Methyl acetate | | | | 0.14±0.01 | |
| 2 | 584 | 1.644 | Ethyl formate | 9.9±0.50[a] | 8.81±0.43[a] | | | 9.54±0.51[a] |
| 3 | 586 | 1.891 | Ethyl acetate | 60.94±3.05[a] | 66.21±3.24[a] | 4.89±0.22[c] | 1.58±0.09[c] | 17.36±0.95[b] |
| 4 | 686 | 2.518 | Ethyl propionate | 0.48±0.02[a] | 0.11±0.01[b] | | | |
| 5 | 686 | 2.540 | Propyl acetate | 0.14±0.01[a] | 0.10±0.01[a] | | | |
| 6 | 686 | 2.620 | Methyl butyrate | 0.03±0.00[b] | | | | 0.84±0.03[a] |
| 7 | 778 | 2.796 | Isopentyl formate | 0.24±0.02[c] | 0.60±0.03[b] | | | 1.00±0.04[a] |
| 8 | 785 | 3.053 | Ethyl butyrate | 0.04±0.00[a] | 0.04±0.00[a] | | | |
| 9 | 785 | 3.247 | Butyl acetate | 9.80±0.50[a] | 9.30±0.42[a] | | | 5.09±0.27[b] |
| 10 | 820 | 4.680 | Ethyl isovalerate | 1.78±0.10[b] | 1.32±0.08[b] | | | 12.66±0.68[a] |
| 11 | 864 | 5.193 | Amyl acetate | 3.97±0.21[a] | 3.80±0.24[a] | | | |
| 12 | 869 | 6.330 | Prenylacetate | 0.02±0.00[b] | | | | 0.24±0.01[a] |
| 13 | 869 | 6.414 | Ethyl 3,3-dimethylacrylate | 0.02±0.00 | | | | |
| 14 | 983 | 7.614 | Ethyl 2-hydroxy-3-methylbutanoate | 0.04±0.00 | | | | |
| 15 | 1029 | 8.875 | Hexenyl acetate | 0.16±0.01[a] | 0.06±0.00[b] | | | |
| 16 | 1043 | 8.900 | Butyl pentanoate | 0.01±0.00[b] | 0.02±0.00[b] | | | 0.17±0.01[a] |
| 17 | 1047 | 9.117 | Hexyl acetate | 0.02±0.00[a] | 0.01±0.00[a] | | | |
| 18 | 1277 | 18.500 | L-Bornyl acetate | | | 0.10±0.01 | | |
| 19 | 1294 | 19.949 | 2-Methylpropyl benzoate | 0.07±0.00[c] | 0.06±0.00[c] | 0.40±0.02[a] | 0.27±0.01[b] | |
| | | | **Alcohol**s | | | | | |
| 20 | 662 | 2.156 | n-Butyl alcohol | 0.10±0.00[b] | | | | 16.41±0.76[a] |
| 21 | 788 | 2.275 | Cyclopentanol | | | | | 0.57±0.03 |
| 22 | 700 | 2.817 | 2-Methyl-1-butanol | 0.12±0.01[b] | 0.32±0.02[a] | | | |
| 23 | 769 | 2.982 | 2-Penten-1-ol | | | 0.12±0.01 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 24 | 995 | 4.659 | 6-Methyl-heptanol | 1.85±0.0[8a] | | | | 0.26±0.01[b] |
| 25 | 858 | 4.934 | 2-Hexen-1-ol | 0.05±0.00[d] | 0.17±0.01[cd] | 0.51±0.03[b] | 1.19±0.07[a] | 0.26±0.01[c] |
| 26 | 860 | 5.020 | Hexyl alcohol | 0.51±0.03[b] | 0.67±0.03[b] | 2.08±0.12[b] | 3.77±0.20[a] | 2.07±0.98[b] |
| 27 | 960 | 7.780 | n-Heptanol | | | 0.10±0.00 | | |
| 28 | 969 | 8.092 | 1-Octen-3-ol | 0.07±0.00[c] | 0.06±0.00[c] | 0.35±0.02[b] | 0.38±0.02[b] | 0.94±0.05[a] |
| 29 | 969 | 8.835 | Citronellol | | | | 1.49±0.06 | |
| 30 | 971 | 9.401 | 3-Ethyl-4-methyl-1-pentanol | | | 0.12±0.01 | | |
| 31 | 1042 | 9.535 | 4-Isopropyltoluene | | | 0.08±0.00[b] | 0.31±0.02[a] | |
| 32 | 1055 | 9.664 | 2-Ethylhexanol | 1.84±0.11[c] | 1.16±0.01[c] | 11.2±0.52[a] | 9.17±0.43[b] | 10.61±0.59[ab] |
| 33 | 1059 | 9.795 | Eucalyptol | 0.07±0.00[bc] | 0.10±0.00[b] | 0.39±0.02[a] | 0.10±0.01[d] | 0.06±0.00[c] |
| 34 | 1060 | 11.095 | 1-Octanol | 0.13±0.01[b] | 0.09±0.00[b] | 0.92±0.05[a] | 0.87±0.03[ab] | 0.33±0.02[ab] |
| 35 | 1063 | 11.157 | Dihydromyrcenol | | | | 1.88±0.10 | |
| 36 | 1082 | 12.093 | Linalool | 0.05±0.00[c] | 0.03±0.00[c] | 0.88±0.04[b] | 0.93±0.04[b] | 2.68±0.14[a] |
| 37 | 1138 | 12.753 | Fenchyl alcohol | 0.10±0.01[b] | | 0.53±0.03[a] | 0.18±0.01[b] | |
| 38 | 1153 | 14.249 | Menthol | | | 0.22±0.02 | | |
| 39 | 1158 | 14.598 | Borneol | | | 0.96±0.05 | | |
| 40 | 1159 | 14.622 | 1-Nonanol | 0.08±0.00[c] | 0.05±0.00[c] | 0.44±0.02[b] | 0.75±0.03[a] | |
| 41 | 1164 | 14.817 | DL-Menthol | 0.68±0.03[b] | 0.85±0.04[a] | 0.65±0.03[b] | 0.47±0.02[c] | |
| 42 | 1187 | 14.914 | 4-Terpineol | 0.15±0.01[c] | 0.12±0.01[c] | 0.80±0.03[a] | 0.27±0.01[b] | |
| 43 | 1198 | 15.422 | (-)-α-Terpineol | 0.07±0.00[c] | 0.05±0.00[c] | 0.51±0.02[a] | 0.38±0.02[b] | 0.06±0.00[c] |
| 44 | 1228 | 17.356 | Geraniol | | 0.99±0.05[b] | 13.28±0.57[a] | | |
| 45 | 1258 | 18.133 | 1-Decanol | 0.06±0.00[b] | | 1.50±0.07[a] | | |
| 46 | 1200 | 18.536 | cis-Anethol | 0.06±0.00[b] | 0.04±0.00[b] | 0.17±0.02[a] | | |
| 47 | 1262 | 18.978 | Thymol | | | 0.10±0.00 | | |
| 48 | 1457 | 24.504 | 1-Dodecanol | | | | 0.37±0.15 | |
| 49 | 1543 | 28.659 | Cedrol | | | 0.07±0.00 | | |
| | | | **Aldehydes and ketones** | | | | | |
| 50 | 508 | 1.589 | Propionaldehyde | | | 2.93±0.14[b] | 2.78±0.12[b] | 3.66±0.20[a] |
| 51 | 543 | 1.725 | Isobutyraldehyde | | 0.16±0.01[b] | | | 0.53±0.02[a] |
| 52 | 555 | 1.815 | 2-Butanone | | | | | 0.92±0.04 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 53 | 643 | 2.008 | 2-Methylbutyraldehyde | | | | 1.57±0.04$^{a}$ | 1.22±0.07$^{b}$ |
| 54 | 644 | 2.279 | 1-Penten-3-one | 0.04±0.00$^{b}$ | | 0.65±0.03$^{a}$ | | |
| 55 | 654 | 2.384 | 3-Pentanone | | 0.17±0.01$^{b}$ | | | 0.84±0.05$^{a}$ |
| 56 | 715 | 2.982 | 2-Pentenal | | | 0.08±0.00 | | |
| 57 | 791 | 3.613 | 4-Methyl-3-pentene-1-one | | 0.07±0.00$^{c}$ | 22.98±1.13$^{a}$ | 21.9±0.99$^{a}$ | 8.49±0.37$^{b}$ |
| 58 | 806 | 3.654 | Hexanal | 0.88±0.04$^{b}$ | | 16.59±0.96$^{a}$ | 17.3±0.87$^{a}$ | |
| 59 | 831 | 4.225 | Furfural | 0.14±0.01$^{b}$ | 0.12±0.01$^{b}$ | 0.12±0.01$^{b}$ | 4.13±0.23$^{a}$ | |
| 60 | 853 | 5.500 | 2-Heptanone | 0.01±0.00$^{c}$ | | 0.22±0.01$^{b}$ | 1.68±0.07$^{a}$ | |
| 61 | 841 | 5.816 | 4-Methylhexanal | 0.02±0.00$^{b}$ | | 0.17±0.01$^{b}$ | 2.07±0.11$^{a}$ | |
| 62 | 913 | 7.358 | 2-Heptenal | | | 0.04±0.00 | | |
| 63 | 982 | 7.509 | Benzaldehyde | 0.07±0.00$^{d}$ | 0.04±0.00$^{d}$ | 0.65±0.03$^{b}$ | 0.92±0.06$^{a}$ | 0.21±0.01$^{c}$ |
| 64 | 1005 | 8.823 | Octanal | 0.03±0.00$^{d}$ | | 0.17±0.01$^{b}$ | 0.21±0.01$^{a}$ | 0.13±0.00$^{c}$ |
| 65 | 1013 | 10.619 | 2-Octenal | | | 0.04±0.00 | | |
| 66 | 1052 | 11.762 | 2-Nonanone | 0.03±0.00$^{b}$ | 0.03±0.00$^{b}$ | | | 0.22±0.01$^{a}$ |
| 67 | 1104 | 12.251 | Nonanal | 1.38±0.06$^{c}$ | 1.39±0.07$^{c}$ | 7.01±0.42$^{b}$ | 9.22±0.51$^{a}$ | 2.18±0.14$^{c}$ |
| 68 | 1112 | 14.194 | (2E)-Nonenal | | | | 0.18±0.02 | |
| 69 | 1151 | 15.300 | 2-Decanone | 0.02±0.00 | | | | |
| 70 | 1204 | 15.807 | Decanal | 1.84±0.01$^{a}$ | 0.50±0.03$^{bc}$ | 0.58±0.02$^{b}$ | 0.47±0.03$^{c}$ | |
| 71 | 1208 | 16.063 | 2,4-Dimethylbenzaldehyde | 0.16±0.01$^{c}$ | 0.10±0.01$^{c}$ | 0.57±0.03$^{a}$ | 0.45±0.02$^{b}$ | |
| 72 | 1263 | 16.358 | 5-Hydroxymethylfurfural | | | | 4.46±0.29 | |
| 73 | 1402 | 22.595 | Dodecyl aldehyde | 0.01±0.00$^{b}$ | | | 0.14±0.01$^{a}$ | |
| 74 | 1420 | 23.826 | (Z)-Geranyl acetone | 0.36±0.02$^{d}$ | 0.58±0.03$^{c}$ | 0.74±0.04$^{b}$ | 1.19±0.06$^{a}$ | |
| | | | **Others** | | | | | |
| 75 | 877 | 4.514 | 3,7-Dimethyl-1-octene | 0.02±0.00$^{b}$ | | 0.61±0.03$^{a}$ | | |
| 76 | 883 | 5.574 | Phenylethylene | 0.05±0.00$^{c}$ | 0.04±0.00$^{c}$ | 0.18±0.01$^{b}$ | 0.27±0.01$^{a}$ | |
| 77 | 1029 | 10.851 | Acetophenone | 0.04±0.00$^{b}$ | 0.03±0.00$^{b}$ | 0.14±0.01$^{b}$ | 3.37±0.20$^{a}$ | |
| 78 | 1231 | 14.992 | Naphthalene | 0.48±0.02$^{c}$ | 0.46±0.02$^{c}$ | 1.63±0.08$^{b}$ | 2.35±0.13$^{a}$ | 0.45±0.02$^{c}$ |
| 79 | 1407 | 22.876 | Cedarene | | | | 0.84±0.05 | |
| 80 | 1668 | 25.545 | Butylated hydroxytoluene | 0.77±0.04$^{c}$ | 1.17±0.06$^{b}$ | 2.53±0.11$^{a}$ | | |

### 3.2. Identification of the volatile compounds in five blueberry varieties

As shown in Table 3, the volatile compounds in the five blueberry varieties were identified. A total of 80 volatiles were identified, including 19 esters, 30 alcohols, 18 aldehydes, 7 ketones and 6 other compounds. The number of identified volatile compounds in each blueberry variety ranged from 30 to 53. WH-M and CB-G had the highest (53) and the second highest number (47) of volatile compounds, respectively, while CB-Y had the smallest number (30) of volatile compounds.

Esters are considered to be contributors to fruity and floral notes (WANG *et al.*, 2009). A total of 21 ester compounds were detected in the five blueberry varieties. Ethyl acetate is a common compound that has a strong fruity aroma. Among the 5 groups, the sum of the esters was higher in WS-M and WH-M blueberries than in the other cultivated groups. Esters were abundant in wild blueberries, contributing 87.66-90.44% of the total volatiles (Table 4). Although 13 esters in total were found in wild blueberries in this study, ethyl acetate, ethyl formate, butyl acetate and amyl acetate accounted for more than 80% of the total esters in WH-M and WS-M. The unique esters of WH-M were methyl butyrate, prenylacetate, ethyl 3-methyl-2-butenoate, ethyl 3,3-dimethylacrylate, and ethyl 2-hydroxy-3-methylbutanoate, with the latter two in agreement with previous results (BEAULIEU *et al.*, 2014). L-Bornyl acetate was only detected in CB-G. 2-Methylpropyl benzoate was detected in all varieties except CB-Y. Esters were not considered to be as important as aldehydes to the aroma in highbush blueberries, while they have been identified as important volatiles in some rabbiteye blueberries, which is consistent with previous results (Du and ROUSEFF, 2014).

The total content of alcohols accounted for 4.7-35.98% of the total volatiles (Table 3). The content of alcohols was significantly higher in cultivated blueberries than in wild blueberries. Of the 30 alcohols identified in this study, 8 were identified in all five varieties: 2-hexen-1-ol, hexyl alcohol, 1-octene-3-ol, eucalyptol, 1-octanol, linalool, 2-ethylhexanol, and (-)-$\alpha$-terpineol. Among them, 2-ethylhexanol was dominant, with relative contents ranging from 1.16% to 11.20% (Table 4). 2-Methyl-1-butanol was detected in WS-M and WH-M. 2-Penten-1-ol, 3-ethyl-4-methyl-1-pentanol, borneol and menthol were only detected in CB-G. The unique alcohols in CP-G and CB-Y were citronellol and cyclopentanol, respectively.

A total of 25 different aldehydes and ketones in blueberry juice were identified, accounting for 3.16%-68.67% of the total volatiles (Table 4). The sum of the aldehydes and ketones in CP-G was significantly higher than that in other varieties, and it was also significantly higher in cultivated blueberries than in wild blueberries. Nonanal and benzaldehyde were the predominant aldehydes found in the five blueberry varieties. WH-M had a significantly higher decanal content than that of the other aldehydes. In all cultivated groups, 4-methyl-3-pentene-1-one was the major component, accounting for more than 20% of the total aldehydes in CB-G and CP-G. 2-Pentenal, 2-heptenal, and 2-octenal were only detected in CB-G. Additionally, (2E)-nonenal, 5-hydroxymethylfurfural and dodecyl aldehyde could be used to distinguish CP-G from the other varieties. 2-Butanone was only detected in CB-Y.

**Table 4.** The aroma-active compounds (ROAV > 1) in different blueberries*.

| No. | Volatile | Threshold (μg/L) | Sensory attributes | Aroma classification | ROAV | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | WH-M | WS-M | CB-G | CP-G | CB-Y |
| 1 | Ethyl formate | 150 | Fruity | 1 | 0.54 ±0.03$^{b}$ | 0.44± 0.02$^{b}$ | | | 1.83 ±0.10$^{a}$ |
| 2 | Ethyl acetate | 5 | Fruity | 1 | 100.00±5.00$^{a}$ | 100.00±4.89$^{a}$ | 13.95±0.63$^{b}$ | 3.43±0.20$^{b}$ | 100.00 ±5.47$^{a}$ |
| 3 | Butyl acetate | 66 | Sweet, banana, | 1,3 | 1.22±0.06$^{b}$ | 1.06 ±0.05$^{b}$ | | | 2.22 ±0.12$^{a}$ |
| 4 | 1-Octene-3-ol | 1 | Mushroom | 4 | 0.57 ±0.00$^{c}$ | 0.45 ±0.00$^{c}$ | 4.99±0.29$^{b}$ | 4.12 ±0.22$^{b}$ | 27.07±1.44$^{a}$ |
| 5 | Linalool | 6 | Sweet lemon | 1 | 0.07 ±0.00$^{c}$ | 0.04 ±0.00$^{c}$ | 2.09 ±0.10$^{b}$ | 1.68 ±0.07$^{b}$ | 12.86 ±0.67$^{a}$ |
| 6 | Geraniol | 40 | Rose | 2 | | 0.19 ±0.01$^{b}$ | 4.74 ±0.20$^{a}$ | | |
| 7 | 2-Methylbutyraldehyde | 1 | Stimulating, coffee, sweet | 1,3,6 | | | | 17.03 ±0.43$^{b}$ | 35.14±2.02$^{a}$ |
| 8 | Hexanal | 5 | Fragrant, grassy | 5 | 1.44 ±0.07$^{c}$ | | 47.33 ±2.74$^{a}$ | 37.53±1.89$^{b}$ | |
| 9 | 4-Methylhexanal | 3 | Fruity, rose | 1,2 | 0.05 ±0.00$^{b}$ | | 0.81±0.05$^{b}$ | 7.48±0.40$^{a}$ | |
| 10 | Octanal | 0.7 | Rose, orange | 1,2,3 | 0.35 ±0.00$^{c}$ | | 3.46 ±0.20$^{b}$ | 3.25 ±0.15$^{b}$ | 5.35 ±0.00$^{a}$ |
| 11 | Nonanal | 1 | Floral, citrus, slightly spicy | 1,2,6 | 11.32 ±0.49$^{c}$ | 10.50 ±0.53$^{c}$ | 100.00±5.99$^{a}$ | 100.00±5.53$^{a}$ | 62.79 ±4.03$^{b}$ |
| 12 | Decanal | 3 | Fruity | 1 | 5.03 ±0.03$^{b}$ | 1.26±0.08$^{d}$ | 2.76±0.10$^{a}$ | 1.70±0.11$^{c}$ | |

*Intensity: 1-fruity, 2-floral, 3-sweet, 4-fatty, 5-fragrant, 6-stimulating

### 3.3. Determination of the aroma active compounds in different blueberries

Considering that volatile compounds have different thresholds and people have different sensitivities to them, the relative content cannot reflect the true contribution that every volatile compound makes to the whole aroma profile. Therefore, we used ROAVs to detect the contribution of volatile compounds to the whole aroma profile (YI *et al*., 2016). Fourteen aroma active compounds were selected from five blueberry varieties, which are shown in Table 4.

There were four aroma active compounds (ethyl acetate, 1-octene-3-ol, linalool, nonanal) with higher ROAVs in five varieties. Ethyl acetate and nonanal possessed the highest ROAVs in wild blueberries and cultivated blueberries, respectively. CB-Y had the highest ROAV summations, which was significantly higher than the other four varieties.

Aldehydes were the most abundant chemical group, with aromatic activity found in five blueberry varieties. 2-Methylbutyraldehyde, hexanal, 4-methylhexanal, 1-octanal, nonanal and decanal contributed to stimulating, fragrant, fruity, rose, floral and fruity aroma notes, respectively. 2-Methylbutyraldehyde was observed only in CP-G. 2-Methylbutyraldehyde has stimulating, coffee, and sweet aroma notes, with a very low threshold (1 μg/L) in CP-G. Aldehydes made a major contribution to blueberry aromas, which is in agreement with previous results (Du and ROUSEFF, 2014; HORVAT and SENTER, 1985).

Alcohols were the next most abundant group, including 1-octene-3-ol, linalool, and geraniol, contributing mushroom, lemon and rose aroma notes. 1-Octene-3-ol and linalool were identified in the five blueberry varieties.

Three esters, including methyl acetate, ethyl acetate and butyl acetate, were aroma active. Ethyl formate had a high threshold value (150 μg/L) and a high relative content. However, its ROAVs were low (0.32-1.53). Ethyl acetate contributed a fruity aroma to the five varieties and possessed the highest ROAV in wild blueberries. Butyl acetate contributed sweet and banana aroma notes. However, it has not been previously reported as contributing to blueberry aroma.

Although wild blueberries had higher contents of volatile compounds, their characteristic aroma notes were less than those of cultivated blueberries. The reason may be that the aroma of fruit is not completely dependent on the concentration of the volatile compound but it is closely related to its threshold. The threshold of volatile compounds found differed greatly among the varieties studied. For example, the relative contents of ethyl formate in the two wild blueberries were higher than those in cultivated blueberries, but the ROAVs were lower because the threshold value of methyl acetate was high (150 μg/L).

Six descriptors (fruity, floral, sweet, fatty, fragrant, and stimulating) were used to provide an assessment of the five blueberries. To reflect the difference in aroma among different blueberry varieties, the ROAV of each blueberry aroma component was taken as the logarithm base 10, and the aromatic series of the five blueberry juices on the spider web diagram are shown in Fig. 4.

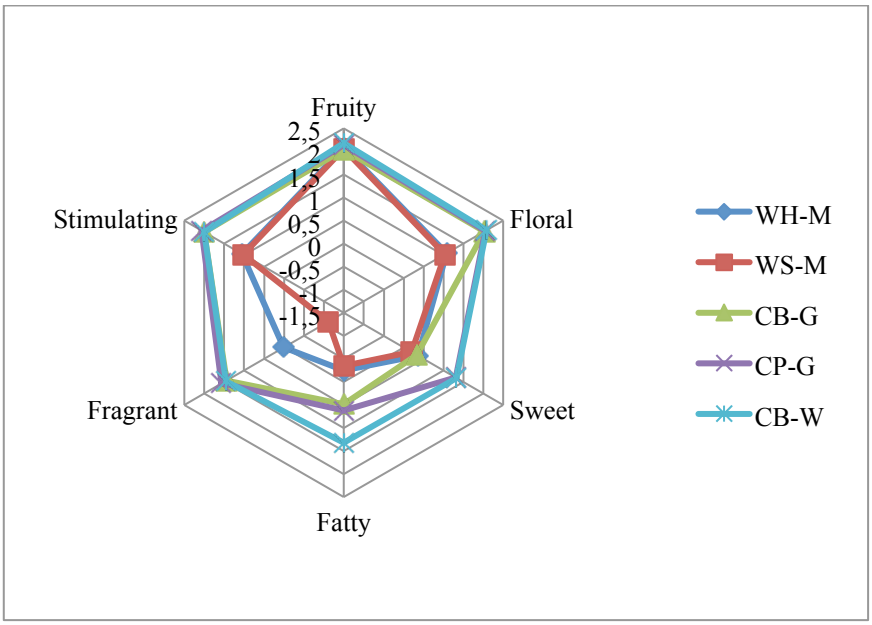


**Figure 4.** Aromatic series in blueberries based on aroma activity values.

The analysis showed that WH-M and WS-M could mostly be described as having fruity and floral notes due to the higher ROAVs of ethyl acetate and nonanal in the samples. CB-G and CP-G had higher values for the attributes fragrant and floral due to their large quantities of hexanal and nonanal. The difference between CB-G and CP-G lies in the fact that CB-G exhibited a greater sweet component. The ROAV of 1-octene-3-ol was higher in CB-Y; thus, CB-Y was perceived to have a fatty aroma. Considering the volatile composition of these blueberries, samples had higher values for the attributes fruity and fragrant due to their large quantities of aldehydes and alcohols.

## 4. CONCLUSIONS AND FUTURE WORK

The P&T extraction method coupled to GC-MS analysis was a quick and efficient method for the evaluation of blueberry volatiles, and the results demonstrated that 3 mL of sample volume purged at 25°C for 11 min were the best extraction conditions. A total of 80 volatiles were identified in five blueberry varieties using the P&T-GC-MS technique. The volatiles of blueberries were composed of mainly aldehydes, alcohols, esters, and terpenes. Among the identified compounds, 12 compounds (ROAV>1), including ethyl formate, ethyl acetate, butyl acetate, 1-octene-3-ol, linalool, geraniol, 2-methylbutyraldehyde, hexanal, 4-methylhexanal, octanal, nonanal and decanal, were considered aroma active. The spider web diagram showed that the sensory characterization of the five varieties was distinct due to the different quantities of volatile compounds.

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