ISEKI Food Association

ISSN: 2182-1054

International Journal *of* Food Studies

Volume 10 | October 2021

International Journal of Food Studies

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INTERNATIONAL JOURNAL OF FOOD STUDIES

Volume 10, ISSUE 2 (2021)
(Published 18 October 2021)

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 282–295

Development of Technology for the Production of Sausage Produce Using Secondary Collagen-Containing Raw Materials

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Received: 24 February 2020; Published online: 18 October 2021

Abstract

One of the main requirements for modern technologies is to expand the range of meat products by creating combined products with a balanced composition of food and biologically active substances. The purpose of the study was to develop a technology for the production of such combined meat products. The research used secondary meat raw materials of the meat processing industry: horse meat, flank and other beef muscle tissue of the second grade, which, after fermentation by a consortium of microorganisms consisting of the following cultures: *Lactobacillus bulgaricus, Bifidumbacterium siccum, Staphilococcus carnosus*, were used instead of the main meat raw materials in the production of sausages. The following indicators of ready-prepared products were studied: organoleptic properties, chemical, vitamin and mineral composition, toxicity and harmlessness, storage duration. The results showed that the use of this consortium of microorganisms in the production of sausage products made it possible to use secondary collagen-containing raw materials for processing. The positive influence of the proposed biotechnological method of processing meat raw materials on the organoleptic, physical-chemical, structural-mechanical, microbiological characteristics and biological value of the finished product was revealed. It was found that the use of a consortium of microorganisms increased the quality of finished products. In addition, the proposed technology has the potential to reduce the cost of production and increase the share of waste-free production in the meat processing industry.

Keywords: Collagen-containing raw materials; Biomodification; Combined meat product; Lactic Acid bacteria

1 Introduction

Currently, in the meat industry, it is possible to produce new types of combined meat products with a balanced composition for both general and special medical and preventive purposes on the basis of biotechnology (Gabitov et al., 2018; Gizatov & Gizatova, 2015; Lamanov et al., 2020; Sultanova et al., 2019). Improving the methods of enzymatic processing of low grade meat raw materials can improve the functional and technological properties, as well as the quality indicat-

 10.7455/ijfs/10.2.2021.a1

ors of ready-prepared products (Antipova et al., 2011; Antipova et al., 2015; Armuzzi et al., 2001; Coton et al., 2012; Fletcher, 2002; Wang et al., 2013).

The traditional field of application of low-grade meat raw materials is canning and sausage production. Canned meat and sausages, saveloys, boiled and boiled-smoked sausages are the most popular meat products, primarily due to their low cost. However, the use of low-grade raw materials in the process of their production leads to a significant decrease in the quality of the finished product. For example, even hard heat treatment in the production of canned food does not get rid of hard connective tissue inclusions, with a negative impact on the consistency of the finished product. In the production of cooked and smoked sausages, where heat treatment is carried out in relatively mild conditions, this disadvantage is even more noticeable.

Taking into account the results of published research, it can be assumed that the most promising areas in the creation and use of microbial consortia are:

- processing of raw meat with a high content of connective tissue (low-grade) to reduce its stiffness, improve functional and technological properties in the process of processing, increase organoleptic parameters;
- acceleration of the process of maturation and salting of meat raw materials.

Production of functional products using a consortium of microorganisms can be implemented at any meat processing plant without the costs of significant capital investments for re-equipment. Full implementation of the proposed technologies will expand the range of functional products against the background of a shortage of dietary protein. Considering the traditional technological schemes for the production of cooked sausages, saveloys and sausages (for example, "Steppe"), it is obvious that the consortium of microorganisms must be introduced at the stage of salting of the raw materials. This solution is optimal, since it ensures the growth of microorganisms within 8 hours, during which time the complete distribution of microorganisms occurs, which significantly increases the efficiency of the consortium. It is recommended that the process of producing products using a consortium of microorganisms is organized according to the following technology. To obtain products (Antipova & Uspenskaj, 2016) that are in demand by the population, it is necessary to select such a ratio of components so that the products have a high nutritional and biological value, and an attractive presentation.

In Italy, *Micrococcus sp., Lactobacillus plantarum* strains were tested to study the organoleptic properties of dry sausages as starter cultures. In England, *Lactobacillus* and *Micrococcus* starter cultures are used in the ratio 50:50 for the production of fermented Lefkas-type sausages (Sufiyanova et al., 2012). Several other crops were used to compare the technological effects: *Petrostreptococcus parubus, L. plantarum, Pediococcus acidilactici*, as well as their combinations with *Streptococcus carnosus* MIII. In all variants of microorganisms tested, the best results are obtained with *Lactobacillus pentosus*. The effect was expressed as a rapid decrease in pH, obtaining a sausage of an attractive colour, a mildly sour taste and a well-pronounced meat aroma (Digaitiene et al., 2012). Optimal variants for organoleptic parameters were obtained using a mixed starter containing 90% *S. carnosus* and 10% *L. plantarum*, in particular in the production of Turkish smoked sausages (Antipova & Uspenskaj, 2016). The role of starter cultures *L. plantarum 4045, Staphylococcus sp., L. plantarum 4045 + Micrococcus 12 and L. plantarum 4045 + Staphylococcus sp.*, and endogenous meat enzymes in the process of lipolysis in dry fermented sausages was studied. Samples with *L. plantarum* had the lowest pH rates, but the content of free fatty acids was higher in the inoculated samples compared to the control ones as mainly endogenous meat enzymes play an important role in the process of lipolysis (Hatakka et al., 2001).

The purpose of this study was to develop functional products from biomodified low-grade raw materials using consortia of microorganisms. Within the framework of this goal, we had the following objectives:

- to study the cultural properties of the selec-

ted microorganisms (i.e. *Lactobacillus bulgaricus, Bifidumbacterium siccum, Staphilococcus carnosus*);

- to study the biochemical properties of the selected microorganisms and the synergy of microorganisms in the consortium;
- to study the functional and technological properties of model minced meat from low-grade raw materials with the addition of a consortium of microorganisms;
- to develop recipes and production technologies for functional products based on biomodified secondary raw materials.

The influence of the consortium on the functional and technological properties of biomodified model minced meat from horse meat, flank and other beef of second grade was studied. The technological scheme of production of functional probiotic sausages using a consortium of microorganisms was described and the quality of finished products was assessed.

2 Materials and Methods

2.1 Sausage production technology

In order to determine the competitiveness of new products in the consumer market, their nutritional and biological value, a comprehensive assessment of their properties was carried out. The following product indicators were studied: organoleptic; chemical, vitamin, mineral composition; microbiological indicators; storage duration.
Samples of beef and horse meat for research were selected according to All Union State standard R 51447, All Union State standard 9792 (Fischer, 2007; Fletcher, 2002; Knol et al., 2001) after which they were made into a combined sample and wrapped in parchment labelled to identify the sample. For the production of sausages in addition to raw meat, we used skimmed cow milk according to All Union State standard 10970; flour according to All Union State standard 26574, not lower than first grade; *Staphylococcus carnosus* (bacterial concentrate freeze-dried) according to Commodity Specification 9229-074-04610209; *L. bulgaricus* (No. 8P-A3 bacterial concentrate freeze-dried in culture medium with the addition of protective sucrose-gelatose-dairy medium) produced by FSUE "SPU" "Microgen" of Ministry of Health, RF, branch in Perm "the Perm SPU "Biomed"; *Bifidobacterium siccum* (No. 1, 791, LVA-3, bacterial concentrate freeze-dried in culture medium with the addition of protective sucrose-gelatose-dairy medium) produced by FSUE "SPU" "Microgen" of Ministry of Health, RF, branch in Perm "the Perm SPU "Biomed"; food additives - carrageenan GPI 200, GPI 521 obtained for imports and approved for use by State Sanitary and Epidemiological Surveillance Agency of Ministry of Health of the Russian Federation; salt according to All Union State standard R 51574 not lower than first grade; hen eggs according to All Union State standard 27583; drinking water by Sanitary Regulations and Standarts 2.1.4.1074; extracts of spices according to Commodity Specification 9169-032-04801346; sodium nitrite according to All Union State standard 4197.
Grinding of raw meat for sausages was carried out using traditional technology. The meat was ground through a perforated plate with hole diameters of 2-3 mm. A leaven of a consortium of microorganisms in the amount of 1ml/ 100 g of raw material was introduced into the ground flank and beef veins. After that, the veins and the flank were separately mixed with table salt in a minced meat mixer. The duration of mixing was 4-5 minutes. Salted raw meat was kept at a temperature of 0-4 ^{o}C for 6-12 hours, depending on the type of sausage. After maturation, fine grinding was performed on a cutter for 6-8 minutes with the addition of the remaining components of spices according to traditional technology. The filling of the casings was carried out with a syringe. Polyamide shells were used. Further, heat treatment was performed in heat chambers. Due to the use of polyamide shells, roasting was excluded from the technological scheme. In heat chambers, sausage loaves were initially kept at a temperature of 60-65^{o}C for 20 to 40 minutes, and then steamed at a temperature of 80^{o}C, until they reached 72^{o}C in the centre of the loaf. After cooking, the sausage was cooled with cold water for 5-10 minutes to a tem-

perature no higher than 8^{o}C. Storage time never exceeded 30 days at a temperature of 2-6^{o}C.

2.2 Determination of meat pH and protein content

The pH value of solutions and meat systems was determined by a potentiometric method using a universal pH-121 ionometer. Each sample of meat weighing 10.00±0.02 g was extracted with distilled water in a ratio of 1: 10 for 30 minutes at 20±5 ^{o}C, mixed and filtered through a folded paper filter. Determination of the mass fraction of proteins in muscle tissues was performed using the Kjeldahl method. 0.2 g of collagen gel was added to the Kjeldahl flask with a capacity of 50 cm^3. The samples were crushed, then using a piece of glass, the suspension was lowered to the bottom of the flask.1-2 cm^3 of concentrated sulfuric acid was added, followed by 1 g of a mixture of copper sulphate and potassium sulphate as a catalyst. The contents of the flask were heated in a fume hood. When the mixture turned brown, the flask was removed from the heat, cooled at room temperature, added 2-3 cm^3 of hydrogen peroxide solution with a mass fraction of 30% and continued to heat until a colorless solution was obtained. The latter was used for quantitative determination of protein (Ammor & Mayo, 2007; Zinina et al., 2016).
The hot sample was cooled, quantitatively transferred to a volumetric flask with a capacity of 250 cm^3, the volume was brought to the mark with distilled water, and the contents were mixed. 5 cm^3 of the resulting mineralized sample solution was added to a measuring flask with a capacity of 100 cm^3, and the volume was re-adjusted to the mark with distilled water. To conduct a colour reaction, 1 cm^3 of the secondarily diluted mineralized sample was introduced into the test tube and 5 cm^3 of reagent 1 and 5 cm^3 of reagent 2 were added sequentially, the contents of the test tube were mixed. At the same time, a control solution was prepared using a control mineralized sample (a sample using distilled water). After 30 minutes, the optical density of the solutions was determined using a photoelectrocolorimeter with a red light filter. The measurement was performed in comparison with the control solution.
Determination of lactic acid was carried out by colour reaction with para-oxydiphenylene (Digaitiene et al., 2012).

2.3 Sausage quality evaluation

10 cm^3 of trichloroacetic acid solution (10% w/v) and 2-4 g of minced meat was added to the mortar and dispersed using the pestle for 10 minutes. The resulting suspension was transferred to a volumetric flask with a capacity of 50 cm^3, using first a 20 cm^3 solution of trichloroacetic acid (10% w/v), and then a few cubic centimeters of distilled water. The flask was left for 30 minutes at room temperature, shaken every 10 minutes, then the volume was brought to the mark with distilled water, the flask was closed with a cork, the contents were mixed well, transferred to centrifuge tubes, and centrifuged with a rotation speed of 50 s^{-1} for 10-15 minutes. The supernatant was drained into a dry flask, 25 cm^3 of clear liquid was taken, transferred to a 100 cm^3 volumetric flask, and the volume was brought to the mark with distilled water. Analysis: to precipitate carbohydrates, 1 cm^3 of copper sulphate solution (20% w/v)was added to 2 cm^3 of the diluted supernatant (distilled water was added using a pipette or burette to bring the volume of the liquid to 10 cm^3., ; 1 g of powdered calcium hydroxide was then added followed by vigorous shaking and left to stand for 30 minutes, shaking from time to time, and then centrifuged. The supernatant was poured into a flask. To perform the colour reaction, 1 cm^3 of the supernatant was transferred to a tube of about 25x200 mm in size; 1 drop of copper sulphate solution (4% w/v) and the tube was placed into ice water. Whilst stirring, 6 cm^3 of concentrated sulphuric acid was added from a microburette, the test tube was placed for 5 minutes in a water bath at a boiling point, and then cooled in cold water to 20^{o}C. 0.1 cm^3 of the vapour - oxidiphenyl solution was added to the test tube, mixed very carefully and thoroughly, and then the test tubes were placed for 30 minutes in a water bath at 30^{o}C, with occasional gentle shaking. After this period, the test tube was placed in a vigorously boiling water bath for 90 seconds, then cooled in cold water and the colour intensity was measured using

a spectrophotometer at a wavelength of 560 nm In 1 cm cuvettes. The control with only the reagents was carried out after precipitation of carbohydrates, which was used instead of 2 cm^3 of our sample centrifugate of 0.3 cm^3 of a solution of trichloroacetic acid and 1.7 cm^3 of distilled water. The A calibration curve was made using lactic acid standards. (Antipova et al., 2001).
To determine the amino acid composition, the products were first hydrolyzed with hydrochloric acid at a concentration of 6 mol / dm^3. The amino acid composition and the content of free amino acids were determined by ion exchange chromatography in an automatic amino acid analyzer AAA T-339 (Czech Republic) (Antipova et al., 2015). Amino acid separation was performed on an analytical column filled with an ion exchange resin Ostion LGFA with step-by-step elution with three sodium citrate buffers with different pH values (3.50; 4.25; 9.50). Peaks of light absorption in the eluate from the column were used to detect the presence of individual amino acids in the hydrolysate, as judged by the location of the peaks, and their quantitative content determined by the area of the peaks.
The fatty acid composition was determined by gas-liquid chromatography (Antipova et al., 2015) followed by GC-MS-C analysis (liquid chromatography-mass spectrometry-computer). Identification and quantitative determination of methyl esters of fatty acids using a Varian-3400 chromatograph with FID detector; column length of 25 m, internal diameter of 0.25 mm. Gas carrier: nitrogen; flow rate: 1.18 cm^3 / min. The temperature of the injector and detector was 250 and 300^{o}C respectively. Rate of temperature rise was from 150 to 300^{o}C/min.
Determination of iron was done by colorimetric method that changes the colour intensity of divalent iron with orthophenanthroline according to All Union State standard 26928 (Rakhimov et al., 2018).
The content of mineral substances (trace elements) was determined by the atomic absorption method on an atomic absorption spectrophotometer, as well as by the calculation method (Antipova et al., 2015; Gabitov et al., 2019). For the determination of calcium and magnesium, trilonometric methods were used (Antipova & Uspenskaj, 2016). Phosphorus was determined by the colorimetric method using a molybdenum-vanadium reagent (Sydykova et al., 2019).
The mass fraction of vitamins was determined according to generally accepted methods, as well as the calculated method (Antipova & Uspenskaj, 2016; Gavrilova et al., 2019; Sydykova et al., 2019; Zinina et al., 2016). Vitamin B_1 (thiamine) by a fluorimetric method; vitamin B_2 (Riboflavin) by a fluorimetric method (luminoflavin variant), vitamin PP (Niacin) by a colorimetric method.
Determination of toxic elements: mercury was determined according to All Union State standard 26927, arsenic according to All Union State standard 26930, lead according to All Union State standard 26932, cadmium according to All Union State standard 26933 (Andreeva et al., 2018; Nesterenko et al., 2018).

2.4 Express Biotest

The determination of safety and biological activity used a method with the test culture *Paramecium caudatum* a free-living, easily cultured single-celled organism. The Express Biotest reacts quite sensitively to the active substances contained in the test samples, and reflects their reaction to the viability of the body. The activity of the test organism's vital processes depends on the quality and quantity of the food substrate. The sample was dried at a temperature not higher than 30 ^{o}C to a constant mass. Then 10 g of suspension, crushed if necessary, was sifted through a 72 mesh sieve into a dish to obtain particles of no more than 225 microns. Three samples of 1 g were taken from the dish and 10 ml of distilled water was poured in. The mixture was kept for 24 hours, shaken 2-3 times, and centrifuged. For further work, we used the supernatant that represented a dilution of the test sample of 1: 10.
Express Biotest included three stages:

Stage I assessment of the biological activity of the samples. 9.9 ml of infusory culture was poured into test tubes. The control sample was filled with 0.1 ml of distilled water. The one with active culture was filled with 0.1 ml of decanted fluid of prepared test sample giving a dilution of 10^{-3}. Serial dilutions

of 10^{-4}, 10^{-5} and 10^{-6} were then made. The state of the infusoria was evaluated after 0.5, 1.0, 3.0, 6.0 and 24.0 hours of cultivation at 25 ^{o}C, determining the number and nature of infusoria movements according to the following criteria: ID – indifference – cells made uniform Brownian movements; BA – bioactivity – cell movements were changed (BC – biocidality, toxic effect: BC-50 – 50±10% of cells died, BC – 100 – 90±10% of cells died (when diluted 10^{-3} – the object had a weakly toxic effect; 10^{-4} – medium toxic effect; 10^{-5} – strong toxic effect; 10^{-6} – very strong toxic effect).

Stage II assessment of the biological activity of the samples by the method of resolving influence. The essence of the method was to identify the biological effect of a sample on the mechanism of adaptation and resistance of the cell using an additional enabling adverse factor. The work used a culture of infusoria from the first stage, which was in contact with different concentrations of the sample under study for 24 hours. The study consisted of determining the time of death of 100% of cells under the action of 8% sodium chloride solution.

Stage III assessment of the biological activity of the samples based on the intensity of reproduction of *Paramecium caudatum*. A culture of infusoria in the exponential growth phase was added to the prepared samples. The density of the inoculate was determined. Then they were cultured for 3 days at 25 ^{o}C. At the end of the cultivation time, the density of the inoculate was determined. The index of reproduction intensity at = 1,000 ± 0.100 showed that the object was not biologically active, at > 1,000 ± 0.100 – the object stimulated cell reproduction, at < 1,000 ± 0.100 – the object inhibited cell reproduction. The value of the index of reproduction intensity in combination with the concentration of a given object in the medium characterized the degree of its influence on the reproduction mechanism.

2.5 Organoleptic evaluation

Before conducting an organoleptic evaluation, tasters (five untrained people, without any special selection) were familiarised with the objectives of the tasting and the requirements of regulatory documentation for the quality of the products being evaluated. Samples of products were presented for tasting in the following order: first of all, products with a weakly expressed smell or subtle aroma, less salty and spicy were evaluated; then products with a moderate smell (aroma) and salinity; after that, products with a strong smell (aroma), salty and spicy. A 10-point scale was used in all sensory assessments. Last of all, in each group of similar products, products were evaluated heated (sausages, saveloys, shpikachki, etc.) or heat-treated (ready-to-eat products, pelmeni, chops and other semi-finished products); the order of their presentation was also determined by the degree of intensity of the smell (aroma) and taste. Indicators of the quality of meat and meat products were determined on the whole (uncut) one firstly, and then on the cut product. An organoleptic evaluation of a whole product was performed on a single product unit. Quality indicators of the whole product were determined in the following sequence:

1. appearance, colour and surface condition - visually, by external inspection;
2. smell (aroma) - on the surface of the product. If it was necessary to determine the smell in the depth of the product, a special wooden or metal needle was taken, inserted into the thickness, and then it was quickly removed and determined the smell remaining on the surface of the needle;
3. the consistency - pressing with a spatula or fingers.

The quality indicators of the cut product were determined in the following sequence:

1. before the assessment, meat and meat products were removed from consumer packaging, shell and twine (clips), bones were removed (if any) and using a sharp knife, it was cut into thin slices perpendicular to

the surface of the product, so as to ensure the characteristic appearance and pattern of this product along the cut;

2. colour, appearance and pattern on the section, structure and distribution of ingredients was determined visually on the newly made transverse and longitudinal sections of the product;

3. smell (aroma), taste and juiciness was assessed by testing slices of meat and meat products. This determined the odour (aroma) and taste (the degree of intensity of salty, sour, sweet, bitter taste, etc.); the strength of fragrant spices, fermentation and smoking; the presence or absence of foreign smell and/or taste, aftertaste; d) consistency - pressing, and cutting, a chewing. Consistency was determined by assessing the density, looseness, tenderness, stiffness, crumbling, elasticity and mass uniformity. The aroma, taste, juiciness of sausages, saveloys and shpikachki was determined hot, for which they were lowered into warm water at 50 ^{o}C to 60 ^{o}C and brought to a boil. The juiciness of these hot sausages, saveloys and shpikachki in a natural casing was determined by making a puncture. Where punctured, a juicy sample produced a drop of liquid. After conducting an organoleptic evaluation of 7 to 8 samples a break was taken for at least 10 minutes. Products were evaluated according to a point system - for compliance of quality indicators with the requirements of regulatory documentation (Antipova et al., 2001).

3 Results

The initial stage of development of sausage production technology was the selection of the optimal combinations and concentration of the consortium of microorganisms. The consortium of microorganisms included strains of the following types of microorganisms: *L. bulgaricus* 354.0 x 10^5 CFU/g, *Bifidobacterium siccum* 290.0 x 10^5 CFU/g, *Staphylococcus carnosus* 400.5 x 10^5 CFU/g.

To activate the cultures of microorganisms, skimmed milk was used, which was autoclaved at 0.5 atm for 20 minuntes before use. The amount of the microbial consortium ferment introduced into the sausage mince was determined experimentally by changing the pH values of the medium and the stickiness of the model minced meat. The results of the studies are shown in Figures 1 and 2.

The results of the pH study showed that the minimum and maximum concentrations of microorganisms in the consortium affected the results negatively; the effect of lowering the pH of raw meat was not achieved at low concentrations, whereas with the highest concentrations the pH decrease was too great, which would lead to the acidification of the meat. Therefore, the optimal value was 1 ml/100 g. The results of previous experimental studies have shown that the action of microorganisms increased significantly the stickiness of minced systems (Armuzzi et al., 2001). In the presence of a consortium of microorganisms, the growth of the adhesive ability was somewhat faster, and achieved higher maximum stickiness values (2.6 -2.7 N / cm^2, depending on the type of minced meat). An increase in the duration of exposure (more than 8 hours) led to some stabilization of the growth of stickiness), which was probably due to the formation of low molecular weight proteolysis products that do not have a high adhesive ability.

According to physical and chemical parameters, the cooked products met the requirements specified in Table 1.

In terms of organoleptic characteristics, the new products were not inferior to the traditional ones. The decrease in the proportion of connective tissue proteins had a positive effect on the organoleptic characteristics of the finished product (Tables 2 and 3), primarily the consistency. There was a decrease in stiffness, increased tenderness, and improved chewability. The accumulation of free amino acids enhanced the taste qualities of the experimental samples.

Differences in the structural and mechanical parameters of control and experimental samples of sausages (Table 4).

Paramecium caudatum test was used to test the toxicity and harmlessness of the resulting cooked products. (Tables 5 and 6).

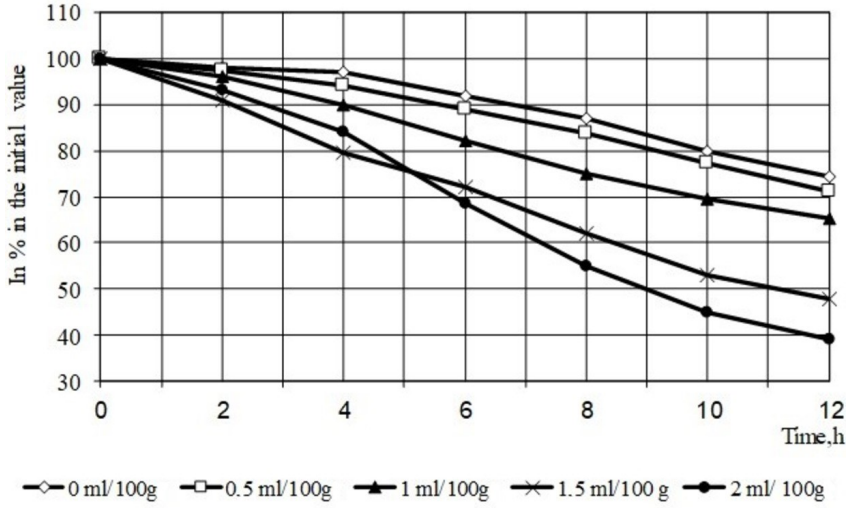

Figure 1: Change in the pH of the medium from the concentration of the consortium of microorganisms.

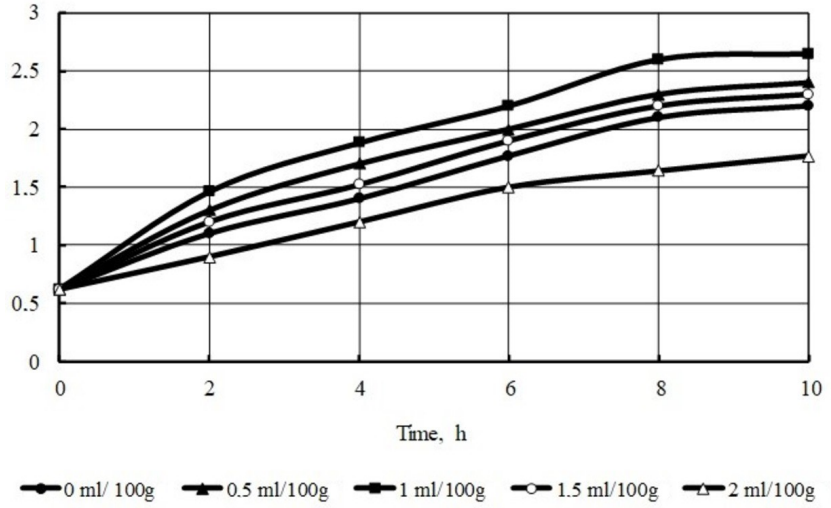

Figure 2: Dynamics of changes in the stickiness of model minced meat according to the concentration of the consortium of microorganisms.

As reported in Table 6, samples did not have any negative effect on the culture of the *Paramecium caudatum*. When the sausage extracts were diluted from 10^{-4} up to 10^{-6} there was no reduction in the viability of the test culture and the index of its biological activity. Thus, sausage products using low-value raw materials and with the addition of a consortium of microorganisms did not show toxicity as assessed by Express Biotest.

4 Discussion

During the development of the production technology, the optimal amount of microorganisms introduced by the consortium into the sausage meat was selected. The optimal amount was 1 l / 100 kg of minced meat. Production of sausages was carried out according to the traditional technology using second grade meat raw materials. According to physical and chemical parameters, the cooked products met the requirements for these types of sausages. In terms of organoleptic characteristics, new products were not inferior to traditional ones. The decline in the proportion of connective tissue proteins had a beneficial effect on the organoleptic properties of final products, primarily on consistency (Cheng & Sun, 2008; Holko et al., 2013; Karam et al., 2013) in terms of the marked reduction of rigidity, increased tenderness and improved chewability. The accumulation of free amino acids enhanced the taste qualities of the experimental samples. When producing food, special attention should be paid to safety, i.e. the absence of substances or concentrations of substances that can cause toxicity in the product. It was found that samples of products did not have a negative effect on the culture of *Paramecium caudatum*. The use of ciliated infusoria to assess the toxicity of human food is based on the fact that the infusoria has a number of enzyme systems similar to higher animals, as well as an acid-base type of digestion. In the presence of toxins, infusoria die. The advantage of the method is the speed of their implementation, good reproducibility and sensitivity, and low cost. The toxicity of finished products was determined by the safety of all infusoria after 24 hours, by its effect on the mechanisms of adaptation and resistance of cells, and by the intensity of reproduction of infusoria after cultivation at 25 ^{o}C for 3 days.

The use of the complex of lactic acid bacteria in the production of sausage products should be recognized as effective and cost-effective, since in the process of adding lactic acid and *Bifidobacteria*, the salting time was reduced and the low-value meat raw materials were softened. The nature of the action of the consortium of microorganisms allows the developed technology to be recommended for introduction into production in order to obtain combined and balanced sausage products.

Similarly, Baka et al. (2011) developed the use of starter cultures as additives to fermented sausages. Thus, the selected starter cultures (i.e. *Lactobacillus sakei 8416, Lactobacillus sakei 4413, and L. sakei 8426, L. Plantarum 7423, and L. curvatus 8427)* were used as starter cultures in addition to control processing in the production of fermented sausages. Starter cultures had rapid growth and prevailed throughout fermentation and maturation, and sensory properties improved compared to the control sample. In addition to the treatment obtained with *L. Sakei* 8416, all other starter cultures prevented lipid oxidation The sausages made with starter cultures *L. sakei* 4413 and *L. Sakei* 8416 had the highest ratings for all sensory properties (Casquete et al., 2011).

In order to improve the food safety of Chinese fermented sausages, Wang et al. (2013) introduced starter cultures of *Lactobacillus sakei* into sausages and studied the impact on sausage quality. The results showed that due to *L. sakei* inoculation, lactic acid bacteria quickly dominated over the microflora and growth of food pathogens such as *E. coli* and Enterobacteria, which were completely eradicated in fermented sausages. The pH of sausages fermented through *L. sakei* significantly decreased. In addition, the nitrite content of *L. Sakei* fermented sausages quickly dropped from 100 parts per million to 9.6 parts per million, while sensory properties improved (Antipova et al., 2011).

Table 1: Characteristics of cooked sausage “Useful”

Name of the indicator	Characteristic and norm for sausage
Mass fraction of moisture, % no more than	73
Mass fraction of sodium chloride, %	2,5
Mass fraction of fat, %	-
Mass fraction of protein, % not less than	10,2
Mass fraction of sodium nitrite, % not more than	0,005
Mass fraction of starch, % not more than	5,5
Residual activity of acid phosphatase, % not more than	0,006

Table 2: Organoleptic evaluation of cooked sausages (Five tasters, in triplicate)

The name of the sample	Indicator						
	Appearance	Appearance and color in the cut	Smell	Taste	Consistency	Juiciness	Overall assessment
Control:							
Sausages “Steppe”	8,2	7,5	7,2	8,4	8,5	7,5	7,9
Saveloys “Steppe”	6,5	5,3	7	7,9	5,8	5	6,2
Cooked sausage “Steppe”	6,5	5,7	6,8	7,6	6,2	5,7	6,3
Experience:							
Sausages “Useful”	8,3	7,6	7,6	8,8	8,4	7,4	7,9
Saveloys “Useful”	6,4	5,3	7,3	8	5,7	4,3	6,2
Cooked sausage “Useful”	6,5	5,9	7,1	7,8	6	5,5	6,2

Table 3: Structural and mechanical characteristics of sausage products

Layer	Control sample		Experimental sample	
	cutoff voltage, x 10-4 Pa	led cutting operation, J / m^2	cutoff voltage, x 10-4 Pa	led cutting operation, J / m^2
Central	2,8 ± 0,09	88,6 ± 2,1	1,55 ± 0,05	74,4 ± 1,9
Peripheral	4,92 ± 0,13	175 ± 3,27	2,01 ± 0,11	98,3 ± 2,27

Table 4: Vitamin and mineral composition of sausage products

Components	Sausage products "Useful" cooked sausage	sausages	saveloys
Vitamins, mg%:			
Folic Acid	0,007	0,005	0,005
B1	0,018	0,017	0,018
B2	0,006	0,007	0,005
B6	0,083	0,04	0,048
Nicotinic acid	0,007	0,006	0,006
Mineral substances, mg%:			
Calcium	98,75	88,35	94
Sodium	7,67	9,07	11,77
Magnesium	32,55	33,85	32,23
Iron	3,09	1,27	1,12

Table 5: Content of heavy metals in the product

Name of the indicator	Cooked	Sausages	Saveloys	Norm
Lead, mcg/g	0,026	0,02	0,021	0,5
Cadmium, mcg/g	0,009	0,004	0,004	0,05
Arsenic, mcg/g	0,089	0,087	0,088	0,1
Mercury, mcg/g	traces	traces	traces	0,03

Table 6: Evaluation of the biological activity of cooked products

The test sample	Index of biological activity in the breeding				
	1:100	1:1000	1:10000	1:100000	1:1000000
Cooked sausage	1,121	1,087	1,004	1	1
Sausages	1,104	1,029	1,014	1,002	1
Saveloys	1,107	1,032	1,02	1,008	1

5 Conclusions

In the course of this research, a technology for producing sausages with a balanced composition of food and biologically active substances was developed. By examining the physical and chemical parameters of the cooked products, we found full compliance with the necessary requirements. At the same time, it turned out that the new products were not inferior to the traditional ones. The decrease in the proportion of connective tissue proteins had a positive effect on the organoleptic characteristics of the finished product. There was a decrease in stiffness, increased tenderness, and improved chewability. The accumulation of free amino acids enhanced the taste qualities of the experimental samples.

When producing food, special attention should be paid to safety, i.e. the absence of substances or concentrations of substances that can cause toxicity in the product. For this purpose, studies were conducted on the content of the elements regulated by the standard. In addition to the analysis for the content of heavy metals, we conducted a biotest for biological activity with *Paramecium caudatum*. The test samples of sausages did not reduce the viability of the test object and the index of its biological activity. Thus, sausage products using low-value raw materials and with the addition of a consortium of microorganisms had high nutritional and biological value and did not show toxicity.

The results of these studies showed that the use of the consortium in the production of meat products allowed the speeding up of the ripening process of meat systems, and also allowed the use of low-value raw meat of low-grade in the fermentation process. It can be concluded that the sausage produced according to the developed technology showed improved functional and technological properties and sensory quality.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 296–310

Response Surface Analysis and Process Optimization of Non-Cereal (Elephant Foot Yam, Taro and Water Chestnut) Snacks

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Received: 31 January 2019; Published online: 18 October 2021

Abstract

The present study was conducted to develop non-cereal starch extruded products. The effects of feed moisture (15-21%), temperature (130-170 °C) and screw speed (120-160 rpm) were evaluated on the physical and functional properties of extruded snacks using response surface methodology. Feed moisture and screw speed increased the bulk density and hardness of extruded snacks. Significant decreases in water absorption index and increases in water solubility index were observed with increases in extrusion temperature. The best conditions were determined by numerical optimization. The optimized value for non-cereal snacks for feed moisture is 18.22%, temperature 155.96 °C, screw speed 142.75 rpm and, desirability is 0.75. Verification of results showed decent agreement between the responses of experimental values at certain optimum conditions and the predicted values.

Keywords: Extruded snacks; Elephant foot yam flour; Taro flour; Water chestnut flour

1 Introduction

At present cereal-based extruded products dominate the market, whereas very few non-cereal-based extruded products are manufactured (Kaushik et al., 2018). Fasts are a part of life of the Indian population in which non-cereal-based foods are consumed. Therefore, the present study will be carried out to develop non-cereal starch-based extruded products for fasting individuals and the general population. Extrusion cooking is a multi-technique process that converts extrudate into specific shape, size and highly expanded products. All the unit operations are performed by a single extrusion machine like mixing, kneading, shearing, shaping, forming and cooking is optional (Bordoloi & Ganguly, 2014).

It is an energy efficient, rapid, continuous process and can be used to produce better quality starchy foods including expanded snacks, ready to eat (RTE) cereals, confectioneries, modified starches, baby foods, pasta, pet foods and extruded crisp breads (Deshpande & Poshadri, 2011). Feed composition, particle size, additives, temperature, feed rate and screw speed affect the pasting, textural, physiochemical and morphological properties of extruded products (Singh et al., 2009). The Indian snack market has been increasing rapidly with an annual growth rate of about 15 to 20% (Ministry of Food Processing Industries, 2015). High tem-

 10.7455/ijfs/10.2.2021.a2

perature and short time extrusion (HTST) cooking has many benefits over low temperature and long-time (LTLT) cooking, because of inactivation of anti-nutritional factors and minimal nutritional loss along with production of unique textural properties like crispness and crunchiness (Moreno et al., 2018).

Extruded foods have been recognized to provide nutritious products and combine quality ingredients and nutrients to produce processed foods that contain detailed levels of each required nutrient (Yagci & Gogus, 2008). Extrusion cooking used in this study was skillful through the application of heat to the blend directly by means of steam inoculation. Frequent changes observed in ingredients through the extrusion cooking process were the gelatinization of starch, destruction of natural toxic ingredients, denaturation of proteins and the diminishing of microbial counts (Balfour et al., 2014).

Parvathi et al. (2016) reported that tubers and edible roots gained third position as human food after cereals and legumes. In several places they also serve as staple foods and sources of cheap energy, especially for the weaker section of the population. Non-cereal-based extruded products were developed using Maori potato flour (Singh et al., 2009). Rodriguez-Miranda et al. (2011) developed taro and maize flour-blended extruded snacks with higher expansion and water solubility indexes. Taro (*Colocasia esculenta*) is a plant generally cultivated in tropical territories for its underground corms. Taro roots contain an abundance of dietary fiber and starches, biomolecules, magnesium, zinc, phosphorous, potassium, manganese, and copper, and vitamins A, C, E, B6 that are basic for human wellbeing (Anuj Saklani & Kumar, 2021).

Puffed snacks using *Amorphophallus paeoniifolius, Typhonium trilobatum* and *Colocasia esculenta* were prepared by Parvathi et al. (2016) and showed good sensory scores. Most snacks developed are tuber and cereal mixes, totally non-cereal-based snacks are very limited in the market. Elephant foot yam (EFY) is a tuber crop developed in Africa, Asia, parts of South America, and the Caribbean and South Pacific islands. Yams are an astounding wellspring of dietary vitality for the vast majority in the developing nations (Krishnan et al., 2010). They are wealthy in minerals like phosphorous, potassium, manganese and in vitamins. In West and Central Africa, yams are prepared into dry yam tubers/cuts and flour. The fundamental application for yam flour has been in bread items and bites. Some species of yam are likewise used for pharmaceutical preparations (Firdouse et al., 2012). Water chestnut (*Eleocharis dulcis*) is an edible water angiosperm and one of the important annual water warm season crops. Water chestnut is an important product in food industry because of its unique taste (Shafi et al., 2016). Water chestnut is used as a nerve tonic from time immemorial. The fruits are used as intestinal astringents, aphrodisiacs, antileprotics, for urinary discharges, fractures, sore throat, anemia, free radical scavenging activity, hepatoprotective activity, antitumor and antioxidant activity (Song et al., 2007).

Response surface methodology (RSM) is an excellent statistical design of experiments for standardized extrusion conditions. It is commonly used to collect data and give valid and objective conclusions (Lohan et al., 2020; Montgomery, 2006). RSM is a process to check the effect of various experimental designs, like extrusion parameters (temperature, screw speed, feed moisture) on the properties (bulk density, expansion ratio, water solubility index and water absorption index) of an extruded snack in a simplified manner (Myers et al., 2016; Whitcomb & Anderson, 2004). Screw speed and feed rate were found to have the least impact on extrudate properties (Anand & Pavuluri, 2009).

In this modern world, the demand of making novel foods is fulfilled by extrusion technology. Preservation and improvement of nutritional value of food during processing (Kaushik et al., 2017) has remained one of the most challenging tasks for researchers (Indumathi et al., 2015; Kaushik, Sachdeva, Arora, & Gupta, 2015; Sachdeva et al., 2015). To make a healthy extruded snack, it is important to wisely select multiple raw materials to achieve a balance between protein and carbohydrates. Extrusion cooking is now one of the most researched arenas in the current era for health and nutrition and is likely to remain so for a long time. Therefore, a process of non-cereal-starch-based extruded product was optimized in this present study.

2 Materials and Methods

2.1 Materials

Ingredients for the preparation of ready-to-eat snack food consisted of elephant foot yam, taro and water chestnut used for the present study was purchased from local markets of Solan, Himachal Pradesh, India. A fine part of the elephant foot yam, taro and water chestnut was scraped, washed, shredded into 1 cm^3 cubes and dried in a mini tray drying oven (Maro Scientific Works Pvt. Ltd., New Delhi, India) for 48 h at 50 ^oC. Dried cubes were milled using a roller-mill (Chopin Laboratory CD-1 mill, France). The flour was sieved through a sieve of 45 mm mesh size to obtain uniform particle size. The flour was then packed in an airtight plastic container for future use.

2.2 Preparation of Sample

For the preparation of non-cereal extruded product, different proportion of elephant foot yam, taro and water chestnut flours were used. First sample concentration was EYF 30%, taro 40% and water chestnut 30%, second sample concentration was EYF 40%, taro 30% and water chestnut 30%, third sample concentration was EYF 30%, taro 30% and water chestnut 40% and fourth sample concentration was equal proportion of EYF, taro and water chestnut. In preliminary trails, the equal proportion extruded products showed superior texture and sensory qualities and were used for further trails.

2.3 Extruder and extrusion cooking

A co-rotating twin-screw extruder (G.L. Extrusion Systems, New Delhi, India) having a barrel with 2 electric band heaters and 2 water cooling jackets received the raw feed and a variable speed feeder was used. The central drive was provided with a 7.5 HP motor (450 V, 3ph, 50 cycles) and a temperature sensor was fixed on the front die plate. The output shaft of the worm reduction gear had torque limiter cooling and the die plate of the die was fixed by a screw nut tightened by a distinct wrench. The programmed cutting blade was settled on a turning shaft. The twin-screw extruder was kept running for a set period of time to steady the set temperatures, and samples were then poured into the feed hopper and the feed rate was adjusted to 4.5 kg/h for easy and non-choking operation. The product was collected at the die end and packed in zip-locked packets for suitable storage.

2.4 Experimental design

Response surface methodology was applied to the experimental data using a commercial statistical package (Design-Expert software version 6.0.8) for the generation of response surface plots and optimization of process variables. A central composite experimental design was used to study the effect of independent variables on dependent variables. Twenty sets of experiments were performed considering three independent variables: feed moisture, temperature, and screw speed on the response variables such as expansion ratio, water solubility index, water-holding capacity, and bulk density. The average from three replicated values of each run was taken as response variable. For the statistical analysis, the numerical levels were standardized to -1, 0 and +1 in table 1. The experimental design in terms of actual levels is given in table 2. Levels of variables were selected based on preliminary trials. The standard scores were fitted to a quadratic polynomial regression model for predicting individual Y responses by employing the least square technique (Myers et al., 2016).
The experimental design involved 21 experiments and numerical optimization was done by conveying the specific areas to each of the responses (expansion ratio was maximized, bulk density and hardness were minimized and water holding capacity and water solubility index were kept in the range). The adjusted R^2 was used to measure goodness of fit. Three optimum formulations having desirability of 1 were determined for each non-cereal extrudate and validated the predicted and actual values of responses at the optimized conditions. The snack quality parameters were desirability function vs. expansion ratio, water solubility index, water holding capac-

Table 1: Independent variables and their coded and actual values used for optimization

Factors	Units	Level		
		-1	0	1
Feed Moisture	%	15	18	21
Temperature	^{o}C	130	150	170
Screw speed	RPM	120	140	160

ity, hardness, and bulk density.
The optimum feed rate was 18.22%, Barrel temperature was 155.96 ^{o}C, Screw speed 142.75 rpm and desirability 0.76. The least deviation in actual values of response against predicted values was found in the formulation presented in table 3.

2.5 Evaluation of textural characteristics of extrudates

Expansion Ratio (ER)

ER was determined by the method of (Ryu & Ng, 2001). The diameter of extrudates was measured at ten different positions along the length of each sample using a digital vernier caliper. The ER of extrudates was calculated as equation 1:

$$\text{Expansion ratio} = \frac{Extrudate\ diameter}{Die\ diameter} \quad (1)$$

Water solubility index (WSI)

Water solubility indexes of samples were determined according to the method described by Kaushik, Sachdeva, Arora, and Gupta (2015). Sample (2.5 g) was dispersed in 30 ml of distilled water using a glass rod and cooked at 90 ^{o}C for 15 min in a water bath. The cooked paste was cooled to room temperature and centrifuged at 3000×g for 10min. The supernatant was decanted into a tarred evaporating dish to determine its solid content. The weight of the dried solids was recovered by evaporating the supernatant for 4h at 110 ^{o}C. The WSI was calculated as equation 2:

$$\text{WSI} = \frac{WS}{WDS} \times 100 \quad (2)$$

Where,
WS: weight of dissolved solids in supernatant
WDS: weight of dry solids.

Water holding capacity (WHC)

Water holding capacities of samples were determined according to the method described by Kaushik et al. (2018). Water holding capacity was determined by mixing 1 g of sample with 15 ml deionized water in pre-weighed centrifuge tubes. After holding for 30 min, the tubes were centrifuged for 10 min at 3000×g. The supernatant was discarded, and tubes were then weighed again. Water holding capacity was expressed as g of water retained per g of sample. The WHC was calculated as equation 3:

$$\text{WHC} = \frac{Fresh\ residue\ -\ Dry\ residue}{Dry\ Residue} \times 100 \quad (3)$$

Hardness

The hardness of extruded snacks was measured using a TMS Texture Analyzer (Food Technology Corporation, Sterling, Virginia, USA) equipped with a 1000 (N) load cell, and a 0.05 (N) detection range.

Bulk density (BD)

The bulk density was calculated by measuring the actual dimensions of the extrudates. The diameter of the extrudates was measured with a vernier caliper and the lengths per unit weight (grams) of the samples were determined (Kaur & Singh, 2005). The BD of the extrudates was calculated as equation 4:

$$Q_d = \frac{4}{\Pi d^2 l} \quad (4)$$

Table 2: Experimental design and results of the central composite design of 20 experiments

Sr. No.	Feed Moisture (%)	Temperature (^oC)	Screw speed (rpm)	Expansion Ratio	Water solubility index (%)	Water holding capacity (g/g)	Hardness (N)	Bulk Density (g/cm^3)
1	15	170	120	2.65±0.56^a	28.19±1.26^a	5.72±1.75^a	62.45±2.49^a	0.26±0.03^a
2	21	170	160	3.09±0.73^a	29.34±1.36^{ab}	6.19±1.56^a	60.23±2.66^{ab}	0.28±0.04^a
3	21	130	120	2.79±0.51^a	29.74±1.34^{ab}	5.71±1.62^b	52.19±2.13^{ab}	0.25±0.03^a
4	21	130	160	3.13±0.75^{ab}	30.55±1.24^a	6.04±1.52^b	51.63±2.19^a	0.29±0.04^a
5	15	130	120	2.69±0.61^a	28.89±1.19^b	5.81±1.67^{ab}	50.46±2.28^{ba}	0.27±0.02^a
6	18	150	140	2.72±0.59^a	28.17±1.28^a	6.08±1.50^a	54.63±2.06^{ab}	0.26±0.03^a
7	15	150	140	2.84±0.65^a	30.53±1.36^a	5.96±1.67^a	53.47±2.14^{ba}	0.25±0.02^{ab}
8	18	170	150	3.12±0.71^{ab}	30.16±1.51^a	5.89±1.43^a	63.13±2.42^a	0.29±0.03^a
9	21	150	140	3.19±0.76^{ab}	30.94±1.42^a	6.13±1.68^{ab}	54.74±2.15^b	0.28±0.03^a
10	18	170	130	3.13±0.69^b	29.47±1.19^{ab}	6.24±1.54^b	61.19±2.49^b	0.24±0.02^a
11	18	150	140	2.75±0.61^a	30.67±1.29^b	5.64±1.47^{ab}	55.74±2.43^a	0.26±0.02^{ab}
12	21	170	120	2.66±0.58^a	29.45±1.31^b	6.19±1.69^a	62.37±2.63^a	0.27±0.03^a
13	18	150	140	2.56±0.55^a	30.13±1.36^{ab}	5.96±1.57^a	56.15±2.84^{ab}	0.26±0.02^a
14	17	150	140	2.87±0.59^{ab}	30.43±1.41^b	5.92±1.43^a	56.34±2.61^{ba}	0.24±0.04^a
15	16	130	160	3.18±0.74^a	29.32±1.23^{ab}	5.73±1.39^{ab}	51.63±2.55^a	0.26±0.02^{ab}
16	15	170	160	2.84±0.66^{ab}	29.41±1.54^{ab}	6.12±1.77^b	60.98±2.23^{ab}	0.28±0.02^{ab}
17	15	150	130	3.05±0.72^b	28.94±1.35^{ab}	6.22±1.62^b	54.36±2.47^{ba}	0.24±0.03^a
18	18	150	140	2.86±0.67^{ab}	29.23±1.14^a	6.11±1.58^{ab}	53.87±2.61^a	0.25±0.03^a
19	18	150	140	2.73±0.69^a	29.56±1.16^a	5.96±1.55^a	54.13±2.39^{ab}	0.24±0.02^a
20	18	130	160	3.17±0.76^a	30.21±1.19^a	5.81±1.49^a	50.76±2.45^a	0.26±0.03^a
Regression				Sum of squares				
Standard deviations				0.20	0.43	0.14	1.12	0.015
P value				0.001	0.001	0.001	0.001	0.001
Adequate precision				3.84	8.874	9.311	14.772	4.952
R^2				0.853	0.844	0.805	0.951	0.822
Adjusted R^2				0.651	0.703	0.629	0.908	0.682
Lack of fit				1.23	1.31	1.68	2.01	2.37
CV (%)				6.77	1.44	2.43	0.60	5.85

Data are presented as means±SEM (n=3)
$^{a-d}$ Means within a column with different lowercase superscript are significantly different ($p < 0.05$) from each other

Where,
Q_d= bulk density (g/cm3),
d= diameter of the extrudate (cm),
l= lengths per g of the extrudate (cm/g)

Statistical analysis

The optimization of product was carried out using Design-Expert software version 6.0.8, however, Microsoft excel, 2014 (Microsoft Corp., Redmond, WA) was used for the calculation of standard error of the mean (SEM). Statistical difference in terms of significant and non-significant values was confirmed by one- and two-way analysis of variance and comparison among means was completed by critical difference values (Kaushik, Sachdeva, & Arora, 2015).

3 Results and Discussion

Independent variables and their coded and actual values used for optimization are summarized in table 1. The data on mean values of physical properties of extruded products along with their standard deviations are summarized in table 2. ANOVA and regression coefficients are summarized in tables 2 and 3, respectively. Models for all parameters were significant, and all parameters were significantly affected by feed moisture, screw speed and temperature. All parameters showed high adequate precision in table 3. Figure 1, 2, 3 and 4 shows the effect of independent variables on the extrusion behavior and product characteristics.

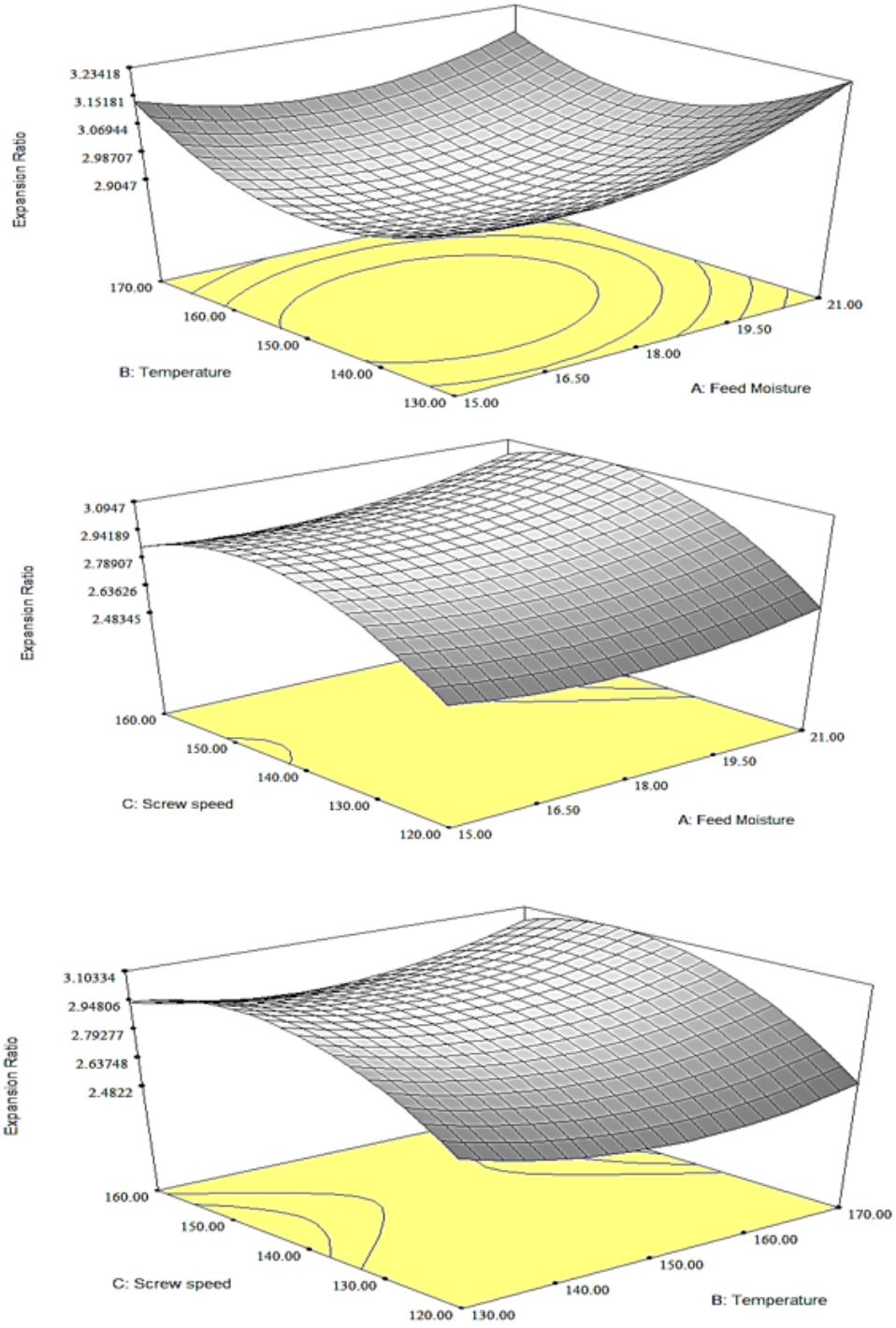

Figure 1: Effect of moisture, screw speed and temperature on expansion ratio

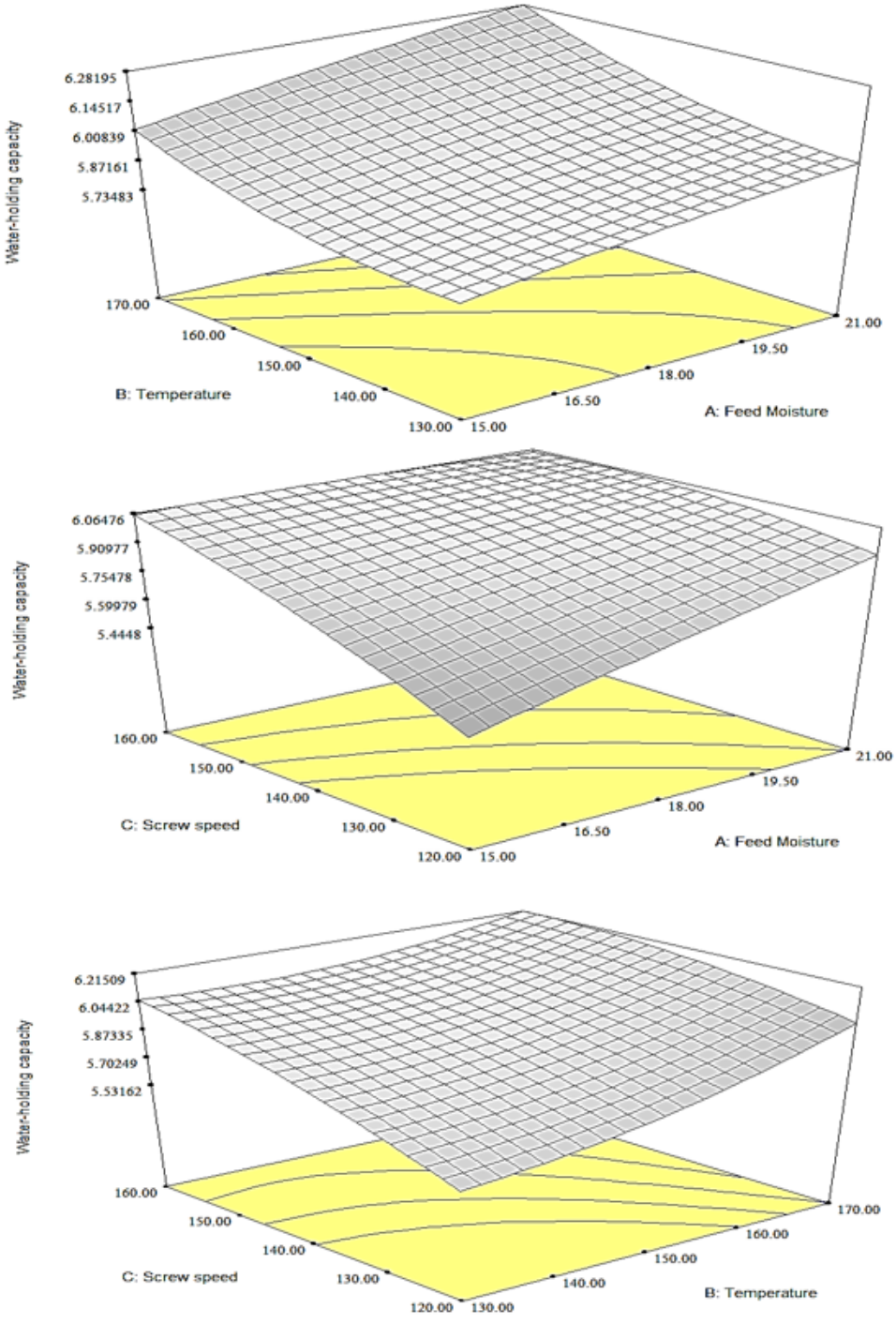

Figure 2: Effect of moisture, screw speed and temperature on water holding capacity

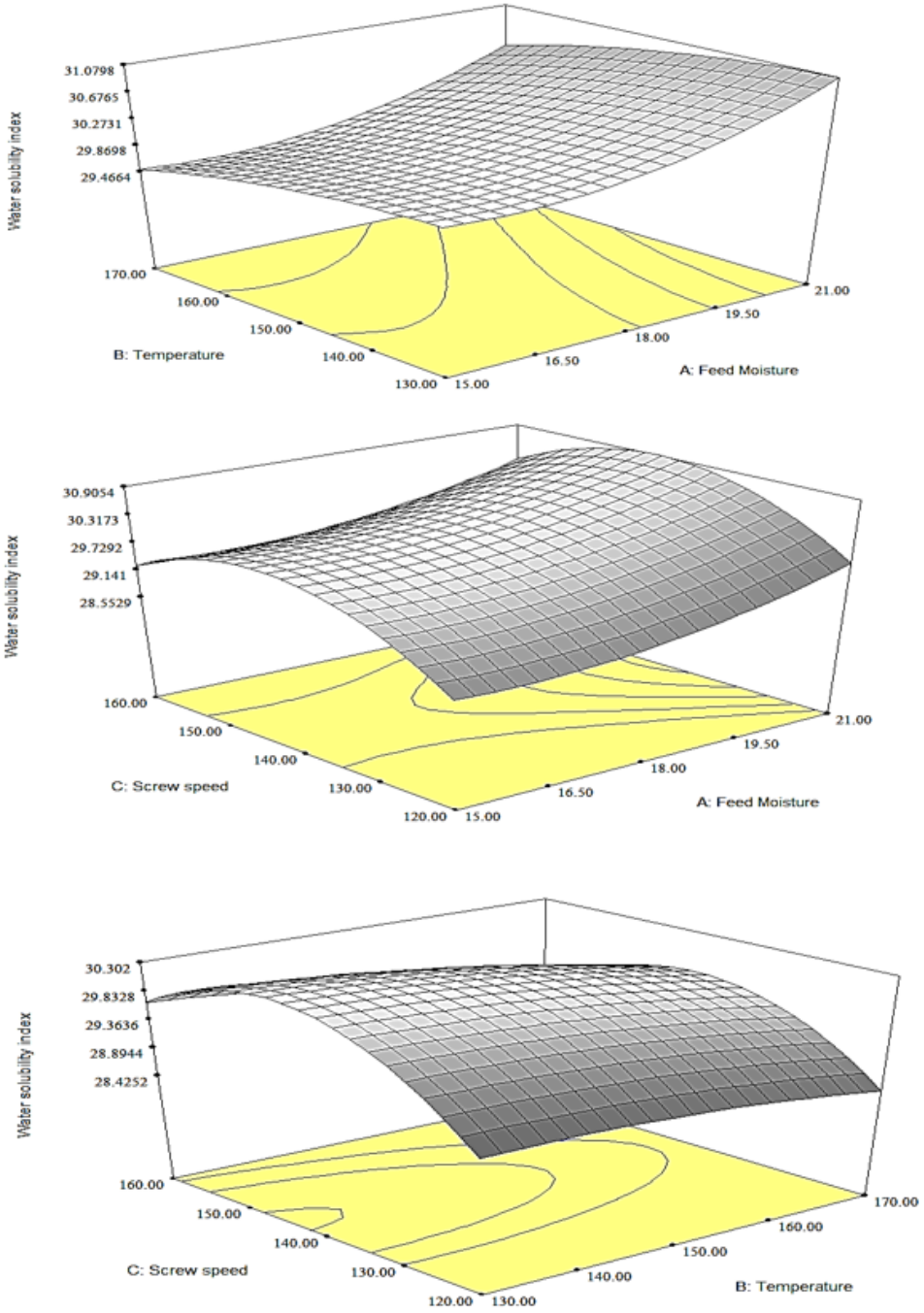

Figure 3: Effect of moisture, screw speed and temperature on water solubility index

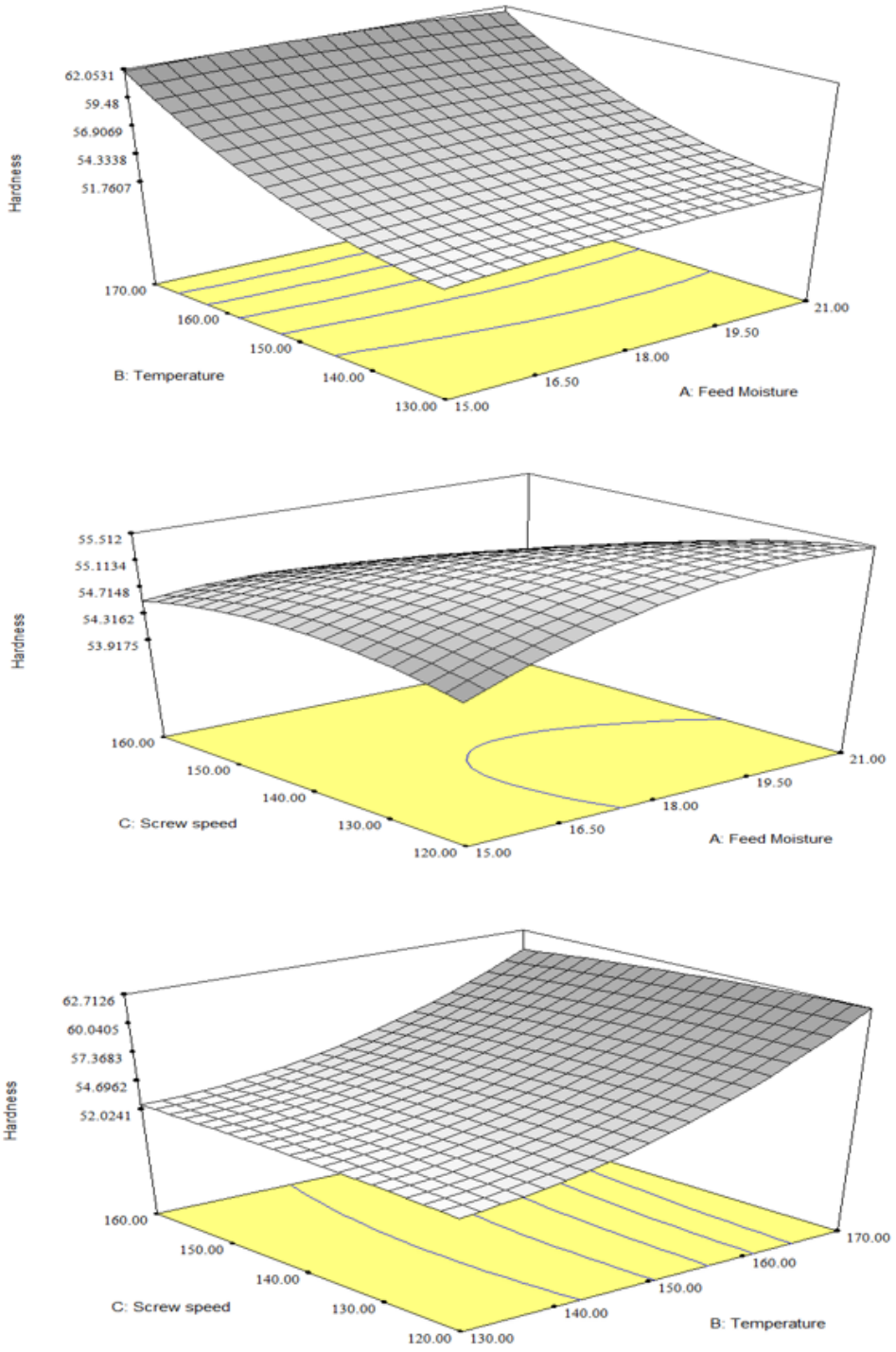

Figure 4: Effect of moisture, screw speed and temperature on hardness

Table 3: Regression coefficients for fitted models

	Regression coefficients				
Parameters	ER	WSI	WHC	Hardness	BD
Feed Moisture (x_1)	-0.30**	-1.07*	+0.32*	+0.11**	+2.20*
Temperature (x_2)	-0.10*	+0.12*	-0.02*	+4.76*	-3.97 ^{n}s
Screw speed (x_3)	+0.15***	+0.79**	+0.11*	-0.38*	+0.01*
Feed Moisture^2 $(x_1)^2$	+0.01**	+0.03**	-1.17*	-0.34*	+1.20*
Temperature^2 $(x_2)^2$	+3.53*	-3.28^{ns}	+1.85*	+2.05*	+1.00*
Screw speed^2 $(x_3)^2$	-6.22^{ns}	-2.56*	-1.26*	-0.28*	-4.20^{ns}
Feed Moisture × Temperature ($x_1 \times x_2$)	-7.08^{ns}	-1.25*	+2.50	-0.29*	-6.25^{ns}
Feed Moisture × Screw speed ($x_1 \times x_3$)	+4.58 *	-6.25^{ns}	-2.04	-0.41***	-2.29*
Temperature × Screw speed ($x_2 \times x_3$)	+1.06*	-3.00^{ns}	-2.00	-0.58*	+1.56*

Level of significance: *P < 0.1, **P < 0.05, ***P < 0.01, ns non-significant

3.1 Expansion Ratio (ER)

Expansion ratio as an index of puffing level is one of the most significant physical characteristics of the extrudates. ER values for the extrudates ranged between 2.56 and 3.18 (table 1; Figure 1). The responses were analyzed using ANOVA and the data are presented in table 1b. The regression model had a coefficient of determination (R^2) and adjusted R^2 value of 0.853 and 0.651, respectively. The CV was found to be 6.77%. Similar results were reported by Ganorkar and Jain (2015) who found the same pattern for ER in extrusion cooking of maize grits at 150-160 ^{o}C product temperature and 47-60 kg/h feed rate. Dogan and Karwe (2003) reported similar results in the extrusion of quinoa at 130-170 ^{o}C, 250-500 rpm screw speed and 300 g/min feed rate.

$$\begin{aligned}&\text{ER Coded Factors} = \\ &0.50(x_1) + 2.05 - 003(x_2) + 0.18(x_3) \\ &+ 0.09(x_1)^2 + 0.14(x_2)^2 - 0.25(x_3)^2 \\ &+ 0.042(x_1 \times x_2) + 0.028(x_1 \times x_3) \\ &+ 0.043(x_2 \times x_3)\end{aligned} \quad (5)$$

3.2 Water solubility index (WSI)

WSI values for the extrudates ranged between 28.19 and 30.94 % (table 1). The responses were analyzed using ANOVA and the data are presented in table 2 and Figure 2. The regression model had a coefficient of determination (R^2) and adjusted R^2 value of 0.844 and 0.703, respectively. The CV was found to be 1.44%. The negative coefficient of the linear term moisture (table 2) indicated that WSI decreased, while positive coefficients of the linear terms screw speed and temperature indicated that WSI increased with the increase in variables. Extrusion at low moisture might result in increases in water-soluble molecules (da Silva et al., 2009). Higher WSI of extrudates was reported with decreasing screw speed (Pardhi et al., 2019). Increasing temperature would increase the degree of starch gelatinization that could increase the quantity of soluble starch resulting in an increase in WSI. The increase in WSI with increasing screw speed was consistent with the results reported by Sebio and Chang (2000).

$$\begin{aligned}&\text{WSI Coded Factors} = \\ &0.48(x_1) - 0.31(x_2) + 0.30(x_3) + 0.34(x_1)^2 \\ &- 0.13(x_2)^2 - 1.03(x_3)^2 - 7.50 - 03(x_1 \times x_2) \\ &- 0.38(x_1 \times x_3) - 0.12(x_2 \times x_3)\end{aligned} \quad (6)$$

3.3 Water holding capacity (WHC)

WHC values for the extrudates ranged between 4.06 and 5.0 g/g (table 1). The responses were analyzed using ANOVA and the data are pre-

sented in table 2. The regression model had a coefficient of determination (R^2) and adjusted R^2 value of 0.805 and 0.629, respectively. The CV was found to be 2.43%. Increase in feed moisture resulted in a significant increase in WHC. It is worth noting that starch granules must undertake a certain degree of conversion to initiate water absorption (Filli et al., 2013). The negative coefficients of the linear terms screw speed and temperature (table 2; Figure 3) indicated that WHC decreases with increases in these variables. It could be expected that more undamaged polymer chains and a greater availability of hydrophilic groups, which could bind more water, resulted in higher values of WHC under low shear conditions with lower screw speed. Higher temperature increased degradation and dextrinization of starch. A negative effect of temperature and screw speed on WHC was also reported by Altan et al. (2008) in barley tomato pomace blend-based extrudates.

$$\begin{aligned}&\text{WHC Coded Factors} = \\ &0.12(x_1) + 0.16(x_2) + 0.18(x_3) - 0.011(x_1)^2 \\ &+ 0.074(x_2)^2 - 0.051(x_3)^2 + 0.015(x_1 \times x_2) \\ &- 0.12(x_1 \times x_3) - 0.080(x_2 \times x_3)\end{aligned} \tag{7}$$

3.4 Hardness

The hardness is the highest force mandatory for a probe to penetrate the extrudates. Hardness can be determined by the availability of hydrophilic groups and capacity of gel formation of the macromolecule (Gomez & Aguilera, 1983). Hardness values for the extrudates ranged between 50.46 and 63.13 (N) (table 2). The responses were analyzed using ANOVA and the data are presented in table 2. The regression model had a coefficient of determination (R^2) and adjusted R^2 value of 0.951 and 0.908, respectively. The CV was found to be 0.60%. Increase in feed moisture resulted in a significant increase in hardness. When the moisture content of feed increased while keeping blend ratio constant, hardness increased (Jha & Prasad, 2003). Hardness can decrease because an increase in the barrel temperature will increase the degree of superheating of water in the extruder, encouraging bubble formation and also decreasing the viscosity of melt (Pardhi et al., 2019).

$$\begin{aligned}&\text{Hardness Coded Factors} = \\ &3.08(x_1) - 1.00(x_2) + 0.51(x_3) - 0.03(x_1)^2 \\ &+ 5.12 - 03(x_2)2 - 6.96 - 04(x_3)^2 - 4.85 \\ &- 03(x_1 \times x_2) - 6.77 - 03(x_1 \times x_3) \\ &- 1.45 - 03(x_2 \times x_3)\end{aligned} \tag{8}$$

3.5 Bulk density (BD)

The density of extrudates varied between 0.24 and 0.29 g/cm^3 (table 1). ANOVA for the model as fitted (table 4) shows high significance ($P < 0.001$) with a correlation coefficient (R^2) and adjusted R^2 value of 0.822 and 0.682, respectively. The CV was found to be 5.85% indicating that experimental data were acceptably explained. The BD increased significantly with the increase in feed moisture content (table 2). The high dependence of BD and expansion on feed moisture would reflect on elasticity characteristics of the starch-based material (Hagenimana et al., 2006). The bulk density was significantly influenced by temperature, screw speed, and water content (Pardhi et al., 2019). Moisture is critical in starch gelatinization, protein denaturation, barrel lubrication and final product quality. A dry extruder can process materials with 8-22% moisture with no additional drying of extrudates. Most extrudate snacks have a moisture content between 8-12% and require additional drying to impart the desired texture and mouthfeel (Navel & Thakor, 2015).

$$\begin{aligned}&\text{BD Coded Factors} = \\ &6.22 - 03(x_1) + 2.00 - 03(x_2) + 6.55 - 03(x_3) \\ &+ 0.01(x_1)^2 + 4.014 - 03(x_2)^2 - 0.017(x_3)^2 \\ &- 3.75 - 03(x_1 \times x_2) - 0.014(x_1 \times x_3) \\ &+ 6.25 - 03(x_2 \times x_3)\end{aligned} \tag{9}$$

Pardhi et al. (2019) standardized the processing parameters of brown rice grits. They reported water solubility index (5.43 to 14.32%), water absorption index (4.72 to 7.81), and bulk

Table 4: Expected and observed values for product responses at optimized processing conditions

Parameter	Expansion Ratio	Bulk density (g/cc)	Water holding capacity (g/g)	Water Solubility index (%)	Hardness (N)
			#Non-cereals in Combination		
Expected Value	2.90	0.27	5.97	29.55	56.78
Observed value	2.81	0.26	5.83	28.47	55.62
CV (%)	6.77	5.85	2.42	1.44	0.60

Data are presented as means±SEM (n=3)

density (0.065 to 0.188/ml). They also reported that with increases in moisture, bulk density, water absorption index and hardness increased, while water solubility indexes decreased; increased temperature, on the other hand, decreased all parameters. (Thymi et al., 2005) utilized tuber starch for the development of extrudates and studied their various physical properties, including bulk density, true density, porosity, expansion ratio, water holding capacity and water solubility index. The influence of process variables on physical properties has been shown to be generally significant in all these studies.

4 Conclusion

The physical properties of elephant foot yam, taro and water chestnut snacks were highly influenced by processing parameters. Lower as well as higher levels of feed moisture and extrusion temperature were found to be critical with respect to expansion and density of snacks. The extruded snacks prepared at optimized processing conditions displayed better physical and functional properties. Elephant foot yam, taro and water chestnut flour, all rich sources of phytochemicals, could be used as functional ingredients in the development of nutritious and healthy extruded snacks. The developed snacks can be consumed even during fasts by the Indian population. The equal ratio of all three ingredients was optimized for snack production.

Acknowledgements

This work was conducted in the School of Bioengineering and Food Technology, Shoolini University, Solan, Himachal Pradesh. All chemicals, glassware and Instruments were provided by the School of Bioengineering and Food Technology, Shoolini University, Solan, Himachal Pradesh.

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Lessons from Japan: Promoting Underutilized Food Crops Through Tourism

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Received: 28 June 2019; Published online: 18 October 2021

Abstract

For the first time in the XXI century, the number of people suffering from obesity worldwide surpassed the number of undernourished people. For many developing countries, this presented a so-called "double burden" of coping for over- and under-nourishment. At a rate of more than 50% of the population in Malaysia being either overweight or obese, the country is facing the most severe dietary crisis in East Asia. Often recognized as one of the healthiest populations worldwide and having a blue zone, Japan has managed to set a yardstick for countries around Asia. Hence the purpose of this study was to uncover the capacity for contribution and successful integration of traditional vegetables into Japanese daily food practices. Semi structured interviews were conducted together with participatant observation with key informants to help understand the role of women in preserving and safeguarding traditional vegetables also known as 'sansai' in terms of farming and food preparation. In order to solve the unhealthy eating patterns among Malaysians, it is timely to start appreciating the contributions of the older generation in terms of wild edible usage and preparatory methods which can help alleviate the double burden of malnutrition among the population.

Keywords: Wild edible food systems; Food and nutrition security; Sansai

1 Introduction

The multidimensional issue of food security presents a global dilemma wherein the demand for food is expected to increase by up to 60% by the year 2050. Evaluating the causes of hunger, malnutrition and obesity requires an evolving understanding that the genetic base of most human caloric intake from plants is dangerously narrow. The current mainstream model of domestication, dominated by Mendelian pedigree breeding, has been maintained at the loss of horizontal, or broad, resistance to plant pests and diseases and a drastic reduction in the variety of plants eaten worldwide. Approximately 60% of the world's energy intake is heavily reliant on the three main staples (rice, maize, and wheat). Worldwide, the number of people suffering from obesity has finally surpassed the number of undernourished people from the year 2000 onward. The "double burden" of nutrition has started affecting many developing countries. These nations have to simultaneously cope with both the over- and undernourishment of their citizens. Since 2000, countries in all regions have experimented with numerous mechanisms to combat this trend, but the results have been inadequate. Malaysia is currently facing the most severe dietary crisis in East Asia, at a rate of approximately 50% of its populace being overweight or obese (Institute For Public Health, 2015). Research suggests that the disruption in work-life balance, household eating

 10.7455/ijfs/10.2.2021.a3

customs, and rapid shift away from indigenous diet are the main culprits, particularly in poor or lower-middle income households (Tee et al., 2018). Japan – a country with consistently good dietary outcomes for its relative level of development - has been the only country in Asia which has been able to withstand this contemporary threat.

2 Literature Review

2.1 Wild Edibles In Maintaining Health and Well-Being

As a tropical country, Malaysia is rich in biological diversity. Out of the 15000 species of vegetable plants available, only 300 species are indigenous to the country and have been used as food (Ministry of Agriculture, 1996). From the copious amount of research focused on traditional Malaysian leafy greens, results have proven that they not only possess higher nutrient content, but also beneficial antioxidants when compared to commercially cultivated vegetables (Asyira et al., 2016). It is sad to see that Malaysians have not accepted and embraced these underutilized plants, despite being healthier and viable alternatives due to their lower calorie content and glycaemic index in comparison to commercially cultivated crops.

Progressive flourishing food systems productively tap into an array of locally available food combinations and traditional food cultures. Japan has successfully kick-started various initiatives to revamp and reintroduce traditional vegetables into the diets of the urban population. This has a lot to do with the fact that the government has managed to preserve both their unique cultural and environmental aspects to the extent that fast food chains such as MOS Burger have also adapted to demands for regional and seasonal traditional produce. In line with their company's name, Mountain Ocean and Sun, they follow the underlying principle that environmental conservation should be tied in together in any food business, as it has a direct impact on health and well-being (Japan for Sustainability, 2006).

From fast food to farmers' restaurants and farm stays, a lot has been done to ensure that traditional vegetables are a vital part of their diets. However, the same cannot be said here in Malaysia. The inclusion of traditional vegetables, or 'ulam,' in the diets of the youth and urban populations is dwindling. Though there are many restaurants trying to feature some of these wild vegetables in their menu, the accessibility of these traditional vegetables is still restricted and confined to a very small and niche market.

As observed in Japan, biodiversity conservation is a key tool to dietary development matters as it is the base for their culinary traditions. The diversity observed in wild edibles and food supplies offers the heterogeneity that is needed to negate the deleterious effects brought about by commercial food to reduce hidden hunger issues and impart the nutrients, taste, and texture frequently absent from repetitive carbohydrate-rich diets typical of altered diets. Introducing these orphan species into urban diets would not only increase the opportunity to produce nutrient-rich food but simultaneously enhance the environmental capacity for future generations to supply themselves.

The romanticised view of local and traditional plant cultivation and preservation has not been fully adopted by flora rich countries due to the loss of cultural heritage, lack of knowledge, and nutritional data, thereby highlighting the socio-economic consequences of modifying its status (Chweya & Eyzaguirre, 1999).

2.2 Rural Tourism

A strong link between biodiversity, culture, and nutrition needs to be built in order for these plants to be successfully introduced into the diets of urban population as seen in Japan. The expression 'eco-' denotes house or habitat in Greek which was then further developed into the term 'turisimoecologico' by Hector Ceballos-Lascurain in 1981 which signifies ecotourism. Green tourism first started out in Europe in the early 1980s and was based on the European agricultural business to improve sustainability. This concept slowly extended its influence through Asia, particularly Japan whereby the Ministry of Agriculture, Forestry and Fisheries initiated a study group on green tourism with an aim to invest-

igate the various agricultural activities available that could benefit both tourist and local inhabitants, as well as raising awareness of traditional culture and the natural beauty Japan has to offer (Bixia & Zhenmian, 2013). The terms ecotourism, green tourism and rural tourism are used interchangeably in Japan which predominantly assimilates the wonders of nature, leisure activities, and agricultural whilst enabling the rural populace to earn a decent living (Kurisu, 2011).

2.3 Women Empowerment

The *Chisan Chisho* movement which promotes regionalism and the local production and utilization of the food kick started in the early 1990s due to the depreciation of agriculture and the rural populace (Taniguchi, 2002). Women who come from farming households mostly dominate the agriculture sector in Japan and are the backbone behind the *Chisan Chisho* movement. Hence it is structured particularly to empower women in local areas by teaching them how to preserve local/regional cuisine while learning to reduce sodium content and add more vitamin-rich food ingredients. It is these women who often take up key roles in green tourism (Kimura & Nishiyama, 2008; Nakamichi, 2010). With such projects, communities all around Japan have started to grow old-fashioned, native food ingredients instead of more profitable non-native varieties. In Malaysia, not much has been done to revitalize these crops and reintroduce them into the diet.

Located in the north of Honshu, Odate is at the north eastern corner of the Akita prefecture, well known for their apple and pear farms. The Akita prefecture has attempted to amplify the alluring charms of the area by reinventing links between regional dietary dishes and the beautiful mountains found in that area. The thing that makes Odate unique is that it merges both nature and food by practicing green tourism whereby farm stays and restaurants collaborate together to revive the consumption of traditional vegetables. Visitors get to learn about agricultural production and nature and eat at restaurants based on local food. Hence, the objective of this work was to showcase and learn the various initiatives taken by Japan and adapt them to a more localized concept and apply them here in Malaysia - particularly in regards to the role of women in preserving and safeguarding traditional vegetables in terms of farming and food preparation

3 Materials and Methods

3.1 Site Selection

Among all the various prefectures which offer farm stays, Odate was chosen as they not only had English speaking tours available for foreign guests, but were also recently awarded the IPRA Golden World Awards 2017 for the Akita Prefecture Tourism Campaign, also the 16th Oorai! Nippon Award and the Discover Mura no Takara by the Ministry of Agriculture, Forestry and Fisheries for being an exemplar village in regards to revitalizing local communities. Even though Akita is well known for its picturesque scenery, hot springs and many other sightseeing attractions, it has been difficult for the local travel industry and tourism agency to take advantage on the endless flocks of tourists to Japan, due to its location, as it is very far away from the bustling capital. As there isn't any local airport and public transportation can take up most of the travelling time, many tourists choose to bypass this location for other famous sightseeing sites. Besides that, Odate is also known for having a huge amount of snowfall during late autumn until early spring. To counteract and lure tourists, the Akita Inu Tourism joined forces to initiate a campaign by leveraging both the Akita dog breed and Japanese Idols.

In summary, some of the selection criteria in regards to the site were based on:

- Number of farm houses available
- Farming activities available
- Accessibility
- Role of local government in promoting green tourism
- Ease of communication

The study method was based on the phenomenological approach over a 5 day period, whereby

participatory observation and site visits to the various farms were undertaken by the researcher. Since language was a barrier, key informant interviewees were selected through Odate Cultural Activity Promotion Committee. Semi structured interviews were then conducted individually among 15 farm mothers identified by the committee and our host Mrs Ishigaki to gain a deeper understanding of the role the women play in ensuring good health among their families. Detailed information about the various types of 'sansai' used were asked, the incorporation of 'sansai' into the diets of local and the urban community, local delicacies and its relation to health and well-being, knowledge transmission among farm mothers and the various initiatives undertaken to ensure knowledge preservation. The questions were asked in English and then translated into Japanese by a translator working with the Odate Cultural Program Committee. Once the informants had answered, the translator would then interpret it back to English. Participant observation was also carried out during the researcher's stay to understand the interactions between human and plants when it comes to foraging. The researcher accompanied a few farm mothers in gathering 'sansai' in nearby areas, engaging them in conversation about the usage of these wild edibles as well as observe the foraging methods employed. The natural habitats of the plants was also recorded. During the five day stay, a different farm mother was assigned during each lunch and dinner to teach the researcher how these 'sansai's' are incorporated into their meals.

4 Results and Discussion

4.1 Food

As Odate is known for their local produce, most of the food featured in Mrs Ishigaki's place were vegetables. The cooking methods were the same throughout with the different farm mothers, though there were a few variations when it cames to pickling. As some 'sansai's' tend to be more fibrous in nature, they were usually boiled to help soften the texture. Others were pickled in rice wine vinegar and other seasonings (wasabi, soy, salt, sugar) or deep fried 'tempura' style. The best types of vegetables to utilize for making tempura include seasonal vegetables that are fresh as it is the main feature of the dish and the sauce is thought to be more of a garnish rather than a taste enhancer. On top of that, root vegetables are usually ideal as it can withstand higher temperatures and maintain its crispy texture due to the lack of moisture present. The Japanese also enjoy eating with their eye, hence colourful dishes were served during meals. The Kawaii culture does not only delight young children but also the elderly (Karasawa et al., 2011). Some of the highlights of the meals include kiritampo, which is an indigenous dish famous in the Akita prefecture and is usually eaten alongside a Japanese style hot pot with chicken stock also known as Kiritampo nabe. This dish is usually featured when visitors visit the locals and is made from freshly cooked rice that has been worked and pressed onto a skewer, glazed with sweet miso. It is then cooked on hot embers and can be eaten once toasted or can be broken into smaller pieces and placed in the hotpot (Japan National Tourism Organization, 2018). Akita is considered to be one of the top rice producing regions in Japan mainly due to its location (facing the Sea of Japan, the island of Tsuhima and also the Oou mountains) that contributes to the high night and day temperature differences. As it is considered to be a really traditional local food, various food companies have started producing kiritampo so that it can be enjoyed all the time.

Some of the other highlights included the Hinai Chicken which the Odate people are extremely proud of as it is a sought after delicacy. It contains high amounts of collagen and many other health benefits (Concierge, Akita). They usually consume the eggs raw, topped with soya sauce over steam rice. Another item which is unique and originates from Akita is tonburi. It is also considered as land caviar due to its greenish colour and is made from the Kochia Scoparia tree. The seeds are then dried, boiled, soaked and rubbed by hand to remove the outer layer. They can be served flavoured with soy (Casey, 2009). However, using it alongside yoghurt, will help mask the sourness of fresh yoghurt.

Another delicacy that flourishes during early winter is the 'hata hata' also known as the fishing sailfin sandfish. The locals generally prefer the females with its egg pouches still intact and can be grilled, cooked or boiled. As with the vegetables, the 'hata hata' is also preserved by boiling it on soy or using salt and rice, so that it can be eaten all year round. The locals can only harvest the fish during winter months as it can only survive in the cold.

> "We do not consume 'hata hata' in the summer. I could eat about 10-20 of this fishes daily. During winter, we have very limited protein options, this is what we usually eat. It has a very rich flavour and can be cooked, dried or pickled easily. The eggs are chewy and have a jelly like structure. It used to be very cheap, but now not so much anymore." Mrs Yamamoto, 67

Kanten (agar) was also used in one of the hot-pot dishes. A quick brief history on its origin, explains that it is a jelly-like dish present at any wedding, funeral, or other ceremony held in Akita's southern region The locals use an extensive variety of ingredients to prepare it, is healthy and has a very long shelf life. Usually the locals prefer using new, exotic fruits and vegetables together with those preserved. In older times, every family would bring their own dish to ceremonies they attended, and kanten dishes were apparently highly valued for being easy to carry and eat.

4.2 Role of Traditional Vegetables

As the winters in Odate tend to be harsh and lengthy, most of the vegetables are pickled using harvested vegetables during summer and autumn. 'Gakko' is an example of a pickled dish made from daikon radish and is usually eaten as a snack while shovelling huge amounts of fallen snow. Other common preservation techniques include smoking and drying these radishes with seasoning.

Edible mountain vegetables also known as 'sansai' play a very significant role in the diets of the locals and are known as power food. Converting cultural to economic capital, these mountain vegetables are indispensable to local communities (Sutton et al., 2016). Many of these vegetables will ripen during spring and survive the long winters. As Odate experiences heavy and long snowfalls compared to the average Japanese city, this then allows the soil to thrive, thus producing nourishing vegetables. Due to the unfavourable conditions, the locals utilize many different techniques of cooking and preserving leafy greens.

> "A lot of the vegetables featured in our diets are 'sansai' especially during spring, summer and autumn. We do get a few mountainous vegetables during winter which are perfect for tempura or the hot pot, but during this season, pickled vegetables are what we utilized more commonly." Mrs Tanaka, 68

> "My husband and children prefer eating 'sansai' and crave for it during the long winter months. There is a richer flavour profile and it's more nutritious than the common vegetables in the market. The people here in Akita are mostly farmers, hence our diets revolve around vegetables. We have a seasonal calendar which showcases the various wild vegetables that thrive during different months." Mrs Sato, 73

As diverse as the Japanese landscape is, the same can be said about their cooking methods. Details on the time frame and the location for gathering these vegetables are posted on various websites and also published as calendars. One thing to note is that there are many different varieties of vegetables, and different villages have different types of 'sansai' cuisines. From once being recognized as famine food, these vegetables have been put on a pedestal in line with local traditions of emphasizing seasonality and to elicit memories of the archetypal hamlets found in Japan. However, not all mountain vegetables can be consumed, as some can be poisonous and harmful to health due

to various substances present in it.
Osawa argues in the preface to his mountain plant gathering guide that the subtle local flavours, wild origins and handpicked variety of mountain vegetables are perfectly suited to a Japanese palate sensitive to the complexities of food fragrance, sweetness, age, heat and moisture. Citing a final reason to the contemporary boom in mountain vegetables, Osawa explains as 'urbanization progresses and the population concentrates in cities, there is a strong trend of people seek the taste of their native place (Osawa, 2003).
The tastes of native places are a nostalgic reference to the foods that evoke warm human networks, local traditions and healthful environments associated with quintessential old villages in Japan. Mountain vegetables often also tend to be bitter in nature, and require a lot of pre-preparation such as soaking or boiling to remove this overpowering flavour and make them more palatable (Makiko, 2011). The other method of cooking 'sansai' is to fry them tempura style. This tends to be preferred by young children.
The mountainous terrain of Japan has allowed local agricultural communities to uncover the hidden potential of natural resources, Satoyama is a term referred to mountain settlements that consist of mountain forests, paddy fields, farmlands and settlements governed by the rural populace (Chen & Qiu, 2012), which was coined during the Edo period. In the past, locals relied heavily on these natural resources to ensure sustenance and biodiversity and just like the early settlers, most of the knowledge available was centered around the forest. Having its roots in environmental conservation, biodiversity preservation and accountable human governance, modern day environmentalists have tried to conjure Satoyama back into the hearts and mind of the people.
In Odate, foraging is still very much a lifestyle of the rural folk. Foraging for 'sansai' requires skills that are taught by the mothers and are refined over time. The amount harvested should also be taken into careful consideration. Usually foragers should pick about 1/3 of each vegetable as overharvesting will lead to soil erosion and the loss of substantial gains. However, during early spring, many visitors from all parts of Japan come down to be part of this. In spite of rules and signs being posted, many of these inexperienced farmers ruin these plants, making them less available to produce a higher yield in the following seasons. Amateurs are often attracted to the various advertisements promoting the benefits of 'sansai' on radio and television, thus encouraging them to make the long trip down. The lack of knowledge among these urbanites are a major sustainability concern. To control the situation, farm mothers have started selling these 'sansai' in small cases, so as to decrease this from happening and preserving the continuity of production and survival of these plants. Also with the years of experience among farm mothers, they can recognize the ripened vegetables better compared to others that require a little bit more time, thus preventing further losses and destruction.
They have visitors not only from Japan on a regular basis, but also from America, Europe, Taiwan, China, Singapore and many other Asian countries. Even when locals stay with them, the farm mothers prefer to converse in their local Akita dialect. Though language might be a barrier, particularly with foreigners, the farm mothers choose to let their actions speak louder than their words by putting their heart and soul into making one feel comfortable and teaching everything they know. The older ladies hold bi–weekly gatherings during mid spring to late autumn to forage the local forest nearby. The mountain vegetables gathered are then brought back and prepared for their families.

4.3 Role of women

Mrs Ishigaki has been working as a farmer all her life and owns an apple farm. This was the same for all the women interviewed, who have been actively exposed to farming at an early age. Women do play a very active role in ensuring that cultural traditions are maintained. Fermented food was featured in every single meal and each family owns their own secret recipe, largely due to the time of the visit (very early spring). As Odate gets really cold during the winter, the people have to rely on fermented vegetables to get their daily dose of fibre, nutrients, and essential vitamins and minerals. Consumption

of these fermented foods is one of the reasons attributed to the longevity of the population.
The farm mothers are representatives of farm developments working to revive the agriculture sector and promote local vegetables. Each activity is tailor made for their guests bearing in mind the different seasons. Some of the activities include harvesting apple, making 'Magge Wappa', a traditional bento box made from cedar, juicing apples, visiting Onsens, immersing in the natural beauty Odate has to offer.
One of their main target functions is to teach children the importance of preserving their lifestyle and culture. The farm mothers have started a theatre group that uses the Akita dialect to showcase their plays which revolve around the local culture, traditions and farming activities. During the cold winter months, young children are taught to pickle vegetables, make apple chips and traditional sweets, which encourages them to see the bigger picture of how produce are being transformed into other goods. Knowledge preservation is a key aspect when tailoring these activities as most farm mother's children are no longer pursuing farming.

> "We regularly have visits from primary school children both from the Akita region and from further prefectures as far as Okayama and Kochi. The farm mothers worked together on a puppet show not too long ago using the Akita dialect. A lot of the activities are created not only to promote fun, but for both children and adults to learn more about the heritage and culture involved in farming. My kids have all moved away, but these days, our grandchildren are slowly taking up agricultural based courses in the universities and are moving back to help us tend to the farms."
> Mrs Ito, 75 years old

As with most farming cities, their own kids have moved to urban, sprawling cities, leaving the aging population behind. Even with all the various activities they do, the farm mothers are still worried that they might not see young blood in the industry, though there has been a significant rise in interest over the past couple of years. The government is also slowly looking into ways to encourage the younger generation to move to farming towns and learn about agriculture.
Accessibility to health care is easy in Japan, but the rising community need is something that needs to be addressed. Academia is the first to jump on this bandwagon to help foster intergenerational discourse and gain deeper understanding on indigenous food culture and the direction its heading in the near future. The prevalence of non-communicable diseases is still relatively small in comparison to other developed countries. The government has looked into various measures and strategies to combat this issue. Blame shifting has occurred, whereby the female counterparts are deemed responsible for the ongoing ignominious failure. As more women have entered the workforce, the struggle between cooking and caring for the needs of their children and being a provider is difficult to maintain. The age-old customs of producing high quality local food have been severed. Domestication of crops has paved way for consumers to purchase these once seasonal produce all year long, hence allowing the younger generation to be oblivious of the difficulty in the past of sourcing it. However, the position of 'sansai' has been re-evaluated as many have started longing for the authentic Japanese 'mother's taste experience' where you can literally feel the tender loving care put in by these farm mothers when they painstakingly prepare these dishes, which can take hours at a time. Apart from that, generation X and millennials can start creating a sense of association and reconnect to their hometowns and it is a lot healthier. These farm mothers are willing to transmit and teach their ancestral knowledge for free and is a recurrent goal among the elderly. Though there has been a resurgence of interest, more still needs to be done to further these objectives.
Farm stays have been capitalizing on the recent yearning and desire of bona fide family recipes that have been passed on from one generation to the next. This goes a long way as many communities worldwide have lost their heritage, but it is different in Japan. Farm mothers in Odate have been working together to explore the various health benefits offered from 'sansai' and how

to utilize them to their advantage. These recipes represent age-old insight that have been perfected by farm mothers to not only evoke a sense of familiarity but to showcase the depth of flavour in Japanese cuisine further reiterating the importance of women as preservers of knowledge and culture.

Women have also been the backbone of the farming and agriculture industry in Japan, though they have never been formally recognized for their contribution. A quick look at Mrs Ishigawa's role: she not only thinks of ways to promote her products but she can also be seen on the farm, making sure that the crops grow. After World War 2, the duality of lifestyle played by women in ensuring the well-being of the household whilst taking up opportunities in agricultural production and turning it into a stable income have become more prevalent, though they are still considered as farmer's wives and the men still play the central roles. The feminization of agriculture has grown exponentially and the number of women in farming has long surpassed men (Uemura, 2012). Although their roles are growing significantly, the lack of dominance and power in managing their own crops have limited their opportunities for capacity building and enhancing their status in life. As developed as Japan is, women empowerment is still lagging behind compared to other developing nations.

The women act as preservation agents in a family. This can be traced back to the past, whereby the women were in charge of domestic duties and the men were the sole bread winners of the family. Though times have changed, the Japanese still hold on to the paternalistic and authoritarian culture (Cheng et al., 2014). People who live in metropolitan cities tend to rely a lot more on convenience food. Many villages have started similar programmes that have been spearheaded by farm mothers, to rejuvenate their agricultural heritage. The term rejuvenation is needed to inject in some vitality into the aging population in Japan, and the decimation of the younger generation in pursuit of better lives away. Studies done by Yan et al. (2008), have highlighted the fine line between environmental protection and income generation when green tourism has been practised.

The farm mothers have started working together with an online marketeer to start marketing their produce to a wider audience. It is considered to be a success as their sales have increased by over 50% within the span of a year. Apart from that, the farm mothers launched various programmes targeted at youths and school children to start creating an interest in them, in hopes that they would continue to preserve their heritage. The farm mothers in Odate have just started the green tourism program targeted at foreigners for about 3 years now and farm stays for the locals for 6 years now. As most of them can only speak a few words, the usage of translation apps have come in handy, especially with tourists who are not well versed with English. English learning programmes have also being introduced by the Odate Cultural Activity Promotion Committee to help farm mothers familiarize themselves with basic English phrases to help ease communication.

The Cheerful Mother shop that works to connect agricultural villages with consumers through produce processing and direct selling, is an initiative between 69 different farm families and is spearheaded by women. Each family brings its own produce to sell that can range from flowers to 'sansai', pickled, fermented food, pre-packed meals alongside a café staffed with female chefs to recreate certain regional dishes. As this is considered a start-up initiative by the locals, it further emphasizes their roles in contributing financially to a family. A delivery system has also been set up targeted specifically at wild vegetables, with short announcements updated regularly on which vegetables are in season. They are picked up almost immediately after. Compared to other farmers' markets around the area, this shop is more specialised as all the farmers have been recognized as eco-farmers. Organic farming has not really picked up pace in Japan, yet food safety remains a priority for its citizens (Nakano, 2011). Eco farming is considered to be a step in the right direction for farmers to start transforming their farm lands to being completely organic. With this programme put in place, eco farmers are required to reduce their reliance on pesticide by 20%, which will then be followed by 70% within the next 5 years, before they make the arduous shift to completely organic. Farmers are given 10 years to be certified as organic

farmers. Many of the uninhabited farm houses are now being turned into active farmlands to revive the dying farming and agri-business industry while actively promoting green tourism in line with governmental policies and local initiatives. They have started farming 'sansai', with these abandoned plots of lands, coupled with the easy maintenance of mountain vegetables as they thrive without any care or the use of insecticide, which provides a win-win situation for both parties. To top it off, the localization of growing 'sansai' can be seen as a boon to burgeoning cultivation.

4.4 Adaptability

One of the key features that these group of women possess is adaptability. As they cater to a diverse group of people yearly, these farm mothers do change their dishes from time to time to suit their guests. As there have been Westerners who have stayed there for longer periods of time, serving up authentic dishes can be a bit too difficult for them to stomach. As such, a few Westernized dishes like yoghurt and pasta are served, alongside Japanese seasoning.

4.5 Marketing activities

Exercise has also been vital in ensuring good and a long lasting life. Radiolethics is performed in the morning for 5 minutes together with music. Odate has been recognized as the best city in 2017 for providing a real authentic experience by JTB company which are the biggest tourism company in Japan. Currently, the committee are aiming for a much higher prize. With the money from the award, Odate Cultural Activity Promotion Committee have started a programme, targeted at foreigners to bring in a steady stream of foreigners who will not only be able to appreciate the diverse landscapes Odate has to offer but also the culture. The activities are tailor made to one's preference but is dependent on the various seasons.

4.6 Linking Japanese initiatives and adapting it in Malaysia

To start including wild edibles into the diets of Malaysians, more has to be done to involve the older generations as they are not only the main spokespersons of the community but also represent key knowledge holders in utilizing these wild edibles. The types of vegetables that can be used as sustenance, nutritive details and method of usage are held by them. As observed in Japan, many of the older generation play indispensable roles in creating new kinds of support networks in aiding the revitalizing of the enjoyment of healthy, social eating environments for children and older adults. Hence for Malaysians to compensate for hyperactive work-life circumstances for working class urban families and develop positive dietary habits for nutrition, a new found appreciation for the older generation and social eating customs needs to be inculcated.

5 Conclusion

Much can be said about the integration of sansei into modern day diets. The adaptability and marketing activities conducted by the cultural committee as well as government interventions have a lot to do with the success achieved. To preserve age-old traditions and to retain a form of cultural identity, these traditional plants should be the centre of a meal, therefore traditional cooking should also be documented. Promoting the usage of traditional plants can only be accomplished by emphasising their importance as well as supplementing further opportunities to boost their consumption. So in order for the urban class to shift away from the modern diet, a link between cultural identity and agricultural production should be renewed and restored.

Acknowledgements

I would also like to thank the Odate Cultural Activity Promotion Committee for hosting us and providing insight into their way of life.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 322–333

Effect of Varying Levels of Acorn Flour on Antioxidant, Staling and Sensory Properties of Iranian Toast

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Received: 3 September 2019; Published online: 18 October 2021

Abstract

Due to the high level of antioxidant activity of acorn fruits, they can be used as an ingredient the production of functional foods. The goal of the this research was to partially substitute wheat flour with varying levels (10% - 50% w/w) of debittered acorn flour and to investigate its effects on the rheological characteristics of the dough, total phenolic content (TPC), staling, colour indices and sensory properties of toast bread. The farinograph degree of softening and water absorption of the dough decreased with increasing the acorn flour content, while the stability and time of development of the dough increased. Resistance and extensibility to deformation of the samples respectively increased and decreased compared to those of the control. Toast bread with 30% acorn flour replacement was observed to have lower staling than the control. The highest TPC (9.44 mg GAE/g) and the lowest peroxide value (0.36 m eq O_2/kg) were obtained for the bread having 30% acorn flour substitution. Moreover, the breads showed darker crumbs with significantly lower specific loaf volumes. Overall, the bread with 30% of acorn flour substitution showed good rheological, staling properties and reasonable anti-oxidant content compared to the control bread as well as the highest sensorial acceptability.

Keywords: Acorn; Toast; Antioxidant; Staling

1 Introduction

Cereals operate an important duty in human nutrition all over the world. Because they include not only large amounts of energy sources, like starch, but also fatty acids, nutritive protein and dietary fibre. The positive effects on blood cholesterol and their dietary fibre content cause an increasing demand for nutritionally enriched breads with whole grains and different seeds (Mousavi & Kadivar, 2018).

Acorns; the fruit of the oak tree; with approximately 500 species, has been mainly distributed in Europe, Asia and North America. Acorn has been used as the nut of the oak tree in various applications such as animals feeding, medicine as well as in the food industry, due to its antimicrobial, antioxidant and nutritional properties (Mohammadzadeh et al., 2013).
Acorn flour content of fat (of which over 80% is unsaturated), different sterols, considerable amounts of electrolytes (phosphorus, zinc, cop-

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per, iron, magnesium and calcium) and proteins, is acceptable from a nutritional point of view (Ghaderi-Ghahfarrokhi et al., 2017). Acorn meal could be a functional nutritional ingredient in foods that include wheat flour such as pastries, noodles, muffins, cookies, breads and desserts with a growing presence in the food industry, to improve the eating habits of personal clients and the general population for well-being benefits and disease prevention (Polimac & Komlenić, 2016). Moreover, acorns contain phenolic compounds like the derivatives of proanthocyanidins, benzoic acid, flavons, chalkons, cinnamic acid, quinones, and amino phenolic compounds with high antioxidant activity, which can break the chain of radical reactions, bind with metals and scavenge free radicals. Increasing the consumption of these compounds leads to a decrease in the risk of digestive diseases and dangerous cancers (Ferrari & Torres, 2003). Dietary supplements, nutraceuticals, organic foods and functional foods as the worldwide nutritional health products (NHPs) have become popular in an extremely fast growing market. In addition to biologically nutritious components acorns possess biologically active substances that enable the utilization of acorns in the preparation of functional foods (Rakić et al., 2004). In spite of the extensive utilization of acorn flour in bakery products, no study has been carried out to consider the effects of the partial replacement of wheat with the acorn flour in Iranian toast production. The aims of this research were (I) to study the rheological characteristics of the dough such as water absorption, development times and stability, resistance to extension and extensibility and (II) to evaluate the total phenolic content, colour indices, staling, peroxide value and sensory properties of the toast breads.

2 Materials and Methods

2.1 Materials

Acorn Flour (moisture content: 4.2 ± 0.1% dry basis (db); 150 μm mean particle size) was provided from a local market in Yasouj city (Kohgiluyeh and Boyer-Ahmad Province, Iran). The wheat samples (Sepahan (HRW)) was obtained from field trials grown at Tarbiat Modares University Agricultural Research Centre, in 2018. Wheat samples were milled in a laboratory mill supplied with different breaks, reduction rollers and sifters. Toast bread improver and yeast were obtained from Kobodena Co., Yasouj, Iran. All other chemicals and reagents used were at least of analytical grade and provided form Sigma Aldrich (St. Louis, MO, USA).

2.2 Chemical tests

The physicochemical properties of wheat and acorn flours, including moisture, protein, ash, fat, total phenolic contents and crude fibre were analysed according to AACC (2000) methods No. 46-12 , 33-17 , 30-10, 44-16 and 08-01 respectively. Energy values were determined according to the general Atwater factor according to Atwater (1902). Energy (kcal) = 9 × (g fat) + 4 × (g protein) + 3.75 × (g carbohydrate). The results were expressed as g per 100 g of dried mass.

2.3 Farinograph and extensograph tests

The attributes of the dough during mixing were determined using a farinograph (Brabender, Duisburg, Germany) according to AACC. No. 54-21 (2000) method. The softening degree, dough development time, water absorption and stability time of the control and three acorn-added samples were obtained. Extensibility and resistance to extension measurements were carried out in accordance with the standard procedure utilizing an extensograph-E (Brabender, Duisburg, Germany). The dough developed utilizing the farinograph-E was split into two 150-g loaves and retained for 45-135 min for proving. The resistance to extension and dough extensibility were obtained so. All evaluations were performed in duplicate at 25 ^{o}C.

2.4 Dough preparation

According to AACC (2000) method, the amount of water measured by the farinograph was added

to the dry ingredients then mixed until homogeneous dough was achieved corresponding to the recipe of Iranian toast. The control contain (100% wheat flour) and five acorn/wheat (0, 10, 20, 30, 40 and 50% acorn flour substituting for wheat flour) dough samples were prepared and utilized in the extensographic measurements to analyse the rheological characteristics of the dough.

2.5 Toast bread making procedure

Wheat flour was mixed with compressed yeast and salt, each comprising 1.5 g/100 g of the total mixture and previously dissolved in water. The amount of water added to each formulation varied according to the farinographic water absorption index, previously determined (Table 1). For each formulation, the ingredients such as toast improver including alpha amylase, glycerol mono-stearate (GMS), ascorbic acid, ammonium chlorate, corn oil, salt, sugar and yeast were mixed in an automatic spiral mixer (Bomann, Clatronic s.r.l., Italy), for 12 min. Instantly after mixing, the dough was left to rest for 15 min at room temperature. After that, the dough was divided into portions 300 g, moulded into cylinder shapes, put in baking pans (8 ×15 × 5 cm) and left to rest for 45 min in a proofing cabin at 30 ^{o}C and 80% RH. Samples were baked for 35 min at 205 ^{o}C in an oven (Self Cooking Center®, Rational International AG), with vapor injection in the first instants of baking. Two hours after removing the samples from the oven they were packaged in perforated OPP film and stored at room temperature (20 ^{o}C, 60% RH) for further analysis. For each sample, two baking experimental tests were performed and four loaves were obtained from each baking test.

2.6 Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) evaluations were performed using a Perkin-Elmer Model DSC 6 (Connecticut, USA). DSC curves determine the thermal properties of starch retrogradation in the toast bread. In this measurement an indium standard was utilized as calibrator calorimeter. Enthalpy (ΔH J/g) was obtained for each endotherm at 22 ^{o}C for 3 days (24, 48 and 72 h after baking). The analyses were carried out in triplicate (Primo-Martin et al., 2007).

2.7 Total phenolic content

Extraction and determination of polyphenols

Concentrated hydrochloric acid: methanol: water (1:80:10, v/v) mixture at room temperature for 5 h extracted soluble phenolics from bread samples. Insoluble phenolics' extraction was performed with methanol and concentrated sulphuric acid (10:1, v/v) for 20 h at 85 ^{o}C according to the method previously described by Hartzfeld et al. (2002). Total phenolic content (TPC) was calculated as the total of the soluble and hydrolysable polyphenolic fractions as mentioned by Perez-Jimenez and Saura-Calixto (2005). TPC was obtained according to the Folin-Ciocalteau procedure as represented by Singleton (Singleton et al., 1999). Data were evaluated as average values (n = 3) and showed as mg of Gallic acid equivalent (GAE)/ g of dry matter (DM) and analyses were conducted in triplicate.

Determination of peroxide value

The evaluation of the peroxide value (PV) was performed according to the AOCS (2003) procedure. Accordingly, 2 g oil, extracted from bread according to the AOCS method 3-54, were weighed in a glass Erlenmeyer flask and mixed with 10 ml chloroform. Glacial acetic acid (15 ml) and a saturated aqueous solution of potassium iodine (1 ml) were added and then shaken and stored in the dark for 5 min. Distilled water (75 ml) was then added and mixed and the free iodine was determined with a 0.01N solution of sodium thiosulphate, using a starch solution (10 g/l) as indicator. All analyses were carried out in triplicate.

Table 1: Effect of substituting acorn flour on mixing properties of wheat flour dough with 70% extraction

Acorn flour level (%)	Farinograph properties				Extensograph properties: Proving time 45 (min)		90 (min)		135 (min)	
	WA (%)	DST(min)	DDT(min)	DS(BU)	RE (N)	EX (mm)	RE (N)	EX (mm)	RE (N)	EX (mm)
0	60.2±3.3^{a}	1.9±0.06^{c}	1.1±0.044^{c}	190±5.6^{a}	112.4±2.5^{e}	201±5.5^{a}	104.3±3.1^{d}	174±1.1^{a}	87.6±2.4^{d}	133±2.1^{a}
10	58.4±2.12^{ab}	2.0±0.55^{c}	1.3±0.02^{b}	100±4.7^{b}	170.9±3.6^{d}	186±3.43^{b}	167.5±2.5^{c}	163±2.3^{a}	141.4±2.03^{c}	121±5.6^{b}
20	57.1±3.14^{ab}	2.3±0.39^{c}	1.3±0.071^{b}	92±3.2^{b}	207.4±4.52^{c}	144±2.6^{c}	256.4±3.8^{a}	125±2.05^{b}	211.5±4.8^{a}	98±4.8^{c}
30	54.3±1.06^{b}	3.0±0.11^{b}	1.4±0.05^{b}	90±3.29^{b}	325.4±2.88^{a}	112±4.5^{d}	268.6±5.4^{a}	110±1.19^{bc}	198.3±6.2^{b}	96±1.5^{c}
40	44.8±3.31^{c}	3.6±0.25^{b}	1.8±0.11^{b}	20±1.25^{c}	277.1±7.62^{b}	92±5.1^{e}	212.3±4.6^{b}	91±2.3^{c}	209.8±5.5^{a}	80±3.4^{d}
50	37.9±2.56^{d}	4.8±0.35^{a}	2.3±0.15^{a}	16±0.9^{c}	211.8±5.12^{c}	85±4.7^{e}	215.1±5.1^{b}	85±2.15^{c}	201.1±4.3^{ab}	82±2.12^{d}

WA, water absorption ; DDT, dough development time; DST, dough stability time; DS, degree of softening; RE, Resistance to extension ; EX, extensibility; BU(Brabender unit). Values are expressed as mean of two replications ±S.D. Different letters show significant differences at a significance level of 5%.

2.8 Bread physical properties

The volume of bread was measured in duplicate utilizing a volume analyser BVM-L370 TexVol Instruments (Viken, Sweden). Crumb bread colour was determined in the CIE $L^*a^*b^*$ system through the reflectance procedure using Color i5 spectrophotometers (X-Rite, USA) adjusted for the following parameters: illuminate D65, measuring geometry d/8, slit width 25 mm, observer 10^o (Marpalle et al., 2014). The colour of all samples was determined 1 h after baking. A mean of four evaluations for L*, a* and b* values were saved. Average values of at least 2 breads were taken for statistical goals. Firmness of bread crumb was evaluated by a puncture test utilizing an Instron 4301 (Instron LTD. High Wycombe, UK). On the test day, crosshead speed was set at 10 cm/min and bread samples were punctured with a 3-mm cylindrical probe. The result of the puncture tests were achieved from force–distance curves and the firmness parameter was taken as the force (N) required to puncture the bread crumb. All analyses were carried out in triplicate.

2.9 Sensory evaluation

The sensory acceptability was performed on the bread samples with the various percentages of acorn flour. Each bread slice (2 cm thick) coded with a number and served to each the panel, consisted of 24 untrained assessors (aged between 21 and 43 years) who evaluated the bread overall acceptability, under normal (daylight) illumination. The measuring of acceptability of the acorn toast bread samples was taken by a nine-point hedonic rating scale, utilized with scores of 1, 5 and 9 representing “dislike extremely”, “neither like not dislike” and “like extremely” respectively (Lin et al., 2009).

2.10 Statistical analysis

Statistical software of SAS v. 9 was utilized for the statistical analysis of the data achieved in this research. All data were subjected to analysis of variance (ANOVA) and the means of the results at a significance level of 5% were compared by Duncan’s multiple range test.

3 Results and Discussion

3.1 Chemical analysis of flour types

Compositions of acorn flour (AF) and wheat flour (WF) are presented in Table 2. One of the important factors influencing the overall quality of flours (colour, flavour, texture) is pH, according to Sabrin (2009). The moisture content of flours is a critical quality factor for transportation, packaging and preservation. Moreover, the moisture content also should be standard. The results illustrated that the pH values of AF and WF were the same (5.64). AF had a lower moisture (4.12%) and protein (4.21%) contents than

WF but higher fat (6.49%), fibre (3.11%) and ash (2.19%) contents. Hadnadev et al. (2011) reported that the fat content of wheat flours was within the range of 0.75–2.34%. Sabrin (2009) introduced acorn is a remarkable food source of calorie because of its high levels of fat. The Amount of fat can affect staling, so flour types with high-fat contents are desirable for utilize in low-moisture food products such as biscuits or cookies. The index of TPC for WF (8.21 mg GAE/100 g) was much lower compared to AF (49.8 mg GAE/ 100 g). Adom et al. (2003) reported that TPC for various wheat varieties was within the limit of 710–860 μmol gallic acid/g. Finally, the AF had a higher caloric value (402.7 $\pm$ 18 kcal/100 g dm) probably as a result of its higher fat content which is in close agreement with Silva et al. (2016). Therefore, due to its higher nutritional value and TPC, AF was added to WF to upgrade the bread functional properties.

3.2 Rheology of the dough

A farinograph analysis was performed to evaluate the effect of different AF percentages substituted for WF on the mixing and develops properties of the dough and is presented in Table 1. Water absorption significantly ($p < 0.05$) decreased as AF substituted WF and the control had the maximum water absorption. The ability to water absorb, applied by farinograph, depends on the protein content and damaged starch, as well as on the power of gluten to entrap water and lead to be positively related to bread yield. Thus, substitution of WF with AF suggested a decrease in the gluten content and subsequent water absorption of the sample.

The dough stability and development time significantly increased ($P < 0.05$), while a significant decrease ($P < 0.05$) in degree of softening was showed. Dough development time is defined as the time required to reach the maximum torque. Extending of dough stability to developing and mixing and confining drop of consistency (softening index), are both essential for dough spreading. The Degree of softening and dough stability time indicates the rate of breakdown and flour strength, with a lower degree of softening and a higher dough stability suggesting a stronger dough, according to Alaunyte et al. (2012). The lower gluten content and the presence of tannins and hydrocolloids in AF led to retardation of water absorption, extending the dough development time. The interaction of the acorn fibre and wheat gluten could improve the gluten network during mixing and cause such a decrease in degree of softening. This is consistent with the result reported by Hu et al. (2017), who reported that the dough with a longer stability time always exhibited a lower weakening index.

The dough properties, including extensibility and resistance to extension at different fermentation times (45, 90 and 135 min) with 10 - 50% AF substitution were determined by extensograph. According to the results presented in Table 3, in each of the samples with an increasing AF substitution level, the resistance to deformation and the extensibility of the dough significantly ($p<0.05$) increased and decreased, respectively. It may result with the increment time of fermentation, the dough lost its resistance to long-term proofing; thus, the proofing time should be decreased.

Since the properties of the flour gluten is directly related to the results of extensograph, the increased dough resistance to deformation at 0-30% substitution can be explained by the interaction between the fibre content of AF and gluten network. The reduction in the samples' gluten content (40-50% substitution) caused a decrease in the dough's resistance to deformation. Decreasing the extensibility of the dough due to the formation of capable gluten and fibre network led to its higher firmness and speeds up tearing, according to Rosell et al. (2001).

A correlation seems to exist between the farinograph and extensograph indices. Comparing the results of the dough properties with the water absorption decrease proportional to the level of acorn flour added, it is revealed that the interactions between the gluten matrix of the dough and added the acorn have been so strong that could increase the consistency of the dough structure.

Table 2: Proximate composition of wheat and acorn flours

Parameters	Acorn flour	Wheat flour
Moisture (%)	4.12	13.12
Fat (%)	6.49	2.38
Ash (%)	2.19	0.76
Protein (%)	11.13	4.21
Fibre (%)	3.11	0.42
Energy (Kcal)	402.7	320.8
Total phenolic content (mg GAE/100g)	49.8	8.22

Reported values are the mean of at least 3 replicates

Table 3: Total phenolic content, peroxide value and staling properties of the breads containing 0-30% acorn flour. Different letters show significant differences at a significance level of 5%.

Acorn flour	Enthalpy (j/g)			Firmness (N)			Total phenolic content	Peroxide value
level (%)	After 24h	After 48h	After 72h	After 24h	After 48h	After 72h	(mg GAE/g)	(meq O_2/kg)
0	1.23 ± 0.01^{a}	2.41 ± 0.05^{b}	3.57 ± 0.33^{a}	102 ± 2.33^{c}	106 ± 3.3^{c}	105 ± 2.51^{d}	0.52 ± 0.04^{d}	5.33 ± 0.61^{a}
10	0.88 ± 0.001^{b}	2.33 ± 0.03^{b}	3.17 ± 0.05^{b}	104 ± 1.12^{c}	112 ± 2.2^{c}	129 ± 3.4^{c}	4.87 ± 0.63^{c}	2.17 ± 0.052^{b}
20	0.54 ± 0.003^{c}	2.37 ± 0.11^{b}	3.22 ± 0.05^{b}	153 ± 3.01^{b}	155 ± 1.88^{b}	178 ± 3.7^{b}	6.32 ± 0.88^{b}	1.03 ± 0.31^{c}
30	0.95 ± 0.01^{ab}	2.98 ± 0.2^{a}	3.64 ± 0.08^{a}	194 ± 0.25^{a}	199 ± 3.63^{a}	231 ± 2.55^{a}	9.44 ± 0.67^{a}	0.36 ± 0.05^{b}

3.3 Bread retrogradation

It has been reported that staling caused reorganization of the gelatinized starch in an ordered crystalline structure known as retrogradation as described by AACC (2000). This re-crystallization leads to staling of cereals and food products. Katina et al. (2006) reported that one way to measure bread staling is measurement of the heat required to melt crystallized starch. The obtained enthalpy was the meaningful parameter for detecting significant differences lead to staling in the bread firmness.

The enthalpy of the control, 10, 20 and 30 % acorn bread after 24, 48 and 72 h of storage are shown in Table 3. The panellists gave the lowest score of overall acceptability to the 40 and 50% acorn bread samples; they were therefore discarded and were not subjected to the staling test.

For fresh bread (on the day of baking), a decrease in the enthalpy in comparison to the control by addition of AF at 10 and 20 % was observed, although an increase was showed at 30% substitution. The exact levels of this addition were not statistically significant in the reduction of the melting enthalpy in comparison with the control. Differences were observed between the enthalpies of the samples with different levels of AF which were proportional to the replacement levels on the second and third day of storage.

The firmness values of the bread in the course of storage are presented in Table 3. In most cases, bread firmness increased significantly over the storage period. However, the addition of AF changed the degree of these modifications. On the day of storage, the loaves with 20 and 30 % AF were comparably as firm as the control. Thus, it implied that after 24, 48 and 72 h of baking, staling of the sample with 20% AF was lower than of the control and this sample went staler compared to the control. The reduction in the recrystallization enthalpy of amylopectin and the rise in firmness could be caused by three factors. I) Partial substitution of starch with AF caused a reduction in the total amount of amylopectin in the system, II) amylopectin could interact with AF and retard its re-crystallization and III) Amylose chains' content could bind with fibres and hydrocolloids and inhibit them from

linking to each other. It seems that the low fat, fibre and hydrocolloid contents of the sample with 20% AF did not restrict staling, because this amount of fat and hydrocolloids was too little to cover the flour particles and consequently staling occurred (Gray & Bemiller, 2003). Therefore, it would control water mobility and preserve starch from going stale and the texture from becoming hard. It should be mentioned that staling is a complex process that includes re-crystallization of amylopectin only as one of many factors.

3.4 Phenolic analysis

Total phenolic content (TPC) and peroxide value (PV) of the bread samples containing various percentages of AF are presented in Table 3. The results suggested that by increasing acorn levels, TPC of the breads significantly increased. Some of the literature reports that tea had polyphenols which, have a noticeable preventable impact on the rice starch retrogradation (Wu et al., 2009). In this research we did not show any significant correlation between TPC of the bread samples and the enthalpy of retrogradation.

Acorn bread samples with peroxide values (PV) of 0-50% were used to evaluate the impact of the temperature of baking on the acorn oil, as well as on the shelf life of the breads (Table 3). As the replacement of the level of AF for WF increased, PV was found to decrease. This indicates that the increment of antioxidants and phenolic compounds decreased the oxidation of the bread compounds during baking and consequently PV decreased.

3.5 Bread properties

The physical properties of the composite bread including various levels of AF substitution are shown in Table 4. It was found that with increasing concentration of AF at the same water content, the volume of the samples decreased 24.39%. Fibres can modify bread loaf volume and the firmness of the loaf. As a rule, the incorporation of fibres in bread reduces loaf volume and increases firmness (Table 3), but the extent of modification depends on the fibre source (Elleuch et al., 2011). Moreover, Kurek and Wyrwisz (2015) revealed that a reduction in the loaf volume of the breads obtained, was the main problem of the addition of dietary fibre to bakery products.

It was found that as the substitution of AF in the mixture increased, the breads became darker as evidenced by the lower L* index of the mixture in comparison to that of the control. Pasqualone et al. (2019) remarked that, the addition of acorn flour caused darkening in biscuits. Browning was due to the phenolic fraction of flour, which is known to easily undergo oxidation by polyphenoloxidase (Pasqualone et al., 2014; Taranto et al., 2012).

The lowest index (63.32) L* was for 50% AF bread and the highest one (91.62) belonged to the control sample as shown in Table 4. An increase in a^* and b^* values of the bread may be due to the inherent dark colour of the acorn meal. In the case of a^*, the addition of AF resulted in its change from negative to positive values, signifying the supremacy of red over green in the bread colour. In all cases, the value of b^* was positive, which corresponds to that yellow was more intensive than blue both in the control and AF-containing breads. Replacement of WF with AF caused an increase in crumb yellowness. The results were consistent with a previous study by Skendi et al. (2018).

The porosity of the breads which, was characterized by digital image analysis, is depicted in Figure 1. The sample with 30% substitution of AF was characterized as the most porous sample. Substitution of 40- 50% AF caused a decrease in porosity to values comparable with or higher than those observed for the control. The reduction in porosity is probably due to a dilution of gluten and the interruption of its structure by the increased AF content, providing impairment in gas retention. The results are consistent with the study reported by Korus et al. (2015).

Sensory evaluation is the key procedure to assess the quality of food and requires a small sample size and less time. The descriptive evaluation of the sensory results of the breads made from WF and various concentrations of AF are presented in Figure 2.

The 40% substitution of AF had a remarkable impact on the appearance of the bread. Further addition of AF up to 50% caused a decrease in

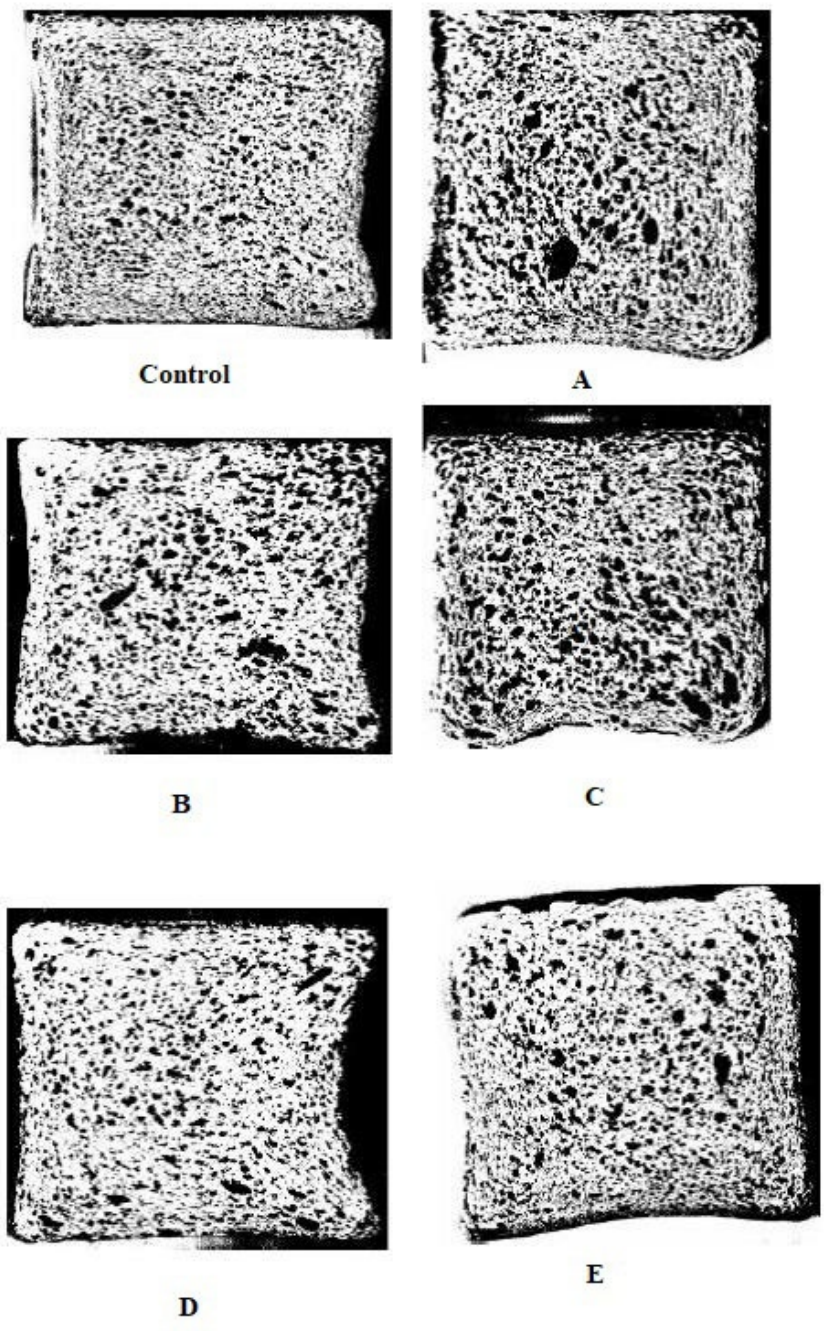

Figure 1: Effect of Acorn Flour addition on the internal structure of toast bread. Control: 100% Wheat bread; A) mixture of 90% refined Wheat Flour (70% extraction rate) and 10% Acorn Four; B) mixture of 80% refined Wheat Flour (70% extraction rate) and 20% Acorn Flour; C) mixture of 70% refined Wheat Flour (70% extraction rate) and 30% Acorn Flour; D) mixture of 60% refined Wheat Flour (70% extraction rate) and 40% Acorn Flour; E) mixture of 50% refined Wheat Flour (70% extraction rate) and 50% Acorn Flour.

Table 4: The results of the proximate analysis and physical properties of the bread samples

Acorn flour level (%)	Bread volume(cm^3)	L*value	a*value	b*value
0	318.67 ± 3.22^a	91.62±1.5^a	-1.21±0.05^d	12.7±0.9^b
10	279.32±1.17^{bc}	87.60±2.3^b	4.31±0.9^c	17.7±0.85^a
20	277.21±2.8^c	73.33±2.03^c	6.85±1.1^b	17.2±0.36^a
30	280.50±1.01^b	70.91±2.03^{cd}	7.12±0.8^{ab}	17.2±0.74^a
40	263.46±1.6^d	67.42±1.1^d	7.75±1.32^a	17.1±1.1^a
50	240.91±2.4^e	63.32±1.3^e	7.6±0.5^{ab}	16.5±1.02^a

Values are mean ± standard deviation (n=3). Mean values in the same row sharing different superscripts were significantly different ($P < 0.05$).

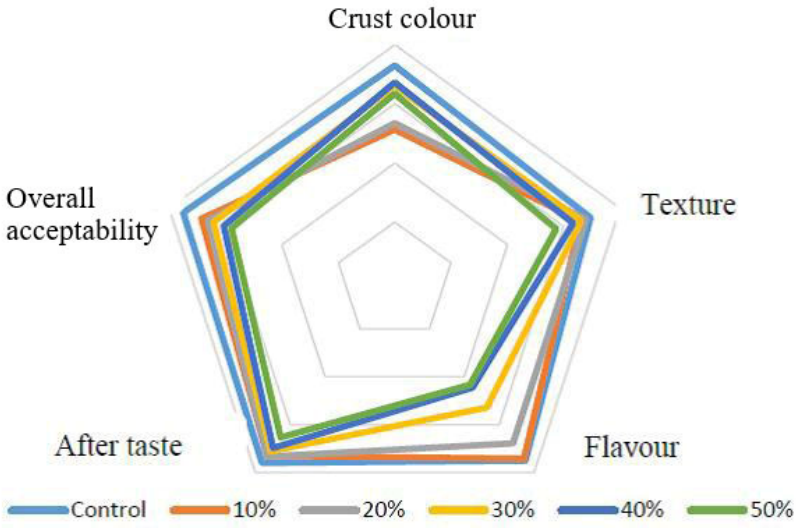

Figure 2: Results of the sensory evaluation of the control and acorn bread samples.

the score of this attribute. Flavour and texture were also more acceptable for 0-30% substitution than other samples. The highest overall acceptability score (7.43) was given to 30% AF bread and in higher levels of replacement, acceptability scores decreased. The bread with 50% AF substitution had the minimum acceptable taste. Therefore, the addition of AF (up to 30%) had a clearly positive impact on the overall acceptance and the sensory attributes of the toast bread.

4 Conclusion

This research was undertaken to see the potential of acorn flour addition on the physicochemical properties of Iranian toast bread, and has achieved new findings. Acorn flour expressed a higher fibre content, protein, fat compared than wheat flour. Adding acorn flour had considerable changes in rheological properties. The degree of softening, water absorption and dough extensibility decreased, while dough stability, development times and resistance to extension increased. The brightness of the bread crust decreased significantly with the increased acorn flour level and

the toast became darker with the acorn flour enrichment, compared to the control. Acorn was found to be higher in antioxidant capability as evidenced by its higher phenolic content and lower peroxide value compared to the control toast. According to the panellist's score in the sensory evaluation and considering the results obtained in this experiment, Iranian toast enriched with 30% acorn flour was suggested as an acceptable and beneficial food.

Acknowledgements

The authors acknowledged the staff of the laboratories of Yasouj University of medical sciences.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 334–345

Portfolio of Beetroot (*Beta vulgaris L.*) Peel Extracts Concentrated by Nanofiltration Membrane

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Received: 26 April 2020; Published online: 18 October 2021

Abstract

Membrane process is an intelligent alternative way of concentration, preferably for organic juices rich in thermolabile natural components. The expectation is to scale up the extraction of desired compounds from agro-industrial wastes through modernized concentration methods. Recovery of betalains, phenolic, and antioxidant from beetroot peel extracts was accomplished by nanofiltration membrane (NF 200) at a recirculation flow rate (400 L h^{-1}) and feed temperature (30 ^{o}C) under constant transmembrane pressure (40 bar). Characterization of betaxanthin, betacyanin, phenolic, and antioxidant activity by spectrophotometric analysis revealed that the final samples contain these compounds respectively: 202.25±3.26 mg·L^{-1}, 360.07±8.43 mg·L^{-1}, 987.79±19.18 mg·L^{-1}, 642.06±14.78 mg·L^{-1}(pure water); 206.62±1.37 mg·L^{-1}, 339.72±2.89 mg·L^{-1}, 972.72±47.49 mg·L^{-1}, 745.97±25.45 mg·L^{-1} (ethanol-water). Final samples exhibit vivid colour and a considerably large amount of desired compounds compared to crude extracts and could have industrial applications.

Keywords: Beetroot; Betalains; Phenolic; Antioxidant; Nanofiltration

1 Introduction

Agro-industrial residues, including beetroot peel, are acknowledged sources of antioxidant-rich compounds such as betalains and phenolic compounds. To maximize the recovery of these valuable compounds with minimal processing cost and utilize them in various fields is quite challenging because of their ease of compartmentalization. Beetroot, a typically known source of betalains and phytochemicals, is rich in therapeutic values and minerals, thereby getting many compliments from consumers as well as food researchers. As stated by Kale et al. (2018), beetroot is a chief source of several macro and micronutrients, for example, carbohydrate (7.59 %), protein (1.35 %), sodium and potassium (72.58 and 30.12 mg/ 100 g of beetroot), and betalain (14.2 mg/ 100 g of beetroot).

Betalain is an active hydrophilic compound mainly found in species of Caryophyllales plant families (Hussain et al., 2018). Determined by the components attached to the main structure of betalamic acid (4-[2-oxoethylidene]-1,2,3,4-tetrahydropyridene-2,6-dicarboxylic acid), two structural groups known as betaxanthin and betacyanin are derived. Vulgaxanthin-I (80 % of betaxanthin), a derivative of betalamic acid conjugated with amines, is responsible for its yellow colour (Sawicki & Wiczkowski, 2018). Red-violet is the predominant colour of betanin (69 % of betacyanin) which is derived from the condensation of betalamic acid with 3, 4-dihydroxyphenylalanine or its glucosyl derivatives (Elbandy & Abdelfadeil, 2008; Miguel,

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Nomenclature

Δt	time required to collect the filtrate (h)
ΔV	volume of filtrate (L)
A	sample absorbance
AA	antioxidant activity
AC	antioxidant capacity ($mg.L^{-1}$)
A_m	active surface area of membrane (m^2)
BC	betacyanin
BX	betaxanthin
C_0	concentration of initial feed ($mg.L^{-1}$)
C_R	concentration of retentate ($mg.L^{-1}$)
DF	dilution factor
EWE	ethanol-water extract
FRAP	ferric reducing antioxidant power
J	permeate flux of pure water ($m^3/(m^2 \cdot s)$)
J_x	permeate flux of sample ($L/(m^2 \cdot h)$)
L	path length (cm)
MW	molecular weight (g/mol)
R_m	membrane resistance (1/m)
S	amount of sample (μL)
TMP	applied transmembrane pressure difference (Pa)
TPC	concentration of total phenolic compounds ($mg.L^{-1}$)
VRR	volume reduction ratio
WE	water extract
α_1	slope of TPC calibration curve
α_2	slope of AA calibration curve
μ	dynamic viscosity of permeate ($Pa \cdot s$)
ε	molar extinction coefficient ($L/(mol \cdot cm)$)

2018; Sawicki & Wiczkowski, 2018). Phenolic compounds found in fruits and vegetables are in the range of simple phenolic acids to complex forms and exhibit antiradical activity to some degree according to their hydroxyl group structures (Shahidi & Ambigaipalan, 2015). As both betalains and phenolics are plant secondary metabolites condensed with antiradical scavenging property (Bucur et al., 2016; Kavalcová et al., 2015), they can defend against oxidative stress and related chronic diseases such as cancer, cardiovascular disease, and neurodegenerative disorders like Parkinson's and Alzheimer's (Mironczuk-Chodakowska et al., 2018).

Membrane filtration is a promising technology with high throughput and low cost if properly applied. Its benefit in biological separation has been known by scientists for decades but it is not quite commonly used. Lately, the trend of modern separation and purification technology brings its application back with some improvements; especially in food processing industries with the purposes of concentration and clarification of beverages including red wine (Banvolgyi et al., 2006), blackcurrant juice (Banvolgyi et al., 2009), apple juice (Vladisavljevic et al., 2003), strawberry juice (Arend et al., 2017), and Indian blackberry juice (Ghosh et al., 2018). Polymeric membranes have low thermal conductivity and high resistance to almost all organic solvents at processing temperatures (Perfilov, 2018). Pressure driven nanofiltration membrane has a higher retention rate of particles with molecular weight between 100 and 1000 Da (Dach, 2008). In addition, nanofiltration is a globally used membrane technology not only for lone filtration but also as a pretreatment for reverse osmosis feed.

Reusing agro-industrial waste products from food industries is one of the effective ways to save the planet by diminishing the waste of fruits and vegetables (dos Santos et al., 2016). Recovery of bioactive compounds from the waste parts of the beetroots, stalk (Maran & Priya, 2016), peel (Kujala et al., 2001; Sawicki et al., 2016; Zin et

al., 2020), and pomace (Vulic et al., 2013), has been accomplished through solid-liquid based extraction methods. Notwithstanding, clarification of beetroot (stalk) extracts has been successfully realized via different membrane filtration processes such as microfiltration and ultrafiltration (dos Santos et al., 2016).
Beetroot peel got our attention for this work as betalain content in the peel is high (Sawicki et al., 2016; Zin et al., 2020). Based on the molecular weight of the targeted compounds, nanofiltration membrane was chosen. The aim of this study was to determine how many levels of nanofiltration concentration could be attained for beetroot peel extracts while ascertaining the reduction in volume. Furthermore, the synergistic effects between betalains, phenolic compounds and antioxidant activity were also explored.

2 Materials and Methods

2.1 Extraction of Beetroot Peel Juice

Beetroots (*Beta vulgaris L.*) were procured from a local market in Hungary and processed right away for extraction. Extraction was accomplished by a single batch type mode at a processing temperature of 22 ^{o}C for 60 minutes with pure water and 15 % (v/v) aqueous ethanol solvent (1:10 peel-to-solvent ratio).

2.2 Membrane Processing

Crossflow filtration process was accomplished by Polyamide Thin Film Composite (NF 200, FILMTEC^{TM} Membrane) using DDS Filtration Equipment (LAB 20-0.72, Denmark). The parameters were as follows: operation temperature (30 ^{o}C), transmembrane pressure (TMP, average 40 bar), and recirculation flow rate (400 L/h) (Figure 1). Concentrated samples were collected from the tank every 500 mL interval of permeate and the time taken to reach each 100 mL of permeate was recorded for future calculation. According to Darcy's law, the flux of pure water J ($m^3/(m^2 \cdot s)$) passing through any type of membrane can be estimated by TMP (Pa) applied for the operation divided by the resistance of the membrane, R_m (m^{-1}) and the dynamic viscosity of the permeate, μ (Pa $\cdot$ s) (equation 1) (Miller et al., 2014).

$$J = \frac{TMP}{\mu \times R_m} \tag{1}$$

Dach (2008) defined volumetric flux (J_x) as the measured volume of permeate at a given time interval. In this experiment, the permeate flux ($L/(m^2 \cdot h)$) of the sample was determined from direct measurement of filtrate volume, *Delta*V (L), divided by the time required to collect the filtrate, Δt (h), and total active surface area of membrane, A_m (m^2) as expressed in equation (2) (Liu et al., 2011).

$$J_x = \frac{\Delta V}{A_m \times \Delta t} \tag{2}$$

2.3 Spectrophotometry Analysis

Centrifugation of the extracts was conducted before spectrophotometric analysis. Determination of desired compound quantity was executed by spectrophotometer (Genesys 5 UV-visible, MILTON ROY, U.S.A) with appropriate dilution. For colour compound detection, absorbance measurements were at 470 nm for betaxanthin compound (vulgaxanthin-I) and 538 nm for betacyanin compound (betanin); and quantified according to equation (3) (Chong et al., 2014; Nemzer et al., 2011).

$$Betalain = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L} \times (mg.L^{-1}) \tag{3}$$

Where A is the absorbance; MW is the molecular weight (g/mol); DF is the dilution factor; ε is the molar extinction coefficient ($L/(mol \cdot cm)$) and L is the path length (cm). Molecular weights and molar extinction coefficients for each betaxanthin and betacyanin are as follow: MW = 308 g/mol and 550 g/mol; ε = 48,000 $L/(mol \cdot cm)$ and 60,000 $L/(mol \cdot cm)$. Quantification of total phenolic compound contents of each sample was completed by the Folin-Ciocalteu colourimetric method at 760 nm and calculated as expressed in equation (4):

$$TPC = \frac{A \times 2500 \times DF}{S \times \alpha_1} \left[\frac{mgGAE}{L}\right] \tag{4}$$

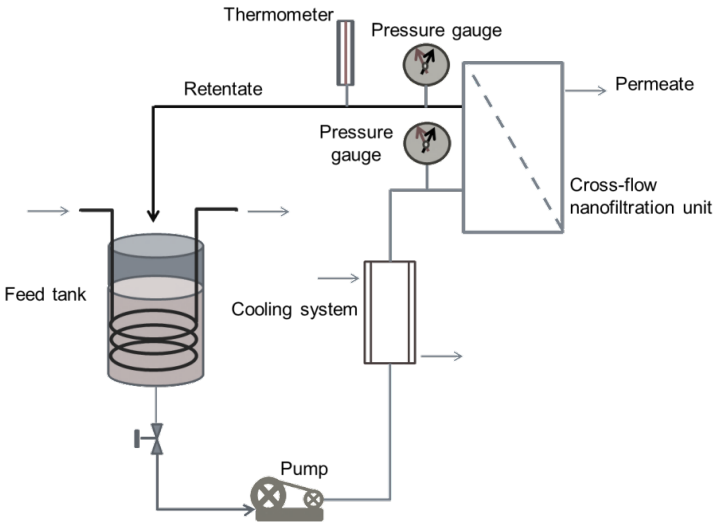

Figure 1: Scheme of cross-flow nanofiltration in batch mode

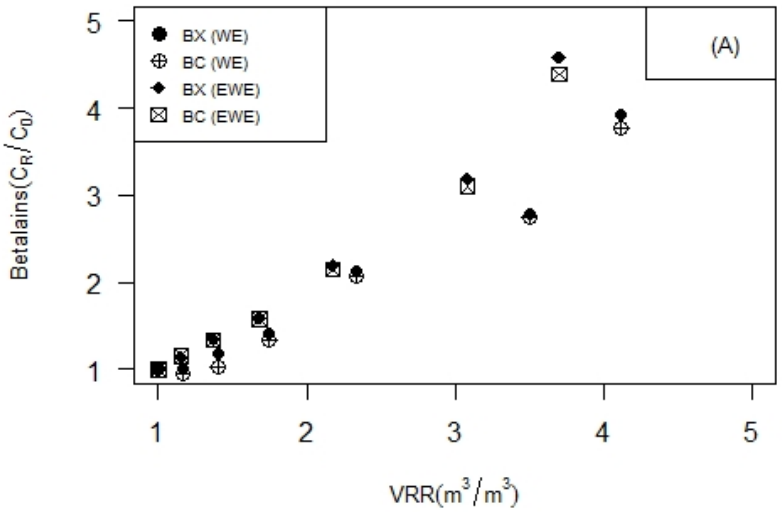

Figure 2: Concentration ratios (C_R/C_0) of respective betalain compounds varied with the volume reduction ratio (VRR)

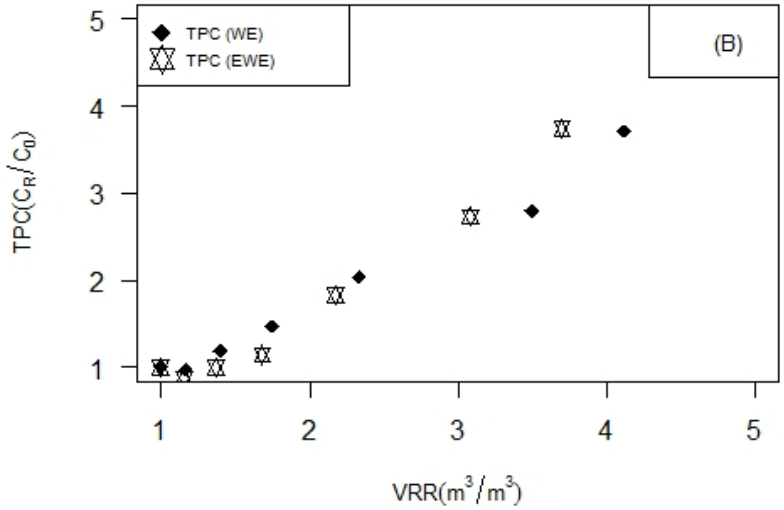

Figure 3: Changes in concentration ratios (C_R/C_0) of phenolic compounds with volume reduction ratio (VRR)

Whereby A is the measured absorbance; DF is the dilution factor; S is the amount of sample (μL); α_1 is the slope of the calibration curve (Koczka et al., 2018). For the antioxidant activity (AA) assay, the ferric reducing antioxidant power (FRAP) method was applied, and the measurement was done at 593 nm (equation 5).

$$AA = \frac{A \times 1550 \times DF}{S \times \alpha_2} \left[\frac{mgASE}{L}\right] \qquad (5)$$

Where A is the absorbance; DF is the dilution factor; S is the amount of sample (μL); α_2 is the slope of the calibration curve (Benzie & Strain, 1996). Data were evaluated by the performing variance analysis model using R statistic software (version 3.6.2). The concentration process of each extract was performed twice, and all sample analyses were performed in triplicate.

3 Results and Discussion

Extraction processes were determined based on our previous experiment in which optimization of the process conditions was focused on achieving the highest betalain compounds, except for peel-to-solvent ratio (Zin et al., 2020). Sample analysis was in triplicate in order to calculate mean values and standard deviations. Analysis of variance showed that a significant level (within and between groups) of all desired compounds was 99.99+% (p-value<0.001). Table 1 represents the outcomes of the spectrophotometric analysis of betalains, phenolic, and antiradical activity. The amount of those respective compounds detected in water crude extract were as follows: 51.46±1.61 mg·L^{-1} (betaxanthin), 95.29±0.58 mg·L^{-1} (betacyanin), 265.96±3.42 mg·L^{-1} (phenolic), 250.40±27.34 mg·L^{-1} (antiradical activity) but, their contents in aqueous ethanol crude extracts were a bit lower than the former: 45.05±1.17 mg·L^{-1} (betaxanthin), 78.38±1.67 mg·L^{-1} (betacyanin), 259.91±29.06

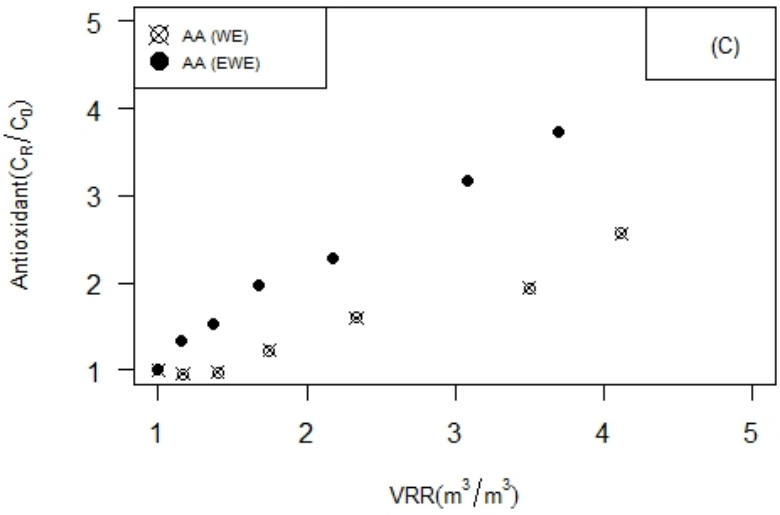

Figure 4: Changes in concentration ratios (C_R/C_0) of antioxidant activity with volume reduction ratio (VRR))

Table 1: Variation of betalains, phenolic contents, and antioxidant activity with volume

	Sample	Betaxanthin ($mg{\cdot}L^{-1}$)	Betacyanin ($mg{\cdot}L^{-1}$)	TPC ($mg{\cdot}L^{-1}$)	Antioxidant Activity ($mg{\cdot}L^{-1}$)
Water extract	Initial feed	51.46 ± 1.61^a	95.29 ± 0.58^a	265.96 ± 3.42^a	250.40 ± 27.34^a
	R (500 mL)	51.72 ± 2.56^a	91.03 ± 0.39^a	259.51 ± 13.32^a	238.01 ± 6.03^a
	R (1000 mL)	60.77 ± 1.89^a	98.36 ± 1.36^a	315.52 ± 1.71^a	242.79 ± 2.3^a
	R (1500 mL)	72.51 ± 1.42^a	127.42 ± 2.06^a	391.68 ± 20.52^a	305.94 ± 27.09^a
	R (2000 mL)	109.4 ± 1.31^a	197.82 ± 4.16^a	542.39 ± 3.69^a	401.14 ± 22.17^a
	R (2500 mL)	143.41 ± 1.45^a	261.62 ± 2.75^a	743.47 ± 25.64^a	485.99 ± 0.00^a
	Final	202.25 ± 3.26^a	360.07 ± 8.43^a	987.79 ± 19.18^a	642.06 ± 14.78^a
Eth - water extract	Initial feed	45.05 ± 1.17^a	78.38 ± 1.67^a	259.91 ± 29.06^a	199.70 ± 3.28^a
	R (500 mL)	51.08 ± 1.98^a	89.56 ± 2.34^a	227.27 ± 10.26^a	266.46 ± 12.31^a
	R (1000 mL)	60.57 ± 1.57^a	103.22 ± 2.94^a	258.70 ± 11.08^a	305.36 ± 8.21^a
	R (1500 mL)	71.74 ± 0.48^a	122.1 ± 1.8^a	296.18 ± 8.55^a	394.08 ± 15.01^a
	R (2000 mL)	98.88 ± 0.68^a	166.47 ± 2.5^a	475.10 ± 15.39^a	456.29 ± 8.21^a
	R (2500 mL)	143.48 ± 1.93^a	240.63 ± 1.93^a	$709.\ 62\pm1.71^a$	634.51 ± 2.46^a
	Final	206.62 ± 1.37^a	339.72 ± 2.89^a	972.72 ± 47.49^a	745.97 ± 25.45^a

Different superscript letters mean significant differences ($p<0.001$)

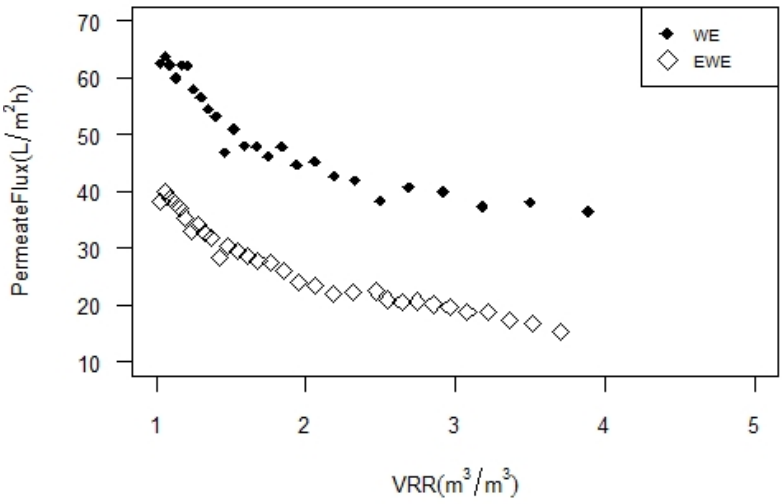

Figure 5: Variation of permeate flux values with volume reduction ratio (VRR) during the concentration process by nanomembrane type NF 200

Table 2: Correlation between the desired compounds and their antioxidant activity

	Variables	Correlation	Regression Statistics
Water extract	AA & BX[a]	0.99	R^2 = 0.9948
	AA & BC[a]	0.99	R^2 = 0.9979
	AA & TPC[a]	0.99	R^2 = 0.9925
	TPC & BX[a]	0.99	R^2 = 0.9966
	TPC & BC[a]	0.99	R^2 = 0.9957
Eth-water extract	AA & BX[a]	0.98	R^2 =0.9540
	AA & BC[a]	0.98	R^2 = 0.9571
	AA & TPC[a]	0.96	R^2 = 0.9277
	TPC & BX[a]	0.99	R^2 = 0.9877
	TPC & BC[a]	0.99	R^2 = 0.9883
	BX (WE) & BX (EWE)[a]	0.99	R^2 = 0.9944
	BC (WE) & BC (EWE)[a]	0.99	R^2 = 0.9889
	TPC (WE) & TPC (EWE)[a]	0.99	R^2 = 0.9886
	AA (WE) & AA (EWE)[a]	0.97	R^2 = 0.9421

Different superscript letters mean significant differences (p<0.001)

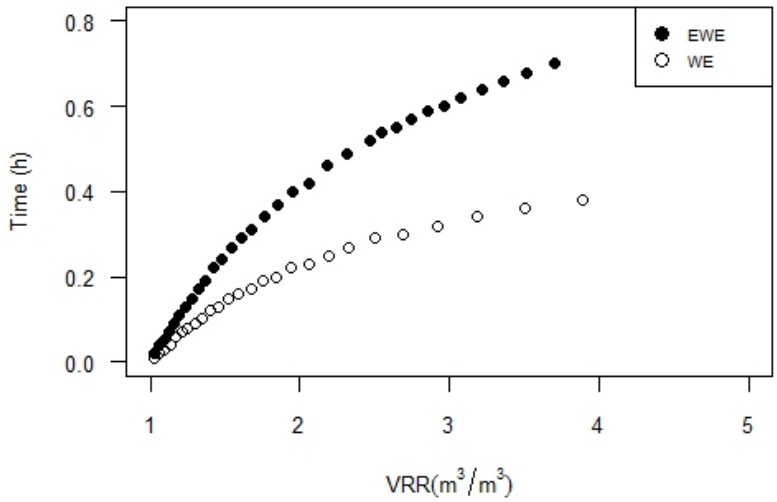

Figure 6: Variation of processing time with volume reduction ratio (VRR) during the concentration process by nanomembrane type NF 200

mg·L^{-1} (phenolic), and 199.70±3.28 mg·L^{-1} (antiradical activity). Since extraction processes were executed at low temperature and short extraction time with mild solvent concentration, the efficiency of the solvent was not significant enough to acquire greater outputs (Sawicki et al., 2016). As active hydrophilic compounds, water seems to be more operative to extract these desired compounds than alcoholic solvents in some cases (Bastos & Gonçalves, 2017).

With high water affinity, betalains and phenolic compounds extraction can mostly be fulfilled by pure water; however, changes in polarity of the solvent by combining with alcohols are also beneficial to accomplish the extraction performance. This can assist in overcoming the disturbance of water-soluble proteins some point which leads to enhancing the recovery of targeted compounds via membrane separation (Delgado-Vargas et al., 2000; Strack et al., 2003).

The amounts of betacyanin compounds were higher in both extracts than betaxanthin since the first compound is more stable in processing than the second one (Bastos & Gonçalves, 2017). Based on the investigation of Nemzer et al. (2011), the ratio of the violet colour compound to that of yellow fundamentally differs along with the varieties of beetroot and processing conditions, albeit the amount of the former compound was always superior compared to the latter one. To achieve homogeneity of raw materials, ground peels were blended prior to extraction; however, the contrast in the amount of these compounds might be attributed to the genotype of raw material and season of harvesting (Chong et al., 2014; Sawicki et al., 2016; Stintzing et al., 2005).

Figure 2 represents betalain concentrations as a function of volume reduction ratio (VRR). As depicted in the figure, all trends of concentration ratio for colour compounds were similar in both extracts. Concentration ratios of betaxanthin (BX) and betacyanin (BC) which were achieved in ethanol-water extract (EWE) were 4.5, while a maximum of 4 was attained in water extract (WE). For TPC, trends of concentration ratio

(3.7) for both extracts were comparable (Figure 3). On the other hand, drastic changes in the concentration ratio of antiradical activity were not shown in water extract displaying the maximum value of 2.6 even though aqueous ethanol could be concentrated up to 3.7 (Figure 4).

The concentration ratios of all desired compounds in ethanol-water retentates surpassed pure water, implying that nanofiltration is more satisfactory for the concentration of aqueous ethanol extract than pure water extract. However, a longer filtration process was required in aqueous ethanol extract, which led to lower flux than pure water. This observation was in accordance with Kim et al. (2002), who observed higher flux in water than in alcoholic solvents as they deduced that differences in molecular weight, dielectric constant, surface tension and viscosity of the solvents have some influence on permeate flux. According to Chong et al. (2014), water can extract the desired compounds with less impurity than alcoholic solvents. The formation of a polymerized layer on the surface of the membrane can also bring a drop in permeate flux, considering that this layer is the effluence of retentate concentration (Jiraratananon & Chanachai, 1996). Bolton et al. (2006) added that flux decline occurs in biotech process stream filtration as a consequence of the accumulation of foulants which creates a cake layer on membranes and causes complete blocking. The amounts of betaxanthin, betacyanin, phenolic, and antioxidants retained in the final aqueous ethanol extract were 206.62±1.37 mg·L^{-1}, 339.72±2.89 mg·L^{-1}, 972.72±47.49 mg·L^{-1}, and 745.97±25.45 mg·L^{-1} respectively.

Figures 5 and 6 typify changes of permeate flux values and processing time with the volume reduction ratio during the concentration process. As interpreted in Figure 5, the permeate flux of ethanol-water extract was considerably lower than that of water extract. Both methods, initially, showed a rapid decrease in permeate flux; yet flux declining rate became almost steady after approximately 12 minutes of processing time. Severe initial fouling is always exhibited in a fresh membrane when TMP is fixed for the whole operation (Miller et al., 2014). Comparatively, the rejection rate is elevated above the threshold flux up to a considerable accumulation of foulants on the membrane surface (Miller et al., 2014).

Along with elevated processing time, the volume reduction ratio improved (Figure 6). The resistance in transport manipulates the processing time, causing permeate flux decline (Miller et al., 2014). In our work, the interference of foulants with the membrane was found to be strong in water extract, which exhibits higher fouling resistance of membrane than aqueous ethanol and even membrane resistance to transport. As discussed earlier, leaching out of some hydrophilic compounds by pure water is more productive than the solvent action, which in turn might be responsible for membrane efficiency reduction. According to Al-Amoudi (2010), natural organic matter like phenolic (aromatic) groups have distinct effects on membrane fouling thereby inducing reversible and irreversible permeate flux decline. Membrane retention for betalains, phenolic, and antioxidant activity was assumed to be 99 % hence they were not detectable in the permeate of either extract. From the regression analysis and the correlational test between the targeted compounds (Table 2), it can be seen that colour compounds and total phenolic compounds detected in each extract are highly correlated (R^2 greater the 0.92 in all cases) with strong antiradical activity. Besides, the significant level within and between water and ethanol-water extracts for beetroot peel was less than 0.001 for each parameter, i.e, betalains, phenolic compounds, and antioxidant activity.

4 Conclusions

Our study showed that a nanofiltration membrane can successfully concentrate beetroot peel juice owing to the considerable amounts of betalains and polyphenols exhibiting desirable antiradical activity, which were in the final retentates as compared to the crude extracts. Since consumers' attention has shifted to safety concerns, the application of phytochemicals in functional food processing instead of synthetic food additives can be expected to thrive. For this reason, our experiment could be helpful to extend the application of membrane filtration processes for the recovery of these valuable compounds from agro-industrial wastes such as beetroot peel, al-

beit, some challenges, such as membrane fouling and its consequence of washing and maintaining costs, need to be taken into consideration.

Acknowledgements

This work was financially supported by the European Union and the European Social Fund (grant agreement no.EFOP-3.6.3-VEKOP-16-2017-00005), Tempus Public Foundation, under the Stipendium Hungaricum Scholarship Program. We appreciate the Doctoral School of Food Science, Hungarian University of Agriculture and Life Sciences for the support to accomplish the work as well.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 346–358

Characterization of Total Phenol and Flavonoid Contents, Colour, Functional Properties from Honey Samples with Different Floral Origins

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Received: 11 May 2020; Published online: 18 October 2021

Abstract

Honey has long been used as a food and has been reported to have potential health benefits. In this work, total phenol content, colour and antioxidant and hepatoprotective activities of honey samples of different floral origins from the State of Hidalgo, Mexico were explored using *in vitro* assays. Hepatoprotective activity was measured by inhibitition of β-glucuronidase; gastroprotective activity was determined by inhibition of urease; antioxidant activity was evaluated by 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methods. All the parameters showed significant differences ($p<0.05$) among honey samples. The total phenolic content ranged from 18.02 to 102.77 mg GAE 100 g^{-1}. The colour ranged from extra light amber to dark amber. Inhibition of β-glucuronidase ranged from 23.70% to 36.00%, while urease inhibition ranged between 7.64% and 63.80%. The antioxidant activity by ABTS was between 44.68 and 441.56 mg AAE 100 g^{-1}, and DPPH showed activities ranging from 35.64 to 573.06 mg AAE 100 g^{-1}. All honey samples contained bioactive compounds and displayed functional properties; therefore, the honeys from this region of Mexico offer attractive characteristics for their potential use in the food industry.

Keywords: Phenols; Flavonoids; Colour; β-glucuronidase; Urease

1 Introduction

Honey is a natural substance produced by honeybees, of the species *Apis mellifera*, from the nectar of flowers and plant secretions. This natural product has several food and clinical applications and contains approximately 200 different chemical components, including fructose, glucose, water, proteins, amino acids, enzymes, vitamins and minerals (Alvarez-Suarez et al., 2010; Rao et al., 2016). Honey is an important food product since it contains bioactive compounds derived from plants and the bees that produce it: honey is rich in phenolic acids and flavonoids, which have a wide range of biological functions and act as natural antioxidants (Kaskoniene et al., 2009). The colour of honey is one of its most distinctive characteristics, and is used as an in-

 10.7455/ijfs/10.2.2021.a6

dication of the presence of phenolic compounds and their derivatives and their potential antioxidant activities (Estevinho et al., 2008).
There are several reports that suggest that the composition and antioxidant capacity of different honeys depend mainly on the floral origin of the nectar collected by bees, other environmental factors and even processing. These factors demonstrate that there is a correlation between the origin and the identified components of the honey (Baharudin et al., 2017; Marghitas et al., 2009; Pimentel-Gonzalez et al., 2016). Recent research indicates that the main factor that gives diversity of colour, flavour and functional properties to honey is its phenolic composition (Hossen et al., 2017). These compounds demonstrate biological activity that have beneficial effects on health (Bueno-Costa et al., 2016; Estevinho et al., 2008). Phenolic compounds and flavonoids can give insight into the botanical origin of honey; therefore, the phenolic composition and antioxidant capacity of honey depend on the floral sources used to obtain this honey, as well as seasonal and environmental factors (Yao et al., 2003).
Current trends show that there are studies using natural compounds aimed at tumour and cancer inhibition; to reduce the need for chemical therapies and surgical interventions (Khan et al., 2017). Polyphenols in honey can chemically prevent different stages of cancer, including tumour formation and growth, through the inactivation of carcinogenic compounds, cell cycle arrest, apoptosis induction, the reduction of angiogenesis and antioxidation (Fresco et al., 2006).
An important function of the bioactive compounds found in honey is prevention of loss of biological membranes and enzymes functionality by elimination of free radicals that would otherwise induce the oxidation of lipids, proteins and DNA, in living systems (Mouhoubi-Tafinine et al., 2016; Niki, 2011). Various plant species have been studied for their capacity to inhibit certain enzymes to confer health benefits. The hepatoprotective effect is attributed to the inhibition of the β-glucuronidase enzyme (Karak et al., 2017), and gastroprotective activity is related to urease inhibition (Biglar et al., 2012). This study was conducted on honey samples with different floral origins by measuring the total phenol and flavonoid contents, the colour, and the functional properties such as hepatoprotective, gastroprotective and antioxidant activities.

2 Materials and Methods

2.1 Honey samples

A total of 24 samples of honey from different regions of the state of Hidalgo, Mexico were obtained directly from beekeepers and evaluated. The samples were collected in the period March-April 2018 from 24 different sites (Table 1). The collection was carried out at room temperature (20-25 ^{o}C) and the samples were later taken to the Institute of Agricultural Sciences of the Autonomous University of Hidalgo State in closed, dark containers. The honey samples were coded and stored in the dark at room temperature until they were analysed. The floral origin of the honey samples was determined according to the methodology developed by Sodre et al. (2007). For a quantitative analysis of pollen, 300 pollen grains were used for each sample in triplicate. Subsequently, visual comparisons of pollen were made using a BX 45 light microscope (Olympus, Japan).

2.2 Total phenolic content

The phenolic content in honey samples was determined using the Folin-Ciocalteu method (Singleton et al., 1999). Honey was diluted 1:10 with distilled water, and the resulting solution was filtered using Whatman No. 1 paper filter. The filtered solution (5 mL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu phenol reagent for 5 minutes, and then 2 mL of Na_2CO_3 solution (75 g L^{-1}) was added. Samples were left to stand for 2 h at room temperature in the dark. After this time, the absorbance was read at 760 nm in a Hitachi U-2000 UV/VIS spectrophotometer (Tokyo, Japan), using water as a blank. A standard curve was prepared with gallic acid in solutions of different concentrations (0-100 mg of gallic acid 100g^{-1}); therefore, results of the phenolic content in honey samples were expressed as mg of gallic acid equivalents (GAE) 100 g^{-1} of honey.

Table 1: Main characteristics of Mexican honey samples

Honey	Collection site	Floral origin
1SA	San Andrés	Cactus
2SF	San Felipe Orizatlán	Cítrus
3AC	Acaxochitlán	Gramineae
4ER	El Real	Conífers
5AT	Atotonilco	Juglans
6HU	Huejutla	Cítrus
7TE	Tehuetlán	Multifloral
8HU	Huautla	Multifloral
9AM	Tenango de Doria	Multifloral
10NR	Tepeji del Rio	Multifloral
11WR	Acatlán	Multifloral
12VZ	Zimapán	Multifloral
13ML	San Felipe Orizatlán	Cítrus
14AB	San Bartolo Tutotepec	Multifloral
15OS	Huejutla	Cítrus
16FA	Zimapán	Multifloral
17JG	Almoloya	Multifloral
18HA	Texcoco	Multifloral
19JJ	Almoloya	Multifloral
20ME	Tepeapulco	Multifloral
21MA	Apan	Multifloral
22VM	Apan	Multifloral
23OR	Zimapán	Multifloral
24MJ	Tlanalapa	Multifloral

2.3 Flavonoid content

The total flavonoid content was measured using the Dowd method as reported by Meda et al. (2005). Approximately 1 g of each honey sample was mixed with 10 mL of pure methanol, and the solution was stirred and then centrifuged at 15,000 rpm for 15 minutes. Then, 2 mL of the suspended liquid was mixed with 2 mL of 2% aluminium trichloride ($AlCl_3$) and left to stand in the dark for 20 minutes. The absorbance was measured at 415 nm in a UV/VIS spectrophotometer (Hitachi U-2000, Tokyo, Japan). A standard curve was prepared of quercetin in different concentrations (0-100 mg of quercetin $100g^{-1}$); therefore, the flavonoid content was expressed in mg of quercetin equivalents (EQ) 100 g^{-1} of honey.

2.4 Colour determination using the Pfund scale

Honey colour was determined by reading the absorbance of a 50% honey solution in water (w/v) read at 635 nm in a 1 mL cell, in a UV/VIS spectrophotometer (Hitachi U-2000) following the method previously reported by Ferreira et al. (2009). The honey samples were classified, after obtaining the readings, using the Pfund scale by converting the absorbance values, applying equation 1 and comparing the results with the honey colour chart.

$$mmPfund = -38.70 + (371.39 \times absorbance) \quad (1)$$

2.5 Antioxidant Activity

2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The antioxidant activity was determined by the discolouration of the ABTS radical (Sigma-Aldrich, Canada), as described by Re et al. (1999): 7 μM ABTS radical was reacted with 2.45 μM potassium persulfate ($K_2S_2O_8$) (Mallinckrodt Chemicals, USA) in a 1:1 ratio and stirred for 16 hours in the dark. When the radical was formed, it was diluted with ethanol (20%) until reaching an absorbance of 0.7 $\pm$ 0.01 at 734 nm. Once the radical was stabilized, 3 mL of this solution was placed in a test tube, and 100 μL of honey extract was added. The mixture was stirred rapidly, and the absorbance was measured after 10 minutes of reaction. The absorbance of the ethanol (20%)-diluted solution was used as the blank. A standard curve was prepared with ascorbic acid at different concentrations (0-100 mg of ascorbic acid $100g^{-1}$); therefore, the results were expressed in milligrams of ascorbic acid equivalents (AAE) 100 g^{-1} of honey.

2,2-diphenyl-1-picrylhydrazyl (DPPH)

To prepare the 0.2 mM DPPH solution, 7.8 mg of DPPH radical (Sigma-Aldrich, USA) was weighed and dissolved in 100 mL of methanol (80%), and the mixture was stirred in the dark for two hours for complete dissolution. Then, 2.5 mL of methanolic DPPH solution was added to a test tube, reacted with 0.5 mL of honey solution, and the mixture was left in the dark for 30 minutes and read at an absorbance of 515 nm. The antioxidant activity was determined using a standard curve with ascorbic acid. The results were expressed in milligrams AAE 100 g-1 of honey (Brand-williams et al., 1995).

2.6 β-Glucuronidase inhibition

This analysis was carried out following the methodology described by Karak et al. (2017) with a few modifications. A dilution of the honey with water was made at 10%, from which 340 μL was taken and mixed with 100 μL of a β-glucuronidase solution (986.4 units mL^{-1} in pH 7 phosphate buffer) (Sigma-Aldrich, USA), and the mixture was pre-incubated at 37 ^{o}C for 15 minutes. Subsequently, 60 μL of p-nitrophenyl-β-D-glucuronide (3.15 mg mL^{-1} in pH 7 phosphate buffer) (Sigma-Aldrich, USA) was added and incubated for 50 minutes at 37^{o}C. The absorbance was measured at 405 nm in a spectrophotometer. A control test was carried out with D-saccharic acid 1,4-lactone following the same steps as analysis of honey samples, and the percentage inhibition was obtained using equation 2:

$$\%Inhibition = \left[\frac{absorbance\ of\ Control - absorbance\ of\ Sample}{absorbance\ of\ Control}\right] \times 100 \quad (2)$$

2.7 Urease inhibition

The urease inhibition test was performed as described by Biglar et al. (2012) with a few modifications. For each honey sample, 100 μL of urease solution (Jack bean urease 5 U mL^{-1}) and 100 μL of inhibitor (a 20% dilution of honey in a 50% ethanol-water solution) were mixed and incubated at 37^{o}C for 10 minutes. Then, a solution of urea prepared in phosphate buffer (pH 6.8) was added and incubated at 37^{o}C for 30 minutes. The urease activity was determined by measuring the production of ammonia using the indophenol method. The reaction was stopped with 600 μL of 4% sulfuric acid. Subsequently, 100 μL of solution A (5.0 g phenol and 25 mg of sodium nitroprusside) and 100 μL of solution B (2.5 g sodium hydroxide and 4.2 mL of sodium hypochlorite in 500 mL of distilled water) were added, the samples were left in darkness for 30 minutes at 37^{o}C, and the absorbance was measured at 625 nm.

2.8 Statistical analysis

For the statistical analysis, a completely randomized design was used. The results were interpreted with analysis of variance, and when significant differences were observed ($p<0.05$) between the different honeys, the mean comparison was performed by the Tukey method using the NCSS 2007 software (USA).

Table 2: Bioactive compounds and honey color

Honey	Total phenols	Flavonoids	Pfund Scale (mm)	Color
1SA	$25.65\pm0.0.49^{c}$	3.95 ± 0.20^{ijk}	105.3	Amber
2SF	18.02 ± 0.49^{a}	4.97 ± 0.52^{l}	71.8	Light amber
3AC	92.32 ± 1.29^{o}	7.63 ± 0.51^{n}	93.6	Amber
4ER	93.16 ± 0.98^{o}	7.01 ± 0.52^{mn}	135.8	Dark amber
5AT	102.49 ± 0.98^{p}	1.28 ± 0.26^{a}	88.7	Amber
6HU	93.73 ± 0.85^{o}	2.02 ± 0.34^{abc}	69.2	Light amber
7TE	102.77 ± 1.29^{p}	3.49 ± 0.52^{ghij}	138.1	Dark amber
8HU	51.10 ± 0.49^{k}	1.79 ± 0.20^{ab}	65.9	Light amber
9AM	43.10 ± 0.97^{ij}	2.25 ± 0.10^{bcd}	42.9	Extra light amber
10NR	49.44 ± 0.33^{k}	6.33 ± 0.07^{m}	110.7	Amber
11WR	38.46 ± 0.33^{fg}	2.37 ± 0.14^{bcde}	59.6	Light amber
12VZ	72.95 ± 0.72^{n}	5.42 ± 0.16^{l}	125.6	Dark amber
13ML	57.30 ± 0.41^{l}	2.77 ± 0.07^{cdefg}	26.2	White
14AB	44.26 ± 0.23^{j}	3.24 ± 0.23^{fghi}	81.8	Light amber
15OS	64.08 ± 0.23^{m}	5.37 ± 0.09^{l}	64.7	Light amber
16FA	71.58 ± 0.22^{n}	3.56 ± 0.21^{hij}	242.3	Dark amber
17JG	35.65 ± 0.38^{e}	3.19 ± 0.07^{efgh}	140.7	Dark amber
18HA	22.34 ± 0.54^{b}	2.11 ± 0.05^{abc}	40.3	Extra light amber
19JJ	33.02 ± 0.62^{d}	3.04 ± 0.09^{defgh}	233.2	Dark amber
20ME	41.10 ± 0.17^{hi}	4.51 ± 0.05^{kl}	122.6	Dark amber
21MA	36.68 ± 0.711^{ef}	2.94 ± 0.07^{cdefgh}	111.5	Amber
22VM	24.33 ± 0.17^{bc}	2.30 ± 0.04^{bcd}	86.1	Amber
23OR	39.15 ± 0.35^{gh}	4.35 ± 0.07^{jk}	175.1	Dark amber
24MJ	41.50 ± 0.49^{i}	2.44 ± 0.29^{bcdef}	100.2	Amber

The results are expressed in means ± standard deviation. Different letters indicate statistically significant differences between the analyzed samples ($p<0.05$). Total phenols are expressed in mg GAE 100 g^{-1}. Flavonoids are expressed in mg QE 100 g^{-1}.

3 Results and Discussion

3.1 Total phenolic content

Significant differences ($p<0.05$) were found between the different honey samples. The samples with the highest phenolic contents were 5AT and 7TE (of Juglan and multifloral origin, respectively), of 102.49±0.98 and 102.77±1.29 mg EAG 100 g^{-1}, respectively (Table 2). Various analyses of the total phenolic content in different honey samples have been carried out worldwide. Our results are similar to those reported by Ruiz-Navajas et al. (2011) who found values ranging between 51.32 and 134.02 mg EAG 100 g^{-1} in honey from south eastern Mexico with warm and tropical climates. The difference in the content of total phenols among the samples analysed is due to the diverse geographical origin, quality and the floral source from which they were extracted (Al-Farsi et al., 2018).

3.2 Flavonoid content

The results for flavonoid revealed that the honey samples with the highest content were 3AC and 4ER both with more than 7 mg EQ 100 g^{-1} (of Gramineae and Conifers origin, respectively). It should be mentioned that none of the samples had values less than 1 mg EQ 100 g^{-1} or more

than 10 mg EQ 100 g^{-1}. The results obtained in this study are comparable to those presented by Meda et al. (2005) in Burkina Faso honey samples with an average flavonoid content of 2.57 EQ 100 g^{-1}, similar to the samples with the lowest content in this study. A study conducted in Brazil by Bueno-Costa et al. (2016) in which they also determined flavonoid content obtained a range of results between 2.98 and 10.46 mg EQ 100 g^{-1}, very similar to the results presented here; however, the upper concentration limit in this Brazilian honey was slightly higher than that obtained in our samples. Unlike the studies mentioned above, Boussaid et al. (2018) reported very low content compared to the other results. Flavonoids are phenolic compounds and are present as various types of structures in honey; among them, the one with the highest content according to Alvarez-Suarez et al. (2012) is quercetin, while Quintero-Lira et al. (2017) reported the major flavonoid was rutin. The concentrations of these compounds are determined by the botanical and geographical origins of the samples, as well as the climate and environmental conditions such as humidity, temperature and type of soil where the plants grew (Al-Farsi et al., 2018). Flavonoid content and pigments derived from these compounds are also directly related to the colour of honey and its different shades (Alvarez-Suarez et al., 2010). Also, phenolic compounds and flavonoids can be used as an indicator of antioxidant activity since they have sequestering and free radical reduction capacity (da Silva et al., 2016).

3.3 Colour evaluation

The results showed that the darkest honey samples classified as "dark amber" were 4ER, 7TE, 12VZ, 17JE, 19JJ, 20ME, 23OR and 16FA (mainly of Conifers and Multifloral origin), with the highest level on the scale reaching 242.3 mm. Most samples had dark tones and fell under the "amber" classification or higher (Table 2). Compared to the results of honey colour in other studies, the honey colour in the samples analysed here showed large variations, with very high to low values on the Pfund scale that depended on when the colour analysis was performed. An analysis performed in Brazil by Pontis et al. (2014) identified honey samples classified as very clear "extra light amber"; however, the study also had samples within the "dark amber" range but with values lower than those found in our samples. Many authors agree that honey colour is directly related to the presence of phenolic compounds and their antioxidant capacity (Bertoncelj et al., 2007; Ferreira et al., 2009) due to the high correlation values in the tests performed. Therefore, the colour, smell and taste of honey are related to the phenolic content and are indicators and characteristics of floral origin (Bertoncelj et al., 2007; Kaskoniene et al., 2009).

3.4 Antioxidant activity

The antioxidant activity in the honey samples was determined by the ABTS and DPPH methods, obtaining results in equivalent milligrams of ascorbic acid (Figure 1). In both cases, significant differences ($p < 0.05$) were found between the analysed samples. In the ABTS test, the highest content were found in the following honey sample: 13ML (405.10±30.83 mg EAA 100 g^{-1}) of a citrus floral origin, and 24MJ (410.31±3.12 mg EAA 100 g^{-1}) and 17JG (441.56±6.25 mg EAA 100 g^{-1}), both of multifloral origin.
In DPPH test the highest content were found in the 12VZ honey sample, of multifloral origin (573.06±10.20 mg EAA 100 g^{-1}), showing significant differences with respect to the rest of the honey samples. Several studies have presented information on the antioxidant activity of honey. El-haskoury et al. (2018) performed tests on honey from Morocco and found average results of 60.94 mg EAA 100 g^{-1}, which is lower than the results obtained in this study; however, they obtained superior antioxidant activity in the ABTS test in relation to DPPH. A Portuguese honey was analysed by Ferreira et al. (2009) using DPPH test, and reported values between 106.67 and 168.94 mg EAA 100 g^{-1} with few samples being characterised as light honey and more as dark honey; a similar trend was also found in a study reported by Estevinho et al. (2008) with similar samples from Portugal. The results of antioxidant activity are directly related to the total phenol content in honey, as determ-

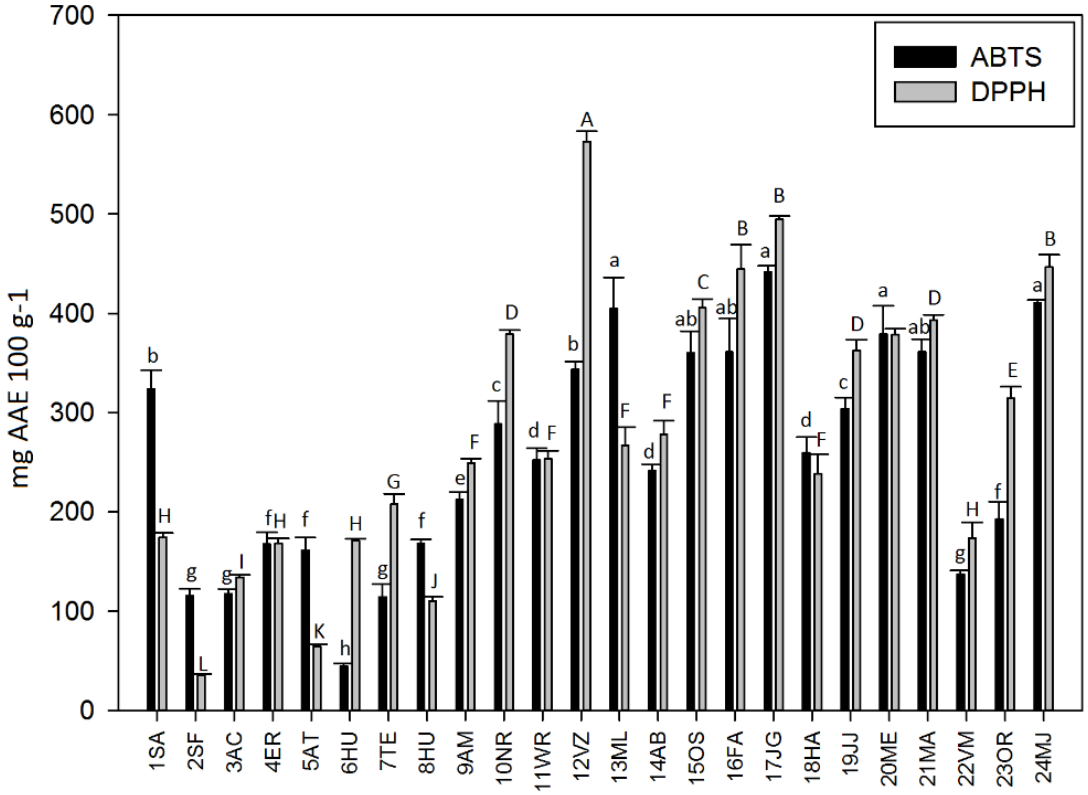

Figure 1: Antioxidant activity of Mexican honeys. The results are expressed in means ± standard deviation. Lowercase letters indicate differences between treatments for the ABTS test ($p<0.05$). Capital letters indicate differences between treatments for the DPPH test ($p<0.05$).

ined by Bertoncelj et al. (2007), who found high correlation factors between both tests, as with the colour intensity.

Based on the determination of the ABTS and DPPH tests performed by Bueno-Costa et al. (2016), it was found that the most appropriate test for antioxidant determination is ABTS since it shows high correlations with the content of phytochemical analytes such as phenols, flavonoids, phenolic acids and carotenoids. The literature mentions that the mechanisms of antioxidant activity in honey include the ability to sequester free radicals, the chelation of metal ions and the oxidizing-reducing capacity of phenolic compounds due to the presence of hydroxyl groups attached to aromatic rings (Bastos & Sampaio, 2013). It is also known that the antioxidant capacity and the colour of honey are affected by the floral source, differences in geographical origin, humidity, soil type and post-harvest conditions (Al-Farsi et al., 2018).

3.5 β-Glucuronidase inhibition

The results shown in figure 2 were obtained from the β-glucuronidase inhibition test. All the honey samples have positive inhibition values higher than 20%. The following samples had the highest percentages: 8HU (33.21±1.96%), 1SA (33.64±1.83%) and 2SF (36.00±0.47%) (mainly

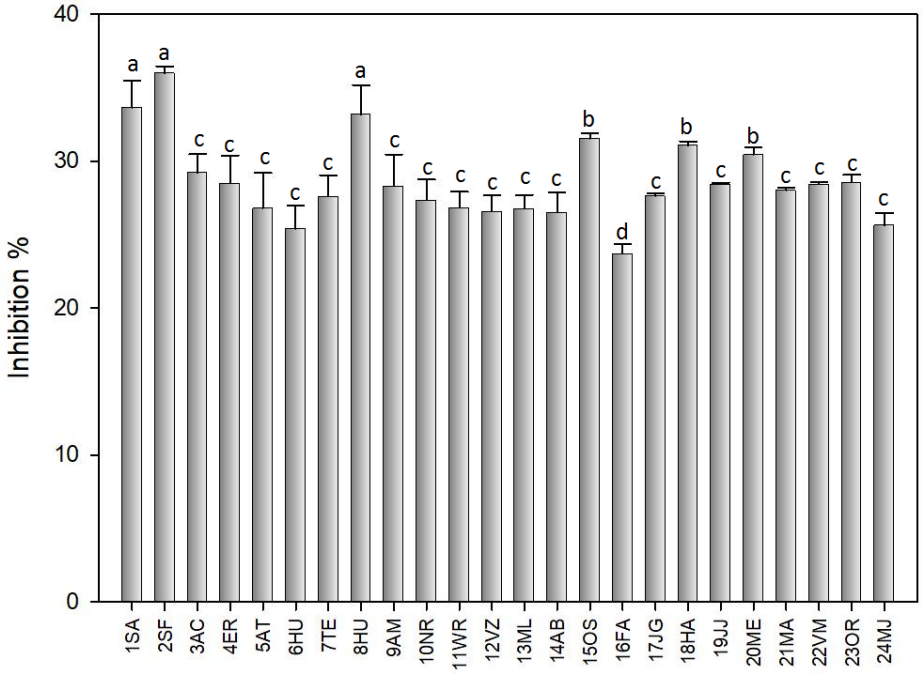

Figure 2: Inhibition percent of β-glucuronidase in Mexican honeys. The results are expressed in means ± standard deviation. Different letters indicate differences between treatments ($p<0.05$).

of cactus, citrus and multifloral origin). The results are similar between different samples that range between 25% and 30%, with some honey samples exhibiting higher peaks. The analyses that have been performed on the inhibition of β-glucuronidase by bioactive compounds are limited. Han et al. (2005) evaluated the hepatoprotective effect of lactic acid bacteria with an inhibition range of 17% to 66% in an in vivo study with rats. *Swertia sp.* plant extracts have been studied as a β-glucuronidase inhibitor; Karak et al. (2017) found an average inhibition percentage of 30.89% from a concentration of 500 μg mL^{-1} of the *S. bimaculate*.

β-Glucuronidase is used as a marker of various diseases because it is a member of the lysosomal glucosidase family, which catalyses the degradation of glycosaminoglycans of normal and cancerous tissue cell membranes (Baharudin et al., 2017). Glucuronic acid conjugates with toxins and diminishes their potency by eliminating them from the body later; this process is limited by high levels of β-glucuronidase (Karak et al., 2017). Liver damage causes an increase in the level of β-glucuronidase in the blood, and liver cancer may be related to this enzyme. Inhibitors of this indicator reduce the carcinogenic potential of toxic compounds. Therefore, inhibitors of β-glucuronidase are suggested as potential hepatoprotective agents (Shim et al., 2000). Some extracts and compounds from plants with hepatoprotective activities are known for their ability to inhibit this type of enzyme, among which are flavonoids (Joshi & Priya, 2007; Kim et al., 1994).

3.6 Urease inhibition

All honey samples exhibited urease inhibition, exceeding 20% in most samples (Figure 3). A clear variation was seen among the samples analysed, showing significant differences ($p<0.05$). Sample 10NR (63.80±0.11%) had the greatest inhibition (of multifloral origin), followed by 15OS (58.73±0.62%) and 4ER (59.24±3.64%). Cur-

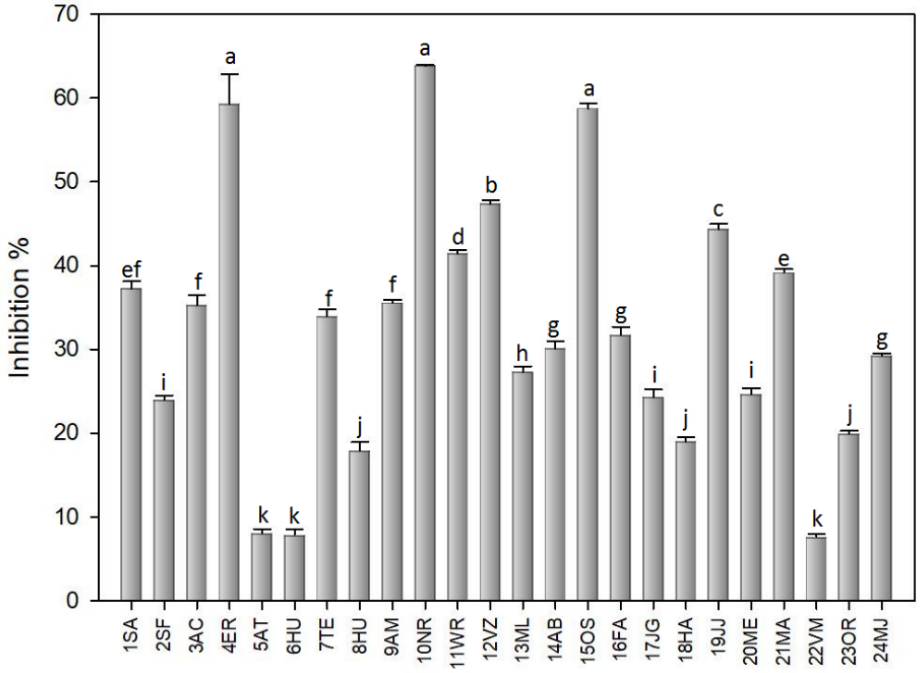

Figure 3: Inhibition percent of urease in Mexican honeys. The results are expressed in means ± standard deviation. Different letters indicate differences between treatments ($p<0.05$).

rent research on urease inhibition is focused on gastric protection and ulcer reduction; however, most studies are based on plant extracts and isolates. On the one hand, Lateef et al. (2012) analysed *Glycyrrhiza glabra* roots and found an approximate average urease inhibition value of 61% with a methanolic extraction and 40% in an extraction with chloroform. These results are within the range of those found in honey. On the other hand, Lin et al. (2005) evaluated the enzymatic inhibition of urease in oregano, and found an approximate value of 40%, which also coincides with results observed here. In the same study, the activity of cranberry was also evaluated, resulting in 9% inhibition.

The study on the inhibition of urease is aimed at determining the presence of *Helicobacter pylori* since this gram-positive bacterium causes chronic gastritis and intervenes directly in the formation of peptic ulcers, carcinoma and gastric lymphoma (Dunn et al., 1997). The gastric mucosa is affected by *H. pylori* since it resists the acidic environment producing urease, which hydrolyses urea-forming bicarbonate and ammonium (Hu & Mobley, 1990; Kuhns et al., 2013). The methanolic extracts of vegetable sources can be considered a natural source for the inhibition of urease, as mentioned by Lateef et al. (2012), so it is possible that these compounds can be transferred to honey, generating a similar effect. The difference in the enzymatic inhibitory activity is mainly due to the type of phenolic compounds in the inhibitor, as verified by Lin et al. (2005), who demonstrated that hydrophobic phenolic compounds such as those present in oregano are superior in urease inhibition than those that are soluble in water, such as those found in blueberries. In honey, phenolic derivatives such as flavonoids behave both ways since they are liposoluble and water soluble (Martínez-Flórez et al., 2002), this is reflected in the varied behaviour of urease inhibition.

4 Conclusion

The honey samples analysed in this study were a good source of phenolic compounds, especially the honey of walnut and multifloral origin, and had good antioxidant activity, as in the case of honeys 17JG and 12VZ (multifloral). β-glucuronidase inhibition values indicated the presence of possible hepatoprotective activity, and some honey samples showed high values of possible gastric protective activity as exhibited by the urease inhibition test *in vitro*. Differences between the honey samples in each test demonstrated that the characteristics of each honey sample were unique, probably due to the compounds they contain derived from different plant origins. This study provides an insight to potential area of opportunity for future research related to the possible beneficial effects of honeys in the region studied, which the native population uses even in traditional medicine.

Acknowledgements

This paper is partially financed by National Funds provided by FCT- Foundation for Science and Technology throughout project UID/SOC/04020/2020.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 359–370

Tetracycline Resistance in Enterococci and *Escherichia coli* Isolated from Fresh Produce and Why it Matters

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Received: 25 June 2020; Published online: 18 Octover 2021

Abstract

The contamination of fresh produce with antibiotic-resistant bacteria is of particular concern as they are often eaten raw and can be a source for foodborne diseases. Tetracyclines have been largely used in humans, animals and plants which might have accelerated microbial resistance to them. Enterococci and *Escherichia coli* can be used as indicators to monitor contamination of the fresh produce with tetracycline-resistant bacteria. The investigation related to this issue is very scarce in Oman.This study aimed at identifying tetracycline-resistant enterococci and *E. coli* in fresh produce at the market place. Thirty-one enterococci and ten *E. coli* were isolated from local (Oman) and imported fruits and vegetables (N= 105). Using the standard Kirby-Bauer disc diffusion method, resistance to tetracycline was found in 6 (19 %) enterococci, isolated from cucumber, lettuce and radish, and 5 (50 %) *E. coli*, obtained from cabbage, lettuce and radish. Genetic analysis revealed the presence of tetracycline resistance genes, *tet*(A) and *tet*(K), in *E. coli* and *tet*(K), *tet*(L) and *tet*(M) in enterococci, including *Enterococcus sulfureus, Enterococcus mundtii, Enterococcus casseliflavus* and *Enterococcus faecalis*. The integron integrase *IntI 1* gene, which is known to facilitate the dissemination of antibiotic resistance genes among bacteria, was detected in 2 isolates of *E. coli* . These results demonstrated the capability of fresh produce to act as a potential source for disseminating tetracycline or possibly other antibiotic-resistant bacteria through the food chain. Thus, control strategies are needed to reduce exposure of the public to such microorganisms.

Keywords: Antibiotic resistance; *E. coli* ; Enterococcus; Integron; Vegetables; Tetracycline

1 Introduction

Tetracyclines (TEs) are amongst the most important antibiotic groups used in medicine (Hernandez et al., 2003). They exhibit a broad spectrum of activity against Gram-positive and Gram-negative bacteria, chlamydiae, rickettsiae, nematodes, mycoplasmas and protozoans. They inhibit protein synthesis in bacterial cells (Hernandez et al., 2003; Markley & Wencewicz, 2018) by preventing attachment of aminoacyl-tRNA to the acceptor (A) site of the ribosome. However, this attachment is reversible rendering TEs to be bacteriostatic (Kohanski et al., 2010; Roberts, 2005).TEs are used to treat infections in humans and animals, preserve harvested fruits and veg-

 10.7455/ijfs/10.2.2021.a7

Nomenclature

ARB	antibiotic-resistant bacteria	HGT	horizontal gene transfer
ARG	antibiotic resistance genes	TE(s)	tetracycline(s)

etables, kill insect pests, and as growth promoters for animals (Hernandez et al., 2003). As resistance to TEs occurred shortly after their first clinical use in the 1940s, new semi-synthetic TEs were generated resulting in the evolution of the TEs scaffold and expansion of their use (Markley & Wencewicz, 2018).

Genetic acquisition of *tet* genes is the major cause of resistance to TEs. The *tet* efflux genes code for membrane-associated proteins that export TE from the cell. Examples include *tet*(A) , *tet*(B), *tet*(C), *tet*(D), *tet*(K), *tet*(L) and *tet*A(P). Ribosomal protection proteins are cytoplasmic proteins that protect bacterial ribosomes from the effect of TEs. The *tet*(M), *tet*(O) and *Otr*A are among these proteins. TEs can also be altered enzymatically, resulting in their inactivation by the cytoplasmic protein product of the *tet*(X) gene (Roberts, 2005). Reduced permeability through alteration or reduced expression of porins or morphological changes and mutation in the ribosome can also lead to tetracycline (TE) resistance (Markley & Wencewicz, 2018).

Horizontal gene transfer (HGT) among bacteria plays an important role in the dissemination of multidrug resistance, especially when antibiotic resistance genes (ARG) are located on mobile genetic elements such as plasmids and transposons. Transposons contain special gene sequences known as integrons. The gene sequence of an integron serves as a site-specific recombination system allowing it to capture or excise novel genetic elements known as gene cassettes that code for various ARG (Roe & Pillai, 2003). The integrase enzyme of the integron can insert gene cassettes at a specific site known as the attI site and then express them (Gillings et al., 2009). Romaine lettuce, alfalfa sprouts and Savoy spinach in Canada were demonstrated to harbor antibiotic-resistant bacteria (ARB) that carried integron DNA (Bezanson et al., 2008). Use of the class 1 integrase-encoding gene (*IntI 1* gene) has been proposed as a generic marker for anthropogenic contaminants because of its common association with antibiotic resistance in pathogenic and commensal bacteria (Jones-Dias et al., 2016).

Enterococcus bacteria have emerged as potential pathogens due to their multiple drug resistance as a result of their capabilities to acquire antibiotic resistance through mutation and HGT (Johnston & Jaykus, 2004). Therefore, antibiotic resistance of enterococci should be monitored to identify foods that can impose a real risk to people (Pesavento et al., 2014). *E. faecalis, E. faecium E. hirea* (Ben Said et al., 2016) and *E. coli* that were isolated from different produce types were found to be resistant to TE (Ben Said et al., 2016; Campos et al., 2013). Many outbreaks have been linked to specific strains of *E. coli* such as *E. coli* O157:H7 in ready-to-eat salads in 2013 (CDC, 2014a) and *E. coli* O121 in raw clover sprouts in 2014 (CDC, 2014b). Treating infections becomes more complicated when the causative agent of an outbreak is resistant to antibiotics as well (O'Flahertya et al., 2019).

The prevalence of antibiotic-resistant bacteria was found to vary among countries due to various factors such as the type of antibiotics used (Chewapreecha, 2014). In Oman, TE-resistant *E. coli* was previously isolated from the oviductal fluid of green turtles (Al-Bahry et al., 2012) and chicken (Al-Bahry et al., 2012) but TE-resistance has not been previously investigated in produce-associated bacteria. The aim of this study was to identify phenotypic and genotypic TE resistance in enterococci and *E. coli* that we previously isolated from different types of locally pro-

duced and imported fresh produce. The *E. coli* isolates were also screened for the presence of the *IntI 1* gene. The results of this study can help in understanding one of the routes of human exposure to TE-resistant bacteria through consumption of fresh produce. This may be used to develop control strategies to reduce exposure of the public to ARB through food consumption.

2 Materials and Methods

2.1 Sample collection and bacterial isolation and identification

Isolation and identification of enterococci and *E. coli* from different local (39 samples) and imported (66 samples) fresh fruits and vegetables were performed as previously reported (Al-Kharousi et al., 2016). About 93 enterococci isolates and 15 *E. coli* isolates were collected. To avoid analyzing duplicate or clonal bacteria, one typical colony (details can be found by searching the manufacturer's website; http://www.oxoid.com/UK/blue/index.asp?c=UK&lang=EN) of the bacterial isolate from each species was selected from each sample (31 enterococci and 10 *E. coli* isolates) for subsequent analysis.

2.2 Phenotypic screening of enterococci and *E. coli* for resistance to TE

The standard Kirby-Bauer disc diffusion method described by the Clinical and Laboratory Standards Institute (CLSI, 2015) was used to test the resistance of enterococci and *E. coli* to TE. Mueller-Hinton agar and TE discs were supplied by Oxoid, UK. *E. coli* ATCC 25922 (TE-susceptible), *Enterococcus faecalis* ATCC 51299 (TE-susceptible) and *E. faecalis* ATCC 29212 (TE-resistant) were used as reference control strains. Enterococci were considered to be resistant if the inhibition zone was $\leq$ 14 mm while *E. coli* was considered resistant if the inhibition zone was $\leq$ 11 mm (CLSI, 2015).

2.3 Identification of TE resistance genes

According to the method developed by Ng et al. (2001), multiplex PCR was used to screen for the presence of 14 *tet* genes in the isolates that showed phenotypic resistance to TE. The sequences of primers are presented in Table 1. In brief, the PCR reaction mix contained template DNA at a concentration of 10 ng/μl and sterile Milli-Q water was added to make a total volume of 25 μl. Primers were supplied by Macrogen, South Korea for *tet* genes as follows: group I; *tet*(B) (0.25 μM), *tet*(C) (0.25 μM) and *tet*(D) (2.0 μM), group II; *tet(A)* (1.0 μM), *tet*(E) (1.0 μM) and *tet*(G) (1.0 μM), group III; *tet*(K) (1.25 μM), *tet*(L) (1.0 μM), *tet*(M) (0.5 μM), *tet*(O) (1.25 μM) and *tet*(S) (0.5 μM), and group IV; *tet*A(P) (1.25 μM), *tet*(Q) (1.25 μM) and *tet*(X) (1.25 μM). PCR beads (puReTaq Ready-To-Go PCR beads) were supplied by GE Healthcare, UK. The thermal profile (Veriti 96-well Thermal cycler, Applied Biosystems, Singapore) for the PCR reaction was as follows: stage 1; denaturation at 94^{o}C for 5 min, stage 2; denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 1 min, extension at 72^{o}C for 1.5 min (35 cycles), stage 3; final extension at 72^{o}C for 10 min and then kept at 4^{o}C.

Enterococci and *E. coli* isolates were screened for the *IntI 1* gene according to the previously reported methods (Gaze et al., 2005; Rosser & Young, 1999). The PCR mixtures were as previously described for the type of primer pairs (described in Table 1). The thermal profile for the PCR reaction of the *IntI 1* gene (1 pmole/μl of each primer) was as follows: stage 1; denaturation at 96^{o}C for 5 min, 55 for 1 min, 70^{o}C for 3 min (one cycle), stage 2; denaturation at 96^{o}C for 15 sec, annealing at 55^{o}C for 30 sec, extension at 70^{o}C for 3 min (24 cycles), stage 3; final extension at 70^{o}C for 5 min and then kept at 4^{o}C. Five-microliter aliquots of PCR products of *tet* and *IntI 1* genes were analyzed by gel electrophoresis with 2 % agarose (Thermo Scientific, TopVision, USA) and 0.5 μg/ml ethidium bromide (Sigma-Aldrich, USA). Gels were visualized by UV using GelDoc (GeneFlash, Syngene, USA). A 100-bp ladder (Fermetas,

O'RangeRuler, Thermo Fisher Scientific) was run on each gel as a molecular size marker. The PCR products of *tet* and *IntI 1* genes were sequenced abroad (Macrogen, South Korea). DNA sequences were aligned and analyzed through the ChromasPro program (Version 1.41, Technelysium Pty Ltd) and compared online with those found at the NCBI using the BLAST program. The DNA sequences were submitted to the European Nucleotide Archive (ENA) to be assigned accession numbers.

2.4 Statistical analysis

Statistical tests were performed using JMP® SAS 14.3, USA to identify significant differences that were considered as $P<0.05$. Chi-square analysis was used to test if TE-resistance differed significantly according to the species and the source of enterococci (local or imported) and the source of *E. coli* (local or imported).

3 Results and Discussion

3.1 Prevalence of enterococci and *E. coli*

Enterococci were recovered from 31 out of 105 samples (30 %: 12 local out of 39; 31 % and 19 imported out of 66; 29 %), including cabbage, cucumber, dates, mango, lettuce, papaya, radish, tomato and watermelon but not banana, pomegranate, carrot or capsicum. *E. coli* was recovered from 10 samples (10 %: 6 local out of 39; 15 % and 4 imported out of 66; 6 %) of cabbage, lettuce and radish. More detailed information on the prevalence and identity of enterococci and the prevalence of *E. coli* can be found in our previous publication (Al-Kharousi et al., 2016).

3.2 TE-resistance of enterococci

Nineteen percent (6 isolates out of 31, from imported samples only) of enterococci were resistant to TE (Fig. 1). These were *Enterococcus casseliflavus, E. faecalis, Enterococcus mundtii* and *Enterococcus sulfureus* (Table 2), isolated from cucumber, lettuce and radish. *E. casseliflavus* had *tet*(L) and *tet*(M). The latter was also found in *E. faecalis*. *E. mundtii* possessed *tet*(L) while *E. sulfureus* harbored *tet*(K) (Table 3).

Enterococci are involved in food intoxication and in spreading antibiotic resistance through the food chain (Abriouel et al., 2008), and they are a leading cause of nosocomial infections (Abriouel et al., 2008; Tian et al., 2019). TE-resistant enterococci were previously isolated from different produce types (Ben Said et al., 2016; Campos et al., 2013). Twenty-nine percent of *Enterococcus faecium* isolated from fresh produce grown in the United States of America were found to be resistant to TE while all *E. faecalis* isolates were susceptible to TE (Johnston & Jaykus, 2004). In this study, resistance to TE was exhibited by six enterococci (19 %) isolated from the imported samples only. These were *E. casseliflavus, E. faecalis, E. mundtii* and *E. sulfureus*, isolated from cucumber (source: United Arab Emirates; UAE), lettuce (source: Iran and Jordan) and radish (source: China). *tet*(K) was harbored by *E. sulfureus* and *tet*(L) by *E. casseliflavus* and *E. mundtii*, whilst *tet*(M) was found in *E. casseliflavus* and *E. faecalis*. The *tet*(K) and *tet*(L) genes code for efflux proteins while *tet*(M) codes for ribosomal-protection proteins (Roberts, 2005).

Statistical tests showed that the type of species of enterococci had no significant effect on their resistance to TE; χ^2 (7, N= 31) = 3.00, P= 0.8853. However, the source (local or imported) of enterococci significantly affected the number of TE-resistant enterococci χ^2 (1, N= 31) = 4.699, P= 0.0302. Actually, all enterococci originated from local samples were susceptible to TE. This may indicate the absence or low levels of TE-resistant enterococci in locally produced fresh produce as compared to those imported from different countries. In particular, TE-resistance was found in enterococci isolated from imported produce from Iran, Jordan, China and the UAE. Information regarding TE-resistance of enterococci associated with fresh produce in these mentioned countries is very limited. The resistance of *Enterococcus* sp. to most antimicrobials was reported to be more prevalent in China than in European or other Asian countries (Liu et al., 2013). The re-

Table 1: Primers used for multiplex PCR for screening tetracycline resistance genes (Ng et al., 2001) and the *IntI 1* gene (Gaze et al., 2005; Rosser & Young, 1999)

Targeted gene	Primers sequence 5'-3'	Amplicon size
Tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
Tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
Tet(C)	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418
Tet(D)	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787
Tet(E)	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	278
Tet(G)	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	468
Tet(K)	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	169
Tet(L)	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	267
Tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406
Tet(O)	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	515
Tet(S)	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667
TetA(P)	CTT GGA TTG CGG AAG AAG AG ATA TGC CCA TTT AAC CAC GC	676
Tet(Q)	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	904
Tet(X)	CAA TAA TTG GTG GTG GAC CC TTC TTA CCT TGG ACA TCC CG	468
IntI 1	ATCATCGTCGTAGAGACGTCGG GTCAAGGTTCTGGACCAGTTGC	892

Table 2: Levels of antibiotic resistance in enterococci (n= 31) and *Escherichia coli* (n= 10) isolated from fresh fruits and vegetablese

Bacteria species	Total #	Susceptible		Resistant	
		Local	Imported	Local	Imported
Enterococcus casseliflavus	13	1	10	0	2
Enterococcus faecalis	7	5	0	0	2
Enterococcus faecium	2	1	1	0	0
Enterococcus gilvus	1	0	1	0	0
Enterococcus hirae	1	1	0	0	0
Enterococcus mundtii	4	2	1	0	1
Enterococcus raffinosus	1	1	0	0	0
Enterococcus sulfureus	2	1	0	0	1
Escherichia coli	10	4	1	2	3

Table 3: Levels of antibiotic resistance in enterococci (n= 31) and *Escherichia coli* (n= 10) isolated from fresh fruits and vegetables

Bacteria No.	Identity (PCR)	Source	Gene	Accession #
1	*E. coli*	Cabbage 1*, Oman	*tet*(A)	LT548573
2	*E. coli*	Cabbage 2, Oman	*tet*(A)	LT548574
4	*E. coli*	Lettuce 2, Jordan	*tet*(A)	LT548575
7	*E. coli*	Radish 1, China	*tet*(A)	LT548576
15	*E. coli*	Radish 2, China	*tet*(A)	LT548579
4	*E. coli*	Lettuce 2, Jordan	*tet*(K)	-
27	*E. sulfureus*	Lettuce 2, Jordan	*tet*(K)	-
35	*E. casseliflavus*	Radish 1, China	*tet*(L)	LT548584
36	*E. mundtii*	Radish 2, China	*tet*(L)	LT548585
25	*E. casseliflavus*	Cucumber 3, UAE	*tet*(M)	LT548581
26	*E. faecalis*	Lettuce 1, Jordan	*tet*(M)	LT548582
29	*E. faecalis*	Lettuce 1, Iran	*tet*(M)	LT548583
1	*E. coli*	Cabbage 1, Oman	*IntI 1*	LT548588
7	*E. coli*	Radish 1, China	*IntI 1*	LT548589

*: Sample number

-: Not available

sistance rate of enterococci isolated from clinical samples, in China, to TE was found to be 49.3 %, and *tet*(M) was detected in all TE-resistant isolates, including 22 *E. faecalis* and 14 *E. faecium* . Likewise, high resistance rates to TE (93 %) were reported in enterococci (*E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum* and *E. casseliflavus*) isolated from chickens and pigs in China (Liu et al., 2013). Enterococci isolated from clinical samples in Iran were also found to be resistant to various antibiotics including TE (Asadollahi et al., 2018). Very limited information is available regarding antibiotic resistance of enterococci (clinical or environmental) in the UAE and Jordan.
Potential sources of contamination of fresh produce with ARB include animals, feces, soil, irrigation water, insecticides, fungicides, inadequately composted manure and human (Olaimat & Holley, 2012). In China, residues of TE and various *tet* genes, including *tet(A)* , *tet*(B), *tet*(C), *tet*(E), *tet*(M), *tet*(O), *tet*(S) and *tet*(X), were found in the irrigation wastewater and soil. Longterm irrigation with wastewater increased the abundance of *tet* genes in soil (Pan & Chu, 2018). Thus, the irrigation of vegetables with wastewater in China (Khan et al., 2008) can be a route for transferring ARB to vegetables. Also, the introduced *tet* genes into soils, if present on the transferable plasmids, may persist for a long time due to HGT and may be transferred to other bacteria or human pathogens (Pan & Chu, 2018). Likewise, in the UAE, irrigation with wastewater was reported to be a possible route for vegetable microbial contamination (Hussain & Qureshi, 2020). In Iran, fresh produce was found to have inadequate microbiological quality. Thus, improvements in vegetable production, assessment of contamination of irrigation water and fertilizers, and methods used for the identification of microbial contaminants will be necessary for a safer supply of fresh produce (Soltan Dallal et al., 2015).

3.3 TE-resistance of *E. coli*

Resistance to TE (Fig. 1) was found in 5 (50 %) *E. coli* isolates (two from local samples; cabbage and three from imported samples; lettuce and radish) (Tables 2 and 3). All of the TE-resistant *E. coli* harbored the *tet(A)* resistance gene. In addition, one *E. coli* that was isolated from lettuce harbored *tet*(K) (Table 3). The *IntI 1* gene was not detected in enterococci but in 2 isolates of *E. coli* ; one originated from local cabbage and the other from radish imported from China (Table 3). The accession numbers that were given to sequences of *tet* and *IntI 1* genes are available at http://www.ebi.ac.uk/ena/data/view/LT548573-LT548593 accessed June 24, 2020). Sequences of the *IntI 1* genes identified in the 2 isolates of *E. coli* were also recorded in the 'INTEGRALL' platform which is dedicated to integrons and can be reached on http://integrall.bio.ua.pt/?acc=LT548588 and http://integrall.bio.ua.pt/?acc=LT548589 (both accessed June 24, 2020).
E. coli can be used as an indicator species to determine the antibiotic resistance state of enteric microorganisms (Teuber, 1999). Resistance to TE by *E. coli* isolated from vegetables was previously reported in other countries (Hassan et al., 2011). All of the TE-resistant *E. coli* in the current study harbored *tet(A)* . One *E. coli* that was isolated from lettuce harbored *tet*(K). Both of these genes are efflux genes that code for membrane-associated proteins that export TE out of the cell (Ng et al., 2001; Roberts, 2005). There was no significant difference in the frequency of TE-resistant *E. coli* isolated from local and imported samples; χ^2 (1, N = 10) = 1.67, P= 0.1967 and this may indicate similar levels of contamination with TE-resistant *E. coli* . Investigation of TE-resistance in produce-associated *E. coli* was not previously studied in Oman. TE-resistance of animal-associated *E. coli* was previously studied by Al-Bahry et al. (2013) who found high levels of TE resistance (97.9 %) in *E. coli* isolated from the colon of chickens which were collected from three poultry farms in Oman, where the most common TE-resistant determinants were *tet(A)* followed by *tet*(B). In this study, the *IntI 1* gene for the class 1 integron was detected in 2 *E. coli* isolates; one obtained from local cabbage and the other from radish imported from China. Jones-Dias et al. (2016) detected the class 1 intergron in Gram-negative bacteria recovered from fresh produce grown in Portugal. The class 1 integron is widespread in Enterobacteriaceae bacteria and often harbors ARG (Jaglic

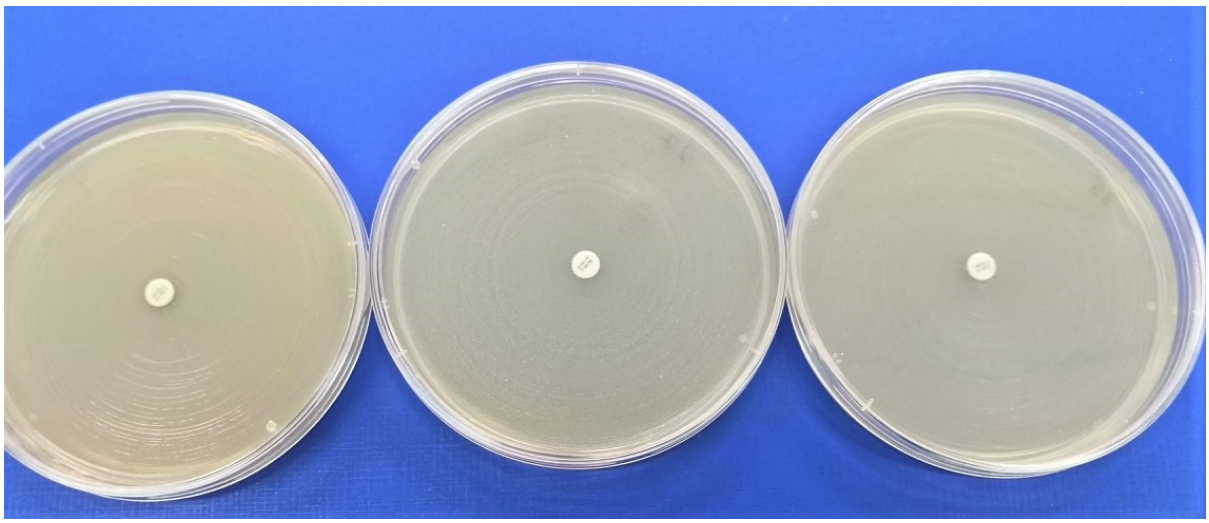

Figure 1: Disc diffusion method showing, from left to right, the resistance of *E. coli*, *E. sulfureus* and *E. casseliflavus* to tetracycline (diameters of growth inhibition zones: 7, 6 and 6 mm, respectively).

& Cervinkova, 2012). It would be interesting to investigate if TE or other ARG are located on the integrons of the 2 isolates of *E. coli* . Ingestion of foods contaminated with ARG, located on integrons, can facilitate transferof these determinants by HGT and thus influence the pool of antibiotic resistance in humans (Bezanson et al., 2008).

3.4 Significance of TE resistance in fresh produce-associated enterococci and *E. coli*

This study demonstrated phenotypic and genotypic TE resistance in enterococci and *E. coli* isolated from fresh produce ready for consumption. As ARB were also reported to be prevalent in humans and animals, as well as in natural (Gasparrini et al., 2020) and food environments, this may suggest that both pathogenic and commensal microbes have played an important role in spreading ARG, and that the food chain may act as a non-negligible route for bridging the antibiotic resistance between the environment and humans (Li & Wang, 2010). Different strains of enterococci and *E. coli* which originate from fresh produce may act as commensals and opportunistic or primary pathogens. Both of them can colonize the human gastrointestinal tract and may cause an opportunistic infection that might happen even years after the ingestion of the contaminated food (Hoelzel et al., 2018). As the results of this study demonstrated the presence of different types of *tet* genes in enterococci and *E. coli* isolated from fresh produce at the market place, these bacteria may colonize the intestine and affect the *tet* resistance pool especially if the *tet* genes can persist in the gut for a long time (Forslund et al., 2013). They also can serve as a vehicle for transfer of *tet* resistance genes in the intestine (Karami et al., 2006) or in the fresh produce environment before ingestion as was demonstrated to occur in lettuce (Jung & Matthews, 2016). In fact, in this study, *tet*(K) was detected in *E. sulfureus* and *E. coli* originated from the same lettuce sample (Table 3). It would be interesting to determine if any genetic event occurred between these 2 isolates in lettuce.

Although previous studies reported high levels of TE-resistance in enterococci (Cauwerts et al., 2007) and *E. coli* (Al-Bahry et al., 2013) from animal sources, DNA is partially degraded by heat, and thus, consumption of raw fresh produce is more likely to deliver a higher concentration of ARG into the gastrointestinal tract

(Hoelzel et al., 2018). Multidrug-resistant bacteria were previously isolated from various ready-to-eat foods (Vincenti et al., 2018), including fresh produce that contained multidrug-resistant enterococci (Johnston & Jaykus, 2004) and *E. coli* (Al-Kharousi et al., 2016). Thus, these food commodities, which usually receive no or minimal heat treatment before consumption, may act as a vehicle for transfer of multidrug-resistant enterococci or *E. coli* to humans (Johnston & Jaykus, 2004). This transfer may pose a risk to public health as drug resistance can decrease drug efficiency, increase the cost of the treatment of infectious diseases and increase the morbidity and mortality rates (Zhang et al., 2006).

4 Conclusions

Half of the isolated *E. coli* (sources: cabbage, lettuce and radish) were resistant to TE and possessed *tet(A)* and *tet*(K) genes. The integron integrase *IntI 1* gene was detected in 2 isolates of *E. coli* . This can potentiate the capability of fresh produce to disseminate antibiotic resistance among bacteria especially when these genetic elements are equipped with ARG. About a fifth of the tested enterococci (sources: cucumber, lettuce and radish) were resistant to TE and possessed *tet*(K), *tet*(L) or *tet*(M) genes. These results demonstrate the contamination of fresh produce with TE-resistant enterococci and *E. coli* . Effective measures are needed to prevent antibiotic-resistant foodborne bacteria from reaching humans.

Acknowledgements

We thank Sultan Qaboos University (SQU), Oman, for providing the funding for this study.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 371–382

Effects of Addition of Swine Skin on the Technological Characteristics of Mortadella Produced in an Industrial Unit

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Received: 8 July 2020; Published online: 18 October 2021

Abstract

The aim of this work was to evaluate the effects of the addition of swine skin on the technological characteristics of mortadella formulations produced on industrial scale. The effects of concentrations of swine skin (1.5 to 5.5 %) and sodium chloride (2 to 3 %) on total protein, total fat, starch, moisture, water activity, sodium, pH and texture profile (hardness, adhesiveness, elasticity, cohesiveness and chewiness) were evaluated and compared to a mortadella formulation without swine skin addition. The mortadella formulations with addition of 3.5 to 5.5 % swine skin and 2 to 2.5 % sodium chloride are in accordance with Brazilian legislation and provided an increase of approximately 12 % in protein content, a decrease of 14 % in sodium content and a water activity less than 0.9488. The swine skin and sodium chloride provided stability to the mortadella and influenced its texture, mainly in hardness, elasticity and chewiness.

Keywords: Emulsified product; Texture profile; Protein; Water activity

1 Introduction

Emulsified meat products, such as mortadella, are among the most widely produced. Their consumption has become popular mainly due to low cost, pleasant taste and variety of products available (Guerra et al., 2011), allowing access to those who are unable to consume the minimum amounts of daily protein (Shimokomaki & Olivo, 2006). Because they are industrialized products, which diversify the use and consumption of meat and add value to less noble cuts, the materials used in production must be of a quality contributing to the action of additives and provide for an increase in shelf life without bringing any damage to health (Godfray et al., 2018).

Bologna-type sausages are widely consumed in many countries and are of great economic importance to the meat industry.

The production of mortadella is characterized by a specific procedure: meat is forced through a special mincing machine, named "exterminator", where a meat mixture is obtained that is not, however, an emulsion (Barbieri et al., 2013). A particularly long and intense cooking process allows the meat to develop its typical organoleptic characteristics and stabilizes the microbiota of the product, ensuring a long shelf life (Barbieri et al., 2013). They are made from beef, swine, chicken or meat mixtures, with addition of different ingredients and flavorings, including extenders or binders and fillers (Fernandez-Lopez

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et al., 2020; Muraoka Junior et al., 2019; Saldana, Siche, et al., 2018).
Brazilian legislation (Brazil, 2015a, 2015b) establishes some quality parameters, including maximum values of water activity (0.955) and sodium concentration (135 mg/100 g) for mortadella sold at room temperature (22 ^{o}C). However, industries are finding it difficult to comply with these established values, thus it is necessary to evaluate alternatives for product preparation that guarantees safety for the consumer.
Swine skin is considered a by-product because it is abundant in slaughter animals, representing a very significant portion of the live animal's weight, in the order of 3.0 to 8.0 % (Ockerman & Hansen, 1994). On, average, 5.5 kg of skin can be removed from one swine carcass (Quevedo, 2005), corresponding to approximately 30 % of the total protein, up to 81% (w/w) of collagen (Reginato & Teixeira, 2009) and about 30 % of fat (Vidal et al., 2020). Fat plays an important role in the manufacturing process as well as in the final texture of mortadella (Saldana, Garcia, et al., 2018). Swine skin can be used to make meat products, such as gelatin and crackling (Ockerman & Hansen, 1994), and it can also act as an emulsifying agent in the food industry (Nagai et al., 2015; Vidal et al., 2020), for example, as used in the manufacture of mortadella (Agostinho dos Santos Alves et al., 2016; Benelli et al., 2015).
In this sense, the aim of this work was to evaluate the effects of the addition of swine skin, a by-product of the meat industry, on the technological characteristics of mortadella formulations produced on an industrial scale.

2 Materials and Methods

2.1 Product formulation

Mortadella formulations were developed in an industry, located in the western region of the State of Santa Catarina, Brazil. Mortadella formulations (n = 3) with swine skin – X1 (1.5 to 5.5 % w/w) and sodium chloride – X_2 (2 to 3 % w/w) were prepared using a 2^2 factorial design matrix (Table 1, run 1 to 7). The other ingredients of the formulations were fixed (undeclared quantities): water, swine meat, mechanically separated poultry meat (MSPM), swine fat and liver, cassava starch, soy protein and additives (sodium lactate, sodium tripolyphosphate, disodium pyrophosphate, sodium hexametaphosphate, sodium erythorbate, monosodium glutamate, mealybug carmine, sodium nitrite, dehydrated glucose and condiments: garlic, nutmeg and white pepper). The fixed operating conditions were mixing time (30 min), stirring speed (60 rpm), raw materials temperature (6 ^{o}C and -12 ^{o}C) and water temperature (22 ^{o}C). The levels were defined based on preliminary tests and in compliance with the technical regulation of quality and identity for mortadella (Brazil, 2000). One traditional mortadella formulation (Table 1, run 8) with no added swine skin (n = 3) was also prepared in order to compare with the other formulations.
The raw materials were crushed in a grinder (Weiller model 1612, Brazil), with a particle size of approximately 8 mm and sent to the mixer (AMFEC model cbm6000, Condado de Norfolk). Then the crushed meat and the other ingredients were mixed for approximately 30 min. The mass was placed under vacuum (Cozzini, VSP3000, USA), with a double vacuum pump to remove air bubbles, and then fed to an emulsion/reduction system (Cozzini, AR 901, USA) which had an output disc of 1.2 mm to refine and homogenize the mass. The mass was stuffed into polyethylene plastic, 0.13 mm thick, diameter of 72 mm and length of 290 mm, with approximately 500 g per unit, and cooked in an oven (Maurer, KSC-36A1, Germany) until the internal temperature reached 74 ^{o}C, monitored by sensor (Maurer, Probe PT-100, Germany). Next, the mortadella was cooled at 25 ^{o}C $\pm$ 1^{o}C until analysis was carried out. As dependent variables (responses) of the 2^2 factorial design and the traditional formulation (without swine skin), the total protein, total fat, starch, moisture, water activity, sodium, pH and texture profile (hardness, adhesiveness, elasticity, cohesiveness and chewiness) were assessed.

2.2 Analytical determinations

The formulations were evaluated in relation to the total protein by method 981.10 (AOAC,

Table 1: Factorial design matrix 2^2 (coded and real values) and response in total protein, total fat, moisture, water activity (a_w), starch, sodium and pH of mortadella formulations.

Run	*Independent Variables**		*Total Protein*	*Total Fat*	*Moisture*	a_W	*Starch*	*Sodium*	*pH*
	X_1	X_2	*(%)*	*(%)*	*(%)*		*(%)*	*(mg/100g)*	
1	-1 (1.5)	-1 (2.0)	$12.58^{d}\pm0.021$	$19.01^{e}\pm0.021$	$55.48^{b}\pm0.030$	$0.9567^{b}\pm0.0001$	$3.39^{b}\pm0.015$	$1134.4^{f}\pm1.966$	$6.67^{c}\pm0.02$
2	1 (5.5)	-1 (2.0)	$13.69^{a}\pm0.068$	$19.72^{b}\pm0.011$	$54.01^{e}\pm0.02$	$0.9488^{c}\pm0.0001$	$3.59^{a}\pm0.021$	$1185.6^{e}\pm1.120$	$6.67^{a}\pm0.01$
3	-1 (1.5)	1 (3.0)	$12.98^{c}\pm0.023$	$18.95^{f}\pm0.100$	$55.26^{c}\pm0.035$	$0.9415^{c}\pm0.0001$	$3.43^{b}\pm0.011$	$1458.0^{a}\pm0.555$	$6.69^{a}\pm0.01$
4	1 (5.5)	1 (3.0)	$13.65^{a}\pm0.404$	$19.62^{c}\pm0.017$	$53.97^{e}\pm0.021$	$0.9401^{f}\pm0.0006$	$3.33^{b}\pm0.021$	$1440.5^{b}\pm1.286$	$6.70^{a}\pm0.01$
5, 6, 7**	0 (3.5)	0 (2.5)	$13.30^{b}\pm0.003$	$19.46^{d}\pm0.011$	$54.67^{d}\pm0.011$	$0.9474^{d}\pm0.0007$	$3.33^{b}\pm0.002$	$1199.0^{d}\pm2.378$	$6.68^{a}\pm0.01$
8***	-	-	$12.04^{e}\pm0.025$	$22.17^{a}\pm0.015$	$58.12^{a}\pm0.025$	$0.9720^{a}\pm0.0011$	$3.08^{c}\pm0.010$	$1377.1^{c}\pm0.888$	$6.71^{b}\pm0.01$

*X_1= swine skin (%), X_2= sodium chloride (%). Fixed independent variables: water, swine meat, mechanically separated poultry meat (CMS), pork fat (undeclared quantities; n = 3). The fixed operating conditions were: mixing time (30 min), stirring speed (60 RPM), raw material temperature (6 ^{o}C and -12 ^{o}C) and water (22 ^{o}C). Mean ± standard deviation followed by equal letters in the same column do not differ statistically in a level of 5 % (Tukey's test).
**Average of the central point.
*** Traditional mortadella formulation (without swine skin).

2019d), moisture by method 985.26 (AOAC, 2019b), total fat by method 991.36 (AOAC, 2019e), sodium by method 976.25 (AOAC, 2019a) and pH by method 981.12 (AOAC, 2019c). Water activity (a_w) was determined using AquaLab (AquaLab model 4TE, USA). The texture profile (TPA) of mortadella (n = 3) at room temperature (22 ^{o}C) was obtained using cubes of 20 mm and a texturometer (Stable Micro Systems model TA. XT, England), with load cell of 10 kg, flat-bottomed stainless steel cylindrical probe (diameter of 31.8 mm), calibration distance of 55 mm from probe height, pre-test speed of 3 mm/s, test speed of 1 mm/s and 3 mm/s, a maximum force required to compress the initial weight by 40 % (the maximum force required to compress the initial weight was determined after experimental tests) and return time of 5 s (Harper et al., 2012).

2.3 Statistical analysis

The results (n = 3) were statistically analyzed by analysis of variance, and then the means were compared via Tukey test using *Statistic* 7.0 software, with significance level of 95 %. Results were also analyzed by Pearson correlation and principal component analysis (PCA) using XLSTAT free software.

3 Results and Discussion

Table 1 shows the matrix of the 2^2 factorial design with coded (real) values of independent variables, traditional formulation (run 8), and the responses of total protein (TP), total fat (TF), moisture (M), water activity (a_w), starch (S), sodium (Na^+) and pH of mortadella formulations.

For protein, the maximum content was 13.69 g/100 g (run 2), with 2 % of sodium chloride and 5.5 % of swine skin. However, the formulation with 3 % of sodium chloride (runs 3 and 4), exceeds sodium values of 13.50 g/100 g provided by ANVISA (Brazil, 2000). Swine skin, sodium chloride and the interaction of variables had a significant ($p < 0.05$) positive influence on protein content. All the runs presented protein content within the standard established by Normative Instruction n^{o} 04 of 2000 (Brazil, 2000). Equation 1 presents a first order coded model that describes total protein as a function of swine skin and sodium chloride concentrations, within the studied ranges. The model was validated by analysis of variance, with a correlation coefficient of 0.99 and $F_{calculated}$ 7.36 times greater than the tabulated F_{value}, which enabled the construction of the contour curve (Fig. 1a), showing that there is an increase in total protein content (13.30 to 13.69 %) when the concentration of swine skin increases (3.5 to 5.5 %), corresponding to an increase of approximately 12 % of protein when

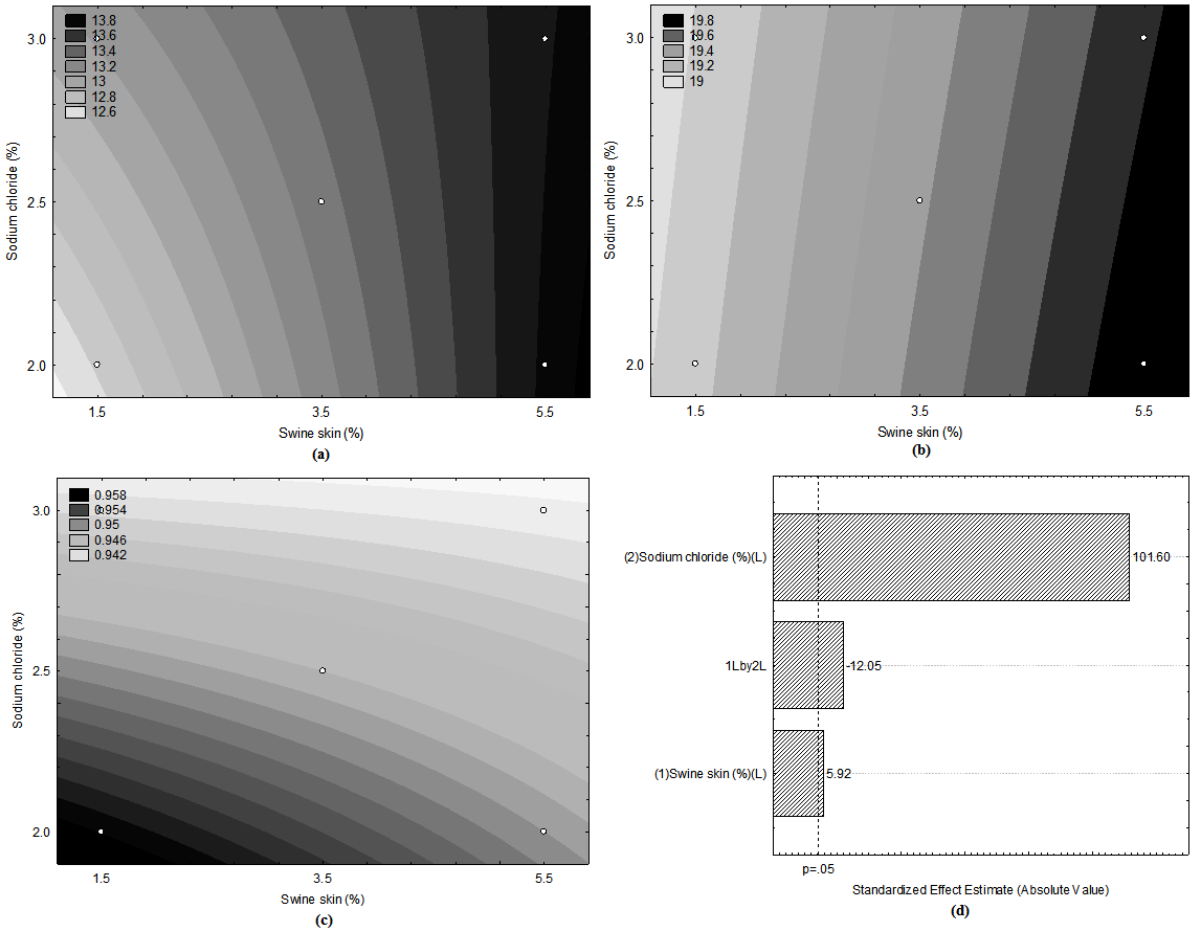

Figure 1: Contour curves as a function of swine skin and sodium chloride concentration for total protein content (%) (a), total fat (%) (b), water activity (c) and Pareto graph (d) with estimated effects (absolute value) of the variables tested for the sodium content of the mortadella formulations, respectively.

compared to the traditional mortadella formulation (Table 1).The values of the present study are higher than those reported by Benelli et al. (2015), which used 6.4 % of swine skin in the preparation of mortadella and obtained 12.09 % of protein.

The increase in protein content observed in the present study, even with addition of less swine skin, may be due to the influence of salt in the solubilization of myofibrillar proteins, responsible for increasing hydration and water retention capacity and stabilization of the meat batter, which reduces losses in the cooking process (Agostinho dos Santos Alves et al., 2017; Bannwart et al., 2014).

$$TP = 13.26 + 0.445X_1 + 0.090X_2 – 0.108X_1 \cdot X_2 \quad (1)$$

Where TP=Total protein (%), X_1 = swine skin (%), X_2= sodium chloride (%). All formulations contained around 19 % of total fat, which is far below the maximum allowed (30%) by Brazilian legislation (Brazil, 2000). The maximum obtained was 19.72 g/100 g on run 2, with 2 % of sodium chloride and 5.5 % of swine skin. This study allowed a reduction of approximately 15 % in fat content when compared to the traditional formulation (run 8), obtaining a healthier prod-

Table 2: Factorial design matrix 2^2 (coded and real values) and response in total protein, total fat, moisture, water activity (a_w), starch, sodium and pH of mortadella formulations.

Run	*Independent Variables**		Texture profile				
	X_1	X_2	*Hardness (N)*	*Adhesiveness (N.s)*	*Elasticity (mm)*	*Cohesiveness*	*Chewiness (N.mm)*
1	-1 (1.5)	-1 (2.0)	26.10^{b}±0.527	-5.79^{d}±0.019	1.66^{c}±0.014	0.842^{a}±0.001	395.14^{a}±2.054
2	1 (5.5)	-1 (2.0)	28.64^{a} ±1.080	-7.17^{c}±0.326	1.58^{c}±0.035	0.847^{a}±0.006	323.77^{a}±2.249
3	-1 (1.5)	1 (3.0)	25.71^{e}±0.774	-3.83^{e}±0.090	1.64^{d}±0.035	0.840^{a}±0.004	312.97^{a}±6.360
4	1 (5.5)	1 (3.0)	27.88^{b}±0.407	-9.66^{b}±0.002	1.69^{b}±0.100	0.847^{a}±0.007	398.89^{a}±10.476
5;6;7**	0 (3.5)	0 (2.5)	26.81^{c}±0.535	-5.85^{b}±0.74	1.64^{c}±0.189	0.834^{a}±0.001	390.27^{a}±14.699
8***	-	-	24.09^{f}±0.225	-27.15^{a}±0.232	1.70^{a}±0.013	0.833^{a}±0.001	180.75^{b}±.544

*X_1= swine skin (%), X_2= sodium chloride (%). Fixed independent variables: water, swine meat, mechanically separated poultry meat (CMS), pork fat (undeclared quantities; n = 3). The fixed operating conditions were: mixing time (30 min), stirring speed (60 rpm), raw material temperature. Mean ± standard deviation followed by equal letters in the same column do not differ statistically in a level of 5 % (Tukey's test).

uct with a lower fat content.

Equation 2 presents the first order coded model that describes total fat content as a function of the swine skin and sodium chloride concentration, within the studied ranges. The model was validated by analysis of variance, with a correlation coefficient of 0.97 and $F_{calculated}$ 8.58 times greater than the tabulated F_{value}, which enabled the construction of the contour curve Fig. 1b demonstrating that the maximum total fat content is located in a region of maximum concentration of swine skin. Sodium chloride did not influence the total fat content.

$$TF = 19.38 + 0.347X_1 \quad (2)$$

Where TF=Total fat (%), X_1 = swine skin (%), X_2= sodium chloride (%). One important consideration is the relationship between the protein and total fat contents. In standard Bologna mortadella from Brazil (Brazil, 2015a) containing around 12 % protein and 25 % of total fat and the traditional mortadella formulation of the present study (Table 1, run 8) the protein/fat ratio is approximately 0.5, and in the runs with swine skin (Table 1, runs 1 to 7), it was about 0.7. The traditional mortadella formulation (Table 1, run 8) contained more fat when compared with runs 1 to 7, a relative reduction of 12 % of fat.

Furthermore, the addition of different concentrations of swine skin (1.5 to 5.5 %) and sodium chloride (2 to 3 %) promoted a reduction in the mortadella moisture. Run 4 (Table 1) with 5.5 % of swine skin and 3 % of sodium chloride had a moisture content of 53.97 % compared to 58.12% for the traditional mortadella formulation.

The lowest value of water activity was 0.9401 (run 4, Table 1). As expected, the more salt that was added the lower the water activity, since water activity is directly related to salt concentration. Thus, as salt concentration increases, water activity decreases.

Swine skin, sodium chloride and the interaction of variables had a significant ($p < 0.05$) positive influence on water activity. Equation 3 presents the first order coded model that describes water activity as function of swine skin and sodium chloride, within the studied ranges. The model was validated by analysis of variance, with a correlation coefficient of 0.99 and $F_{calculated}$ 33.43 times greater than the value of the tabulated F_{value}, which enabled the construction of the contour curve shown in Fig. 1c, demonstrating that the minimum water activity content is found in a maximum range of sodium chloride. The swine skin provided an increase in protein content of mortadella and contributed to the reduction of water activity.

$$a_w = 0.947–0.002X_1–0.006X_2 + 0.002X_1 \cdot X_2 \quad (3)$$

Where a_w = water activity, X_1 = swine skin (%), X_2= sodium chloride (%). Orsolin et al. (2015) studied a_w during the storage of bologna mortadella, and obtained values ranging from 0.969

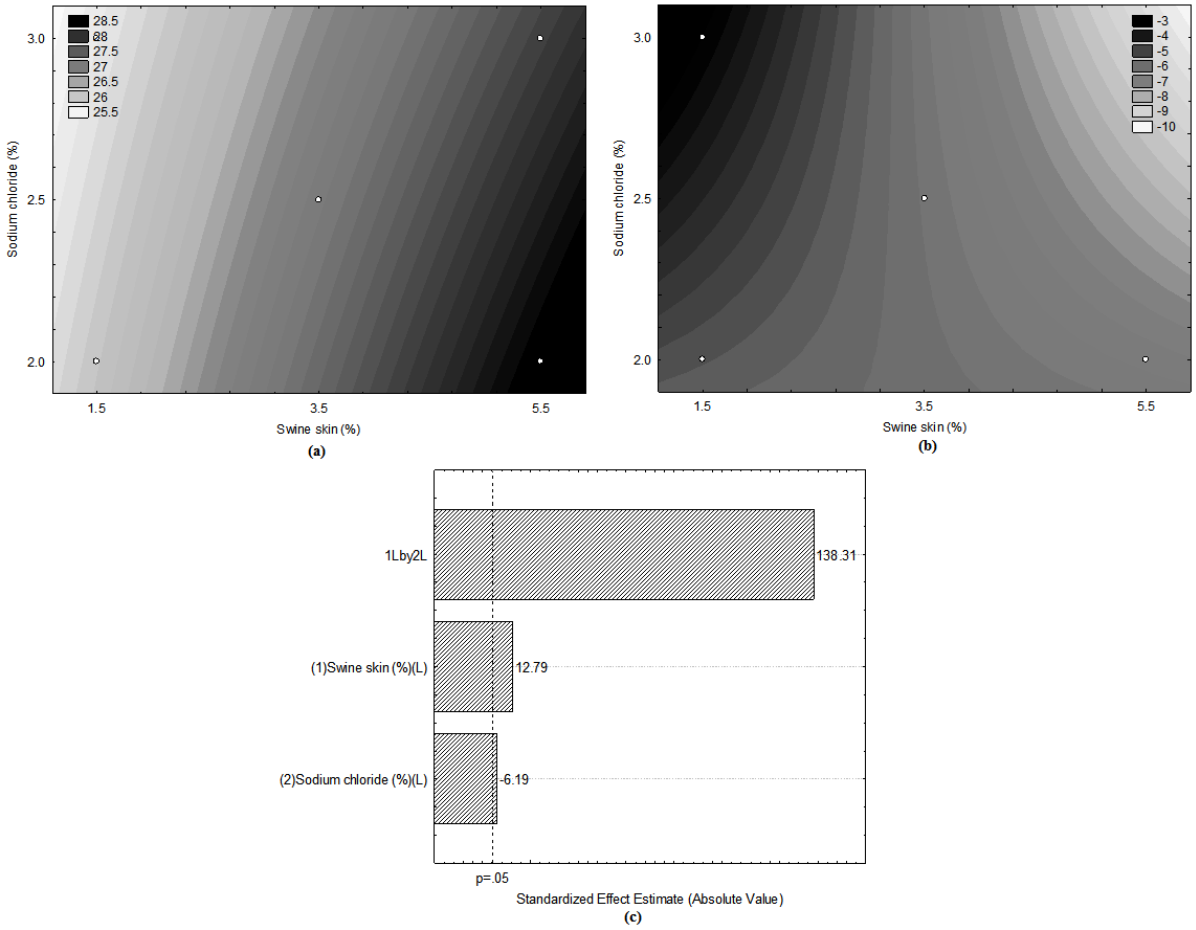

Figure 2: Contour curves as a function of the concentration of swine skin and sodium chloride for hardness (N) (a) adhesiveness (Ns) (b) and Pareto graph (c) with the estimated effects (absolute value) of the variables tested for the chewiness of mortadella formulations, respectively.

to 0.975. Fiorda and de Siqueira (2009) studied mortadella with meat mechanically separated from poultry and swine, and found a_w values of 0.957. It is noteworthy that the water activity values mentioned are higher than those found in the present study (Table 1 and Fig. 1c).

Starch content ranged from 3.08 to 3.59 g/100 g (Table 1), being in accordance with the current legislation (Brazil, 2000), and the swine skin and sodium chloride variables had no influence on this parameter.

The sodium content (Table 1) observed in all formulations varied from 1134.4 to 1458.0 mg/100 g, and is similar when compared with mortadella from the Brazilian market, which usually contain between 1063 and 1480 mg/100 g of sodium (Brazil, 2012; Martins et al., 2015).

Fig. 1d shows a Pareto graph with the estimated effects of variables in the 2^2 factorial design for sodium content. The sodium chloride variable had a positive influence ($p < 0.05$), demonstrating that as sodium chloride content increases, sodium values in the formulations also increase. Formulations with 2 % (runs 1 and 2) and 2.5 % sodium chloride (runs 5, 6 and 7) present relative reductions of 17 to 13 % in sodium content when

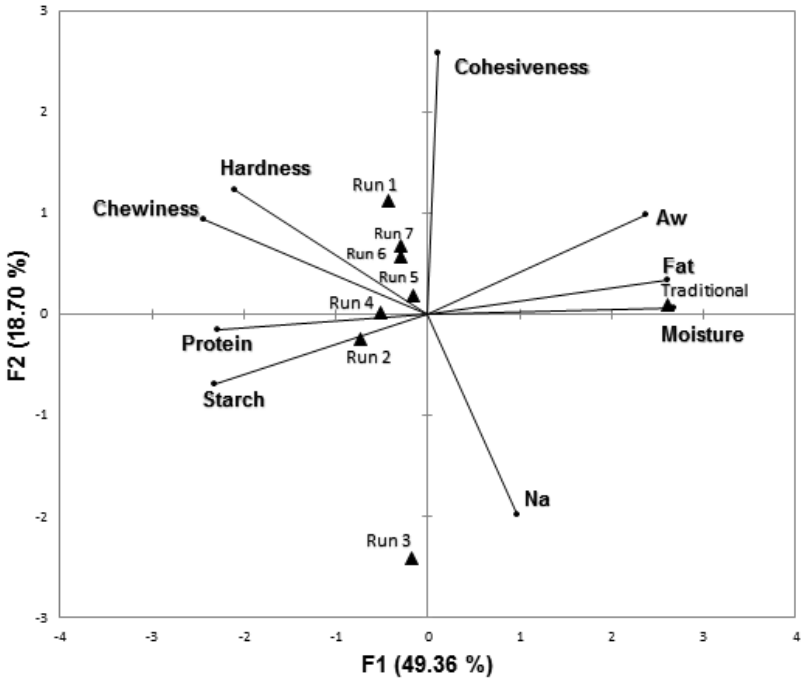

Figure 3: Principal Component Analysis (PCA) for the variables total protein, total fat, starch, moisture, water activity (a_w), sodium, pH, hardness, adhesiveness, elasticity, cohesiveness and specific chewiness of mortadella formulations (7 planning tests complete factorial 2^2).

compared to the traditional formulation (run 8). Sodium chloride is recognized as a multifunctional ingredient in meat products and is normally used at concentrations of 1.5 to 2.5 % (w/w) to intensify flavor, inhibit microbes, extend the shelf life and solubilize myofibrillar proteins (Ordóñes et al., 2005).

The addition of sodium chloride and swine skin had no influence on pH, with values of approximately 6.7 (Table 1). The pH values were considered acceptable for this type of meat product (Agostinho dos Santos Alves et al., 2017; Pietrasik & Janz, 2010). According to Toldrá (2010), a_w less than 0.98 and pH around 6.00 are considered ideal for emulsified meat products so as to guarantee their microbial and structural stability.

Table 2 shows the matrix of the 2^2 factorial design with the coded (real) values of the independent variables studied and the responses for the texture profile in terms of hardness, adhesiveness, elasticity, cohesiveness and chewiness of mortadella formulations.

The swine skin and sodium chloride did not significantly influence elasticity and cohesiveness in runs 1 to 7, however, the traditional mortadella formulation (run 8) showed greater elasticity. Sodium chloride and swine skin addition had no influence ($p > 0.05$) on elasticity and cohesiveness, with mean values of 1.64 mm and 0.83, respectively (Table 2) which were similar to those of the traditional formulation (without addition of skin).

Hardness was influenced by the variables studied, with a highest mean value of 28.64 N (2.0 % sodium chloride and 5.5 % swine skin - run 2) compared with 24.09 N for the traditional formulation.

Equation 4 presents the first order coded model that describes hardness as a function of swine skin and sodium chloride, within the studied ranges. The model was validated by analysis

of variance, with a correlation coefficient of 0.98 and $F_{calculated}$ 10.21 times higher than the tabulated F_{value}, which enabled the construction of the contour curve shown in Fig. 2a, demonstrating that the maximum hardness is found in the highest concentrations of swine skin and lowest sodium chloride.

$$H = 26.97 + 1.18X_1 – 0.287X_2 \quad (4)$$

Where H=Hardness (N), X_1 = swine skin (%), X_2= sodium chloride (%). Swine skin and sodium chloride variables had a negative influence on adhesiveness, and the traditional formulation (without swine skin) had a significantly lower adhesiveness value ($p < 0.05$). Equation 5 presents the first order coded model that describes the adhesiveness as a function of swine skin and sodium chloride concentration variables, within the studied ranges. The model was validated by analysis of variance, with a correlation coefficient of 0.97 and $F_{calculated}$ 1.87 times greater than the tabulated F_{value}, which enabled the construction of the contour curve shown in Fig. 2b, demonstrating that there is an increase in the adhesiveness parameter with low levels of swine skin and by increasing the levels of sodium chloride.

$$AD = -6.28 – 1.80X_1 - 0.134X_2 – 1.11X_1 \cdot X_2 \quad (5)$$

Where AD=Adhesiveness (N.s), X_1 = swine skin (%), X_2= sodium chloride (%). Chewiness was positively influenced ($p < 0.05$) by the addition of swine skin and the interaction with sodium chloride (Fig. 2c), and the chewiness values were higher than those of the traditional formulation. According to (Saldana, Garcia, et al., 2018), instrumental texture properties of mortadella are affected by the fat level of the formulation. The reduction of fat promotes changes in the microstructure of mortadella, generating products with more disorganized structure.

Fig. 3 presents the Principal Component Analysis (PCA) for variables total protein, total fat, starch, moisture, water activity, sodium, pH, hardness, adhesiveness, elasticity, cohesiveness and chewiness of mortadella formulations.

In Principal Component Analysis (PCA), variables are represented as vectors, which characterize the runs that are located close to them. The longer the vector, the better the explanation of the variability between the characteristics evaluated. The first (F1) and second (F2) dimensions explained 68.06 % of the total variance. The main component 1 (PC1) accounted for 49.36 %, while the main component 2 (PC2) accounted for 18.70 %.

The values obtained by means of Pearson's correlation (Table S1) confirmed the relationship between the variables observed in PCA, where fat showed a positive correlation with protein (r = 0.922) and cohesiveness (r = 0.645), and a negative correlation in relation to moisture (r = -0.907). Protein showed a negative correlation ($p < 0.05$) in relation to moisture (r = -0.946) and water activity (r = -0.556). A negative correlation of water activity in relation to sodium content (r = -0.870) and pH (r = -0.734) was observed. Positive correlations between hardness and chewiness (r = 0.627), cohesiveness and chewiness (r = 0.634), chewiness and elasticity (r = 0.935) and chewiness and cohesiveness (r = 0.600) were also observed.

Collagen and fat in swine skin are complementary emulsifying agents, providing stability to the emulsified mass, and acting on texture, especially on hardness, adhesiveness and chewiness. In addition, they stabilize emulsions by binding water and fat, also assisting in the taste and preservation of the product, through the gelling of myosin (Benelli et al., 2015; Choe et al., 2013). The concentration of protein available for gelation is fundamental to the determination of rheological properties of the matrix formed during the processing of embedded meat, and determines the texture and stability of meat products (Sun & Holley, 2011).

Swine skin, which contains collagen in its composition, increases the protein content and can also act as a complementary emulsifying agent to stabilise the emulsified mass (Ordóñes et al., 2005). Collagen protein is used in the food industry to improve elasticity, consistency and stability of food (Olivo & Shimokomaki, 2002). The development of a new product, with addition of swine skin, achieved a nutritional improvement, mainly by increasing protein content. In addition, swine skin and sodium chloride contribute to the reduction of water activity in meat products thus preventing the risks of microbial growth (Dar &

Light, 2014). During cooking, there is a partial unfolding of protein structure which is accelerated by increasing temperature (> 65 to 70 ^{o}C) and cooking time. As a result, there is an aggregation of the unfolded regions of the protein and formation of a three-dimensional network that prevents coalescence of fat globules (Tornberg, 2005; Visessanguan et al., 2000).
Water retention by the protein structure is favored in the presence of chloride ions. Chloride affects cooking performance, and the juiciness and softness of the product. Chloride ions are much more important than sodium ions for achieving an increased water binding by meat proteins as well as accelerating the formation of color in cured products and increasing the reaction rate of nitrite to nitric oxide. Water activity is significantly reduced with the addition of salt, favoring microbial inhibition and a longer shelf life. Sodium ions are responsible for the characteristic flavor and for intensifying flavors (Devine & Jensen, 2004; Ordóñes et al., 2005; Sebranek, 2009).

4 Conclusion

Mortadella with a high concentration of swine skin (3.5 to 5.5 %) tends to have a high protein and fat content, approximately 13.7 and 19.7 %, respectively. These parameters are negatively correlated, mainly with moisture and water activity. The collagen present in swine skin gives stability to the product, influencing its texture, mainly with regard to hardness, cohesiveness and chewiness.
Swine skin is considered a by-product of high protein content which is abundant in the meat products industry. The collagen present in swine skin can be used as an alternative emulsifying agent in the manufacture of emulsified products to add value and increase the protein nutritional value, as well as reducing the cost of production.

Acknowledgements

This study was financed in part by the National Council for Scientific and Technological Development – Brazil (CNPq), Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) – Finance Code 001 and Research Support Foundation of the State of Rio Grande of Sul – Brazil (FAPERGS).

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 383–397

Characterization of Pasteurized Milk Spoilage by Electronic Nose in Relation to its Selected Quality Parameters

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Received: 6 August 2020; Published online: 18 october 2021

Abstract

Pasteurized fresh milk requires an accurate estimation of shelf life under various conditions to minimize the risk of spoilage and product losses. Milk samples were stored for 56 h in an oven at 25 °C and for 15 days in a refrigerator at 4 °C. Samples were analyzed using an electronic nose (e-nose), total bacterial count, titratable acidity and pH to determine the quality of milk. Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA) were used to analyze e-nose data of milk stored at 25 °C, and 4 °C. A clear shift in quality was identified by the e-nose, which also appeared in the total bacterial count after 24 h and 12 days for storage at 25 and 4 °C, respectively. On the other hand, titratable acidity exceeded the normal limits of 0.14 % - 0.21 % after 24 h for storage at 25 °C (0.247 ± 0.006 %) and after 15 days for storage at 4 °C (0.25 ± 0.01 %). If pH was a good indicator of quality for samples stored at 25 °C, it showed no clear trends for samples stored at 4 °C. Based on the microbial count data and e-nose output, the milk had a shelf life of 0.3 day (i.e. 8 h) when stored at 25 °C. Shelf life was extended to 9 days when stored at 4 °C.

Keywords: Pasteurized milk; Shelf-life; Spoilage; Electronic nose

1 Introduction

Beneficial bacteria are important in the production of fermented dairy products, while pathogens and spoilage bacteria have detrimental effects on milk quality and dairy products. The shelf life of pasteurized milk is around 14 days at chilled temperature and is affected by many factors, such as raw milk quality, production system, hygienic, and storage conditions (Bondoc, 2007). However, shelf life decreases with an increase in storage temperature. To determine the shelf life of pasteurized milk stored under different conditions, different quality indicators, such as microbial counts, titratable acidity (TA), pH and enzyme activity (i.e. lipase and protease) need to be performed (Ziyaina et al., 2018).
Food can be characterized by its aroma, which is related to the volatile organic compounds (VOCs). The mixture of VOCs represents the aroma characteristics of dairy products; therefore, consumers typically sniff milk to estimate its quality or freshness in the headspace of the milk container. The VOCs are thus a very important component of the sensory quality of processed and stored milk. Specific combinations of VOCs form a kind of fingerprint, which can be used as an indicator of food quality and safety.

 10.7455/ijfs/10.2.2021.a9

The VOCs in the headspace of milk were used to predict ageing and off-flavours development (Ali et al., 2003). The increase in the concentration of some aldehydes, ketones, alcohols, and esters during the storage of pasteurized milk indicates poor quality. Furthermore, Vallejocordoba and Nakai (1994a) found that the shelf life of pasteurized milk was better estimated by the analysis of the VOCs than by bacterial count.

Normally, the VOCs are qualitatively and quantitatively analyzed using gas chromatography (GC). Vallejocordoba and Nakai (1994a) successfully assessed the VOCs using dynamic headspace/GC to predict the shelf life of pasteurized milk. Recently, Rashid et al. (2019) found that acetone, butanone, pentanal, and ethanol were good indicators of spoilage of pasteurized milk during storage. This was done using headspace solid-phase micro-extraction (HS-SPME) coupled with a flame ionization detector (GC-FID).

Different types of chemical sensors and commercial systems (e.g. electronic-nose and electronic-tongue) can also be used to detect milk spoilage. Recently, electronic noses (e-nose) have been successfully used to monitor microbial growth and shelf life of milk (Kalit et al., 2014). Using an array of five sensors, rancidity of ultra-heat treated (UHT) and pasteurized milk showed a good correlation with ageing of milk (Capone et al., 2001). In addition, a gas-sensing system was successfully used to distinguish between unspoiled milk and contaminated milk with selected bacteria or yeasts (Magan et al., 2001). The e-nose signals of sterile milk inoculated with *Pseudomonas fluorescens or Bacillus coagulans* were correlated with both the microbial loads and sensory scores of the milk (Korel & Balaban, 2002). The e-nose has also been used to assess the bacterial growth and shelf life of milk when stored at 5 ^{o}C and at room temperature (i.e. 25 ^{o}C) (Labreche et al., 2005).

There are limited studies on the use of e-nose for the shelf life determination of milk. In addition, in some countries, the milk may be subjected to high temperature (i.e. 45-50 ^{o}C) during handling and storage. Therefore, predicting the exact shelf life of milk is necessary with varying temperatures of handling or storage (Ziyaina et al., 2018). It is also important to assure good quality of milk for the production of other products, such as yoghurt and cheese. In both cases, fast quality assessment methods are needed for milk. Therefore, the aim of this study was to evaluate the application of the e-nose in monitoring pasteurized milk spoilage during storage at 25 ^{o}C and 4 ^{o}C in comparison to traditional means of quality estimates.

2 Materials and Methods

2.1 Experimental Design

Pasteurized milk (i.e. 500 mL, Tetra Pak carton) spoilage was monitored using two storage temperatures, 25 ^{o}C and 4 ^{o}C. The first set of pasteurized milk samples was stored at 25 ^{o}C in a Labcon oven (LABOCON Corporation, USA) for 56 h (i.e. 0, 8, 12, 24, 32, 41, 48, 56 h) or 2.3 days (0, 0.3, 0.5, 1.0, 1.3, 1.7, 2.0, 2.3 days). The second set of pasteurized milk samples were stored at 4 ^{o}C in a refrigerator (Samsung, China) for 15 days and analyzed every three days (i.e. 0, 3, 6, 9, 15 days). The milk samples were analyzed using the e-nose, microbial count, pH, and TA. At the end of predetermined storage times, one milk carton was unsealed, and three sterile plastic containers were filled with 50 mL of milk to determine pH, TA and microbial count (i.e. triplicate analysis). For e-nose analysis, five vials (15 mL) were filled with 5 mL milk. Controls were used before the start of the storage period.

2.2 Materials

Cartons of pasteurized milk (500 mL each) were collected from the College of Agricultural and Marine Sciences pilot dairy plant, Sultan Qaboos University, Oman. The high temperature short time pasteurization (HTST) was applied in the dairy plant (72 ^{o}C for 15 s). Milk samples were collected from the dairy plant after production and transferred to the Food Processing Laboratory on the day of production.

2.3 E-nose Analysis

A Cryanose 320 (Sensigent Company, California, USA), equipped with 32 sensors, was used in this

study. Five mL of pasteurized milk sample was placed in a 15 mL glass vial (Supleco, Bellefonte, PA, USA) sealed with a rubber septum cap. Vials were kept at 4 ^{o}C until analysis. First, the e-nose sensors were calibrated with air and the e-nose parameters were optimized using similar procedures as Rahman et al. (2018): baseline purge (10 s), sample draw (5 s), air intake purge (20 s), and sample purge (40 s). To measure the volatile profile of the stored milk headspace, the vial septum was punctured with a needle connected to the e-nose. This initiated the collection and transfer of the volatiles from the headspace to the sensors of the e-nose. Five replicates were done for each storage time.

2.4 Optimization of Volatile Compounds Collection

The optimization procedure included four predetermined times (5, 15, 30 and 50 min) and three predetermined temperatures (30, 35, and 40 ^{o}C) of extraction. A water bath (Fisher Scientific (Cambridge) Ltd, England) was used to control the temperature of the vials during the extraction. The vials with milk samples took 5 and 3 min to reach the desired temperature from their storage temperature, i.e. 4 and 25 ^{o}C, respectively. The milk sample stored at 4 ^{o}C was considered as a standard. The experiment was replicated ten times, with each vial considered as a single replicate.

2.5 pH measurement and Titratable Acidity

Measurements of the milk pH were taken using pH/Mv/^{o}C meter from EUTECH Instruments, Singapore. The TA analysis was performed in triplicate. Approximately 50 g of stored milk samples in glass bottles were used for TA analysis. The milk sample (9 g) was placed in a conical flask and three drops of phenolphthalein solution (1 %) were then added. It was then titrated using 0.1 N NaOH (Sigma-Aldrich, Switzerland) until a pink colour appeared. The titration volume (i.e. V) of NaOH was recorded and TA was then calculated (Wehr et al., 2004) according to the following formula.

$$\% \ acidity = \frac{N(NaOH) \times V(NaOH) \times (0.09)}{Sample \ weight} \times 100 \quad (1)$$

2.6 Microbial Count

Milk samples were placed in sterilized plastic containers and transported immediately to the laboratory for measuring the total plate count. The procedures were performed according to Wehr et al. (2004). Peptone maximum recovery diluent was used. Under sterile conditions, several dilutions of milk samples were prepared, and triplicate dishes were prepared for every dilution. The Petri dishes were placed in the incubator (BINDER, Germany) at 32 ^{o}C for 48 h. After 48 h, total bacterial colonies were counted using a colony counter (Gallenkamp Co. Ltd., England).

2.7 Statistical Analysis

E-nose data were analyzed using Principal Component Analysis (PCA) (Past Software version 2.17 c) (Hammer et al., 2001) and Linear Discriminant Analyses (LDA) with R package (RStudio, 2019). The microbial count and TA were assessed using ANOVA followed by Tukey's posthoc test ($\alpha = 0.05$) for mean separation in R. In order to determine the optimal volatile release conditions, a non-metric Multidimensional Scaling (MDS) was used.

3 Results and Discussion

3.1 Total Bacterial Count and Acidity

Total Bacterial Count (TBC) (expressed as $\log_{10}$ cfu mL^{-1}) showed no significant change until 8 h (0.3 d) of storage at 25 ^{o}C when total microbial count was 2.42 $\pm$ 0.20 $\log_{10}$ cfu mL^{-1} (Fig. 1A) (p <0.05, Tukey's test). This initial low microbial count was below the acceptable limit of pasteurized milk of 4.3 $\log_{10}$ cfu mL^{-1} (Food and Drug Administration, 2017). A significant increase in the TBC (7.7 $\pm$ 0.07 $\log_{10}$ cfu mL^{-1})

was observed after 24 h, which then exceeded the spoilage limit of 5 log10 cfu mL^{-1}(p <0.05) (Ziyaina et al., 2019). Similar values of TBC for pasteurized milk stored at ambient temperature after 24 h of storage was observed as 8.18 $\log_{10}$ cfu mL^{-1} (Labreche et al., 2005). The shelf life of the pasteurized milk used in this study was shown to be approximately 8 h (i.e. 0.3 d) at 25 ^{o}C, which was similar to the results of Lucknakhul et al. (2014) (i.e. 0.35 d at the 25 ^{o}C). In contrast, pasteurized milk stored at 19, 15 and 13 ^{o}C had an increased shelf life of 24 h (1 d), 36 (1.5 d) and 72 h (3 days) respectively when the microbial count exceeded the spoilage limit of 5 $\log_{10}$ cfu mL^{-1} (Ziyaina et al., 2019). Considering the Gompertz model for microbial growth curves, the lag periods (t_L) of the current study were 6.0 and 0.3 day (s) for storage temperatures 4 and 25 ^{o}C, respectably, while maximum growths (μ_{max}) were 0.6 and 12.4 ($\log_{10}$ cfu mL^{-1}1) day, respectively.

The TA increased exponentially as a function of storage time (R^2: 0.971) (Fig. 1B) and reached 0.25 ± 0.01 % after 24 h. Similarly, Ziyaina et al. (2018) noticed an increase (< 0.20 %) in TA (above 5 $\log_{10}$ cfu mL^{-1} of TBC) after 24 h of storage at 19 ^{o}C. On the other hand, the pH decreased linearly during storage (Fig. 1B) and after 24 h, decreased to 6.43, below the normal pH range of milk (6.6-6.8). Similar findings were reported by Lucknakhul et al. (2014) at 25 ^{o}C. On the other hand, after 24 h the TA increased (i.e. 0.34 ± 0.0 % - 0.53 ± 0.02 %) above the normal range 0.14 to 0.21 % (Walstra et al., 2005). A similar result was also observed by Lucknakhul et al. (2014). The sourness of pasteurized milk stored at ambient temperature was attributed to either the activity of mesophilic microorganisms, which normally survive pasteurization. Alternatively, the putrefaction of pasteurized milk could cause sourness due to the presence of psychrotrophic bacteria (such as *Pseudomonas* species), which may be the result of a re-contamination after pasteurization (Al-Qadiri et al., 2008).

There were no significant changes in TBC for the milk stored at 4 ^{o}C until the sixth day (i.e. 2.48 ± 0.04 $\log_{10}$ cfu mL^{-1}) of cold storage (Fig. 2A). Then TBC increased significantly in the ninth day (4.89 ± 0.21 $\log_{10}$ cfu mL^{-1}) exceeding the permitted limit of grade A pasteurized milk (i.e. 4.3 $\log_{10}$ cfu mL^{-1}) (Food and Drug Administration, 2017). However, spoilage onset is typically considered when the bacterial count exceeds 5.0 $\log_{10}$ cfu mL^{-1} (Ziyaina et al., 2018). Others reported that the milk reached the end of its shelf life when the total bacterial counts reached values of 6.0-7.0 $\log_{10}$ cfu mL^{-1} (Harding, 1995; McAuley et al., 2016). Our milk had a bacteriological shelf life of approximately 9 days at 4 ^{o}C. In contrast, the shelf life of pasteurized milk stored at 6 ^{o}C was 4 days as observed by Lucknakhul et al. (2014).

Recently, Rashid et al. (2019) studied the shelf life of pasteurized milk at different temperatures (e.g. 4 and 7 ^{o}C) for 19 days. The bacterial count after 9 days of storage at 4 and 7 ^{o}C were 3.27 and 3.64 $\log_{10}$ cfu mL^{-1}, respectively. Furthermore, the bacterial count increased to 3.72, 4.41, and 5.46 $\log_{10}$ cfu mL^{-1} at 4 ^{o}C after storage for 12, 14, and 16 days, respectively. Another study by Labreche et al. (2005) showed that pasteurized milk stored at 5 ^{o}C for 9.2 days reached TPC of 7.1 $\log_{10}$ cfu mL^{-1}. Their finding is higher than our measured microbial count after 9 days of storage (i.e. 4.89 ± 0.21 $\log_{10}$ cfu mL^{-1} at 4 ^{o}C). The variations of the reported results may be due to the varied sources of milk, processing conditions, and post-handling of pasteurized milk and the different temperature conditions. In addition, microbial count, heat stable enzymes (i.e. lipase and protease produced by psychotropic bacteria) of raw milk, and the activity of native plasmin and lipoprotein could play a role in determining the shelf life of milk.

The pH and TA values of milk stored at 4 ^{o}C are presented in the Figure 2B. The pH values showed no significant changes until it reached 6.70 at the end of day 15. It was within the normal pH range of fresh milk 6.6-6.8 (Walstra et al., 2005). Similarly, at 5 ^{o}C storage, the change of the pH value was within the acceptable range at 16 (Ziyaina et al., 2018) and 14 days (Sadhu, 2018). At higher storage temperatures, such as 13, 15, and 19 ^{o}C, the pH reached at 6.5 within 3, 2, and 1 day(s) storage, respectively (Ziyaina et al., 2018).

The TA is a better indicator of shelf life of pasteurized milk compared to pH (Ziyaina et al., 2018). The current study showed insignificant changes in the TA (Figure 2B) until day 9 (i.e.

from 0.160 ± 0.01 to 0.177 ± 0.11 %). This finding is within the acceptable range of TA (i.e. 0.14 to 0.21 %) of fresh milk (Walstra et al., 2005). Similarly, Slewa and Azhar (2018) reported 0.16 % and 0.17 % TA after 1 and 3 days of storage at 6 ^{o}C. However, a significant increase in TA to 0.18 and 0.25 %, was observed in the current study after 12 and 15 days, respectively and this trend was similar to the bacterial counts. The sharp increase in acidity after 12 days was likely due to the fermentation process of lactose to lactic acid.

3.2 Optimization of VOCs Headspace Collection

The optimum conditions of volatiles released in the headspace are illustrated in Figure 3. All increased duration and increased temperatures of extraction enhanced the response of all sensors compared to the standard. The standard response was considered as the pasteurized milk stored at 4 ^{o}C. Each plot shows the effect of storage time with extraction at 30 ^{o}C (Fig. 3A), 35 ^{o}C (Fig. 3B) and 40 ^{o}C (Fig. 3C), respectively. The overall responses of the sensors increased with time, and 30 min at 40 ^{o}C showed the highest responses (i.e. optimum) (Fig. 3C and Fig. 4), but longer volatile release time (i.e. 40 and 50 min) at 40 ^{o}C did not show any further increase in the response (Fig. 3C). Therefore, 30 min and 40 ^{o}C were considered as the optimal conditions for volatile release. Published research shows that temperatures of 20-50 ^{o}C and times of 3-90 min were usually used for volatile release. For example, Oliveros et al. (2005) used 40 ^{o}C for 30 min in order to create a homogeneous headspace, and they mentioned that 38 – 40 ^{o}C allowed for easier comparisons. In this study, temperatures higher than 40 ^{o}C were not used due to the possibility of generating new volatiles as a result of overheating. Vazquez-Landaverde et al. (2005) also pointed out that higher temperatures (45 ^{o}C to 75 ^{o}C) could lead to generating new volatiles in milk as an effect of heating.

The response of the sensors was also analyzed by non-metric Multidimensional Scaling (MDS) (using a Euclidean distance) to quantify the optimum conditions. If we consider as optimal the largest possible distance in the nose-response space, the optimum is reached when the distance is the largest on the MDS plot, which was 40 ^{o}C and 30 min (Fig. 5). The high distances, from the standard (marked as S) in the MDS biplot, indicates high response. Similarly, Groenen and Borg (2015) used MDS to visualize the optimum conditions (i.e. time and temperature) of volatile release.

3.3 E-nose Output of Fresh Pasteurized Milk Stored at 25 ^{o}C

The 6 days of measurements (i.e. 0, 0.3, 1, 1.3, 2, and 2.3) formed 6 natural clusters, marked A-F on the PCA coordinate plot (Fig. 6). The visual clusters B, C, D, E, and F shifted to the left compared to the initial storage (i.e. cluster A). The cluster B for 0.3 day showed only a marginal shift from cluster A, while other clusters (i.e. C, D, E, and F) showed considerable shifts. This change in position in the PCA plot could be correlated with the significant increase in the microbial count observed in the samples corresponding to the additional storage duration, which may have led to the formation of additional volatiles. The e-nose response of cluster D shifted down compared to cluster C followed by further lift up (i.e. clusters E and F). There was a minimal change in microbial count between clusters C, D, and E compared to cluster F. Similarly, using PCA, Capone et al. (2001) identified three classes of milk as rancidity increased during storage. They also observed that the classes of milk shifted to the left as rancidity occurred. Similarly, in the case of fish fillet storage, Di Natale et al. (2001) was unable to classify samples within 1 and 7 days, while at day 11 a clear separation of the samples was observed on the PCA plots.

3.4 E-Nose Output of Pasteurized Milk Stored at 4 ^{o}C

On the PCA plot (Fig. 7), two distinct groups of points emerged (A and B). There were no particular trends in the location of all the data points

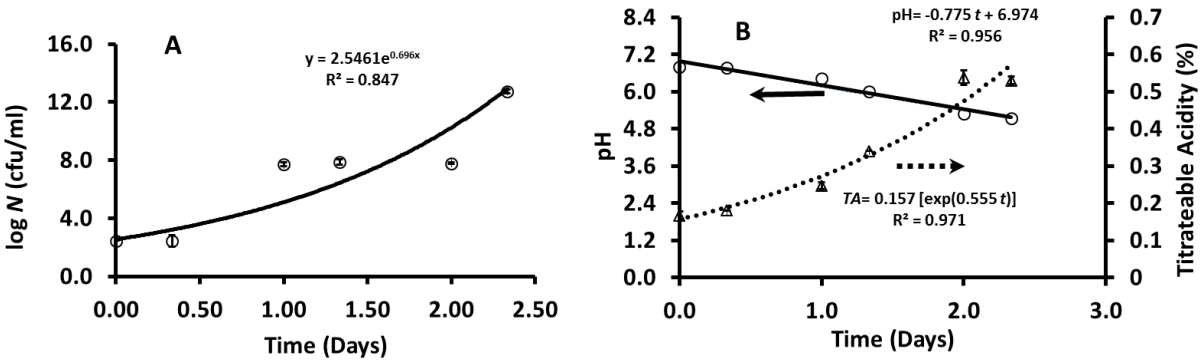

Figure 1: Total Bacterial Count (A) Titratable acidity and pH (B) of fresh pasteurized milk stored at 25 ^{o}C for 56 hours (2.3 days). (Data are plotted as means ± 2SD).

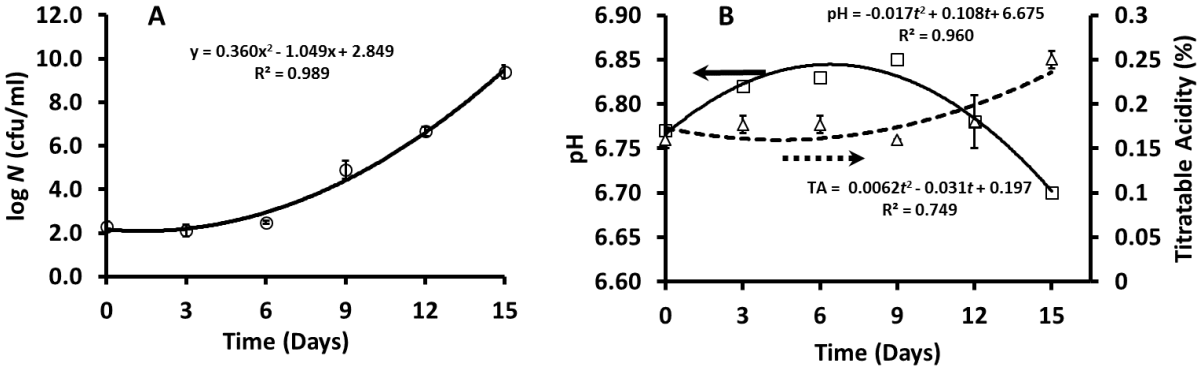

Figure 2: Total Bacterial Count (A) Titratable acidity and pH (B) of fresh pasteurized milk stored at 4 ^{o}C for 15 days. (Data are plotted as means ± 2SD).

after 0, 3, 6, 9, and 12 days of storage but there was a large jump after 15 days (Fig. 7). A linear discriminant analysis (LDA) was applied to analyze the e-nose data (i.e. multiple regression of day as a function of 32 sensors' signals). Four discriminant functions (LD1, LD2, LD3, and LD4) were obtained, which described variances as 72.96, 14.79, 7.11, and 1.78 % respectively. Based on the values of the coefficients, sensors 5, 18, 1, 31, 25, 23, and 20 showed high contribution to separate the clusters (i.e. highest responses to VOCs responsible for spoilage). Figure 8 shows the bar plot of the first discriminate scores as a function of storage days. Samples on day 0, day 3, and day 6 had a negative contribution to the discriminate function (DF) (i.e. there was negligible response of spoilage sensors). In contrast, samples at day 12 and day 15 had a positive contribution to the DF (i.e. major spoilage occurred). On day 9, the responses contributed to the discriminate function both positively and negatively; and this could be considered perhaps a transition phase of the major volatiles (Fig. 8). On the biplot of LDA as a function of LD1 and LD2 (Fig. 9), six clusters (i.e. A, B, C, D, E, and F) were identified each representing different storage days (i.e. 0, 3, 6, 9, 12, and 15 days, respectively). Clusters A, B, C, and D were clearly separated from the clusters E and F (i.e. moved from left to right). The clusters E

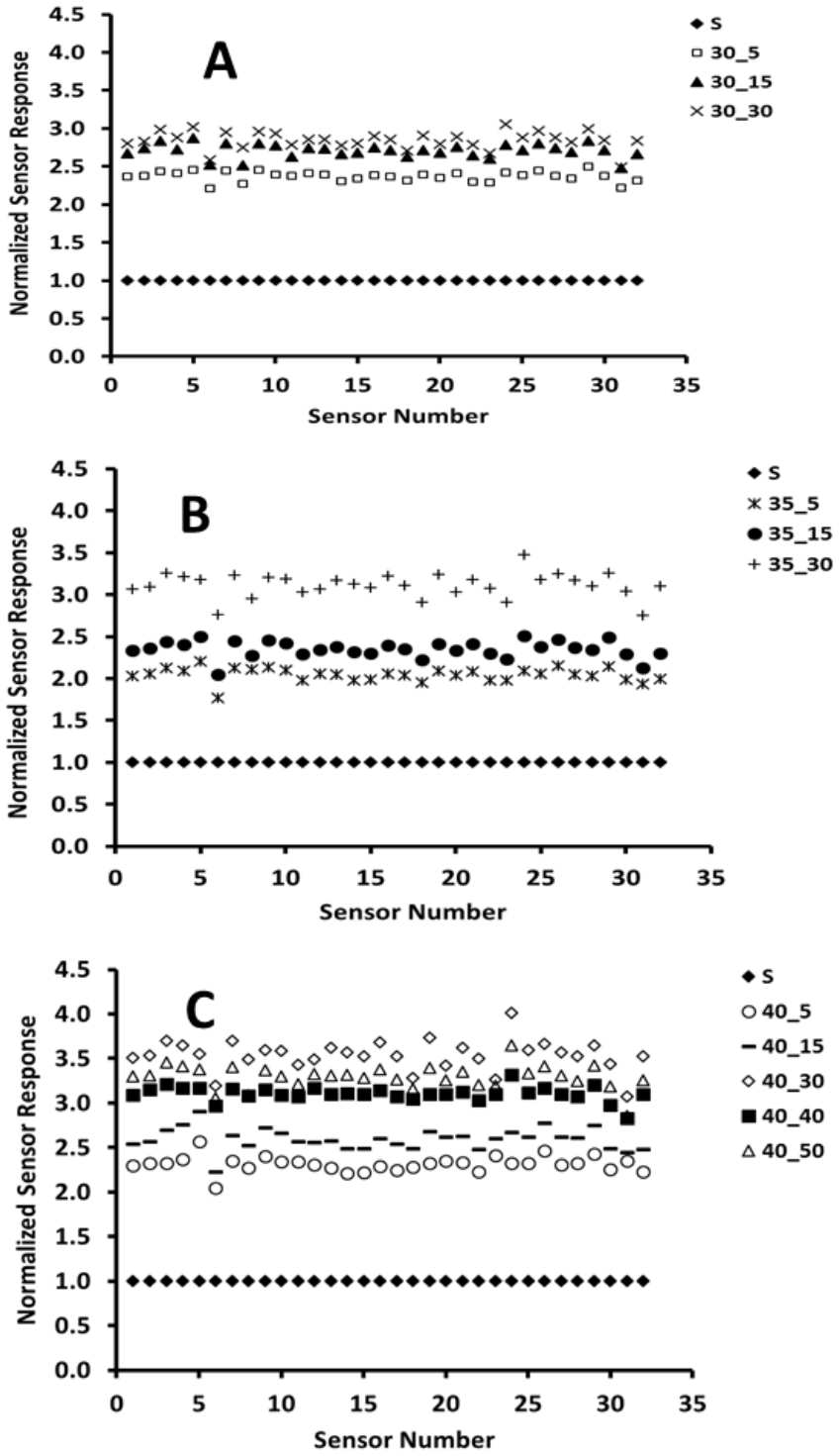

Figure 3: Normal analysis of e-nose signals of milk heated for specific times at specific temperatures: (A) 30 ^{o}C, (B) 35 ^{o}C and (C) 40 ^{o}C.

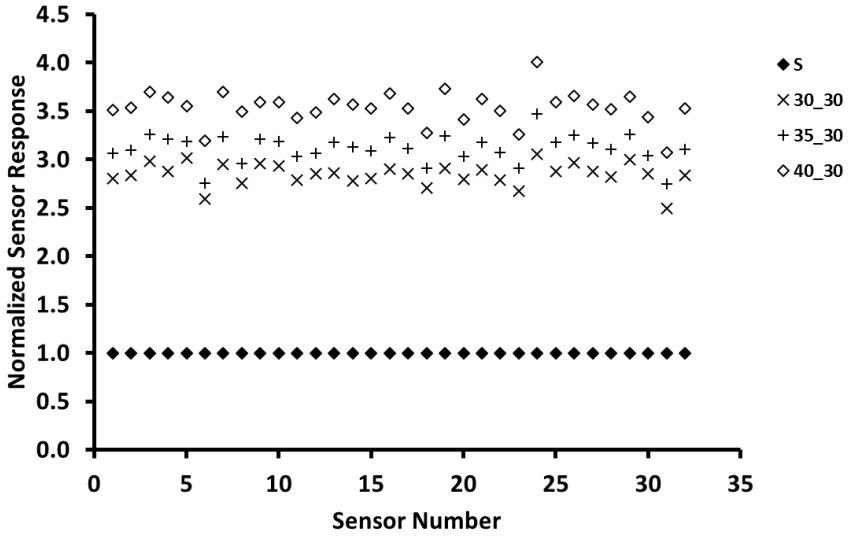

Figure 4: Normal analysis of e-nose signals of milk heated for 30 min at specific temperatures.

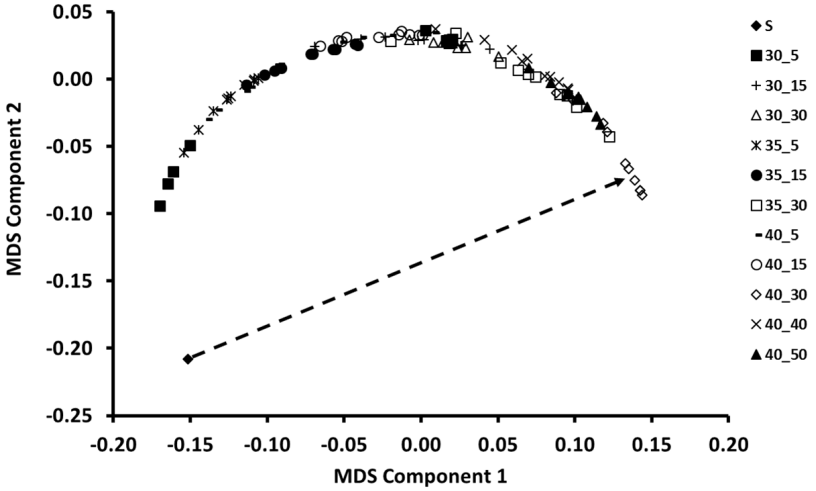

Figure 5: Biplot of MDS (Euclidean) analysis of e-nose signals of milk heated for specific times at specific temperatures (Arrow shows the distance between the reference and optimum measurement condition).

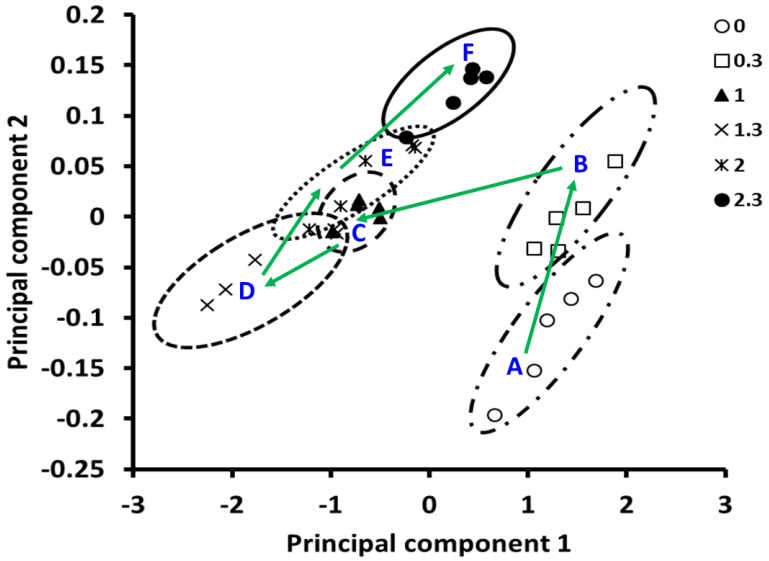

Figure 6: Plot of 2 first principal components of the e-nose signals of milk stored at 25 ^{o}C as a function of time (56 hours or 2.3 days of storage) (Circles show the groupings).

and F showed an onset shift from day 9, which could be related to the beginning of changes in the volatile profile of milk. The same transition was also observed when the microbial count indicated the onset of spoilage. Thereafter, a clear and considerable shift of clusters to the right side of the biplot was observed for samples collected on days 12 and 15. This shift was related to the significant increase in the bacterial count, which was likely due to the formation of new volatile compounds. Rashid et al. (2019) detected six compounds in fresh pasteurized milk stored at 4 ^{o}C (i.e. 2 ketones, 2 alcohols, and 2 aldehydes). The numbers of compounds increased to 12 (3 ketones, 7 alcohols, and 2 aldehydes) at the end of a 19 days storage period. In the present study, a significant change in volatiles was observed on day 12 instead of day 19. This variation between the current study and the literature data presented above could be due to the initial microbial contamination (types and microbial counts) in the fresh milk.

It was clearly observed that LDA was a better classifier compared to the PCA. This was due to the linear assumption of PCA which is not designed to separate groups. It is only designed to show the directions of maximum variability along with a series of the independent axis (i.e. it removes the correlations between variables). However, LDA is a linear classifier and calculates the best linear combination of variables in separating the known groups. Therefore, LDA has the ability to handle complex linear responses of e-nose signals. In addition, it improves the discrimination by maximizing the separation between the groups while minimizing the variance within the groups and within the inter-groups (Tohidi et al., 2018). Furthermore, the LDA was successfully used to predict the shelf life of pasteurized milk (Vallejocordoba & Nakai, 1994b).

3.5 Quality Grade Assessment of Pasteurized Milk

The e-nose could also be used to classify different quality grades of milk in addition to the discriminating milk during storage periods. In this study, three grades of pasteurized milk (i.e. stored at 4 ^{o}C) were proposed based on the volatiles measured by the e-nose and the bacterial count (Table 1 and Fig. 10): excellent, good,

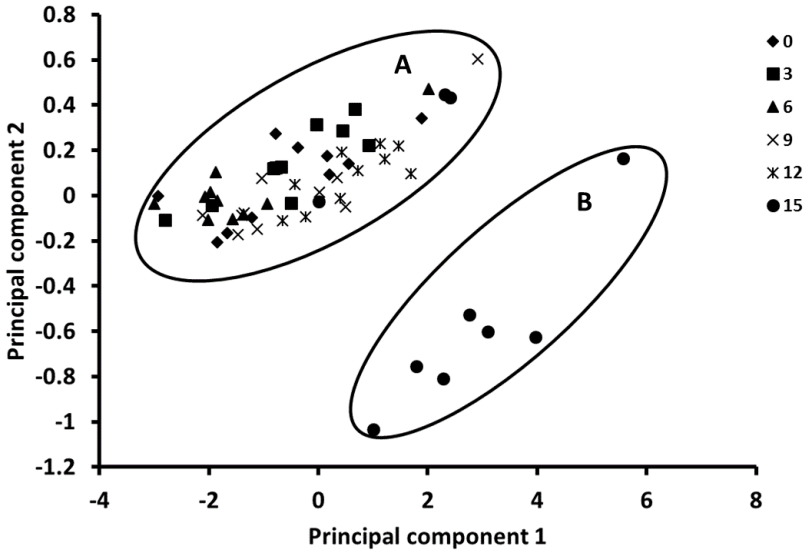

Figure 7: Biplot of principal components analysis of e-nose signals of milk stored at 4 ^{o}C as a function of time (15 days of storage). (Circles show the groupings).

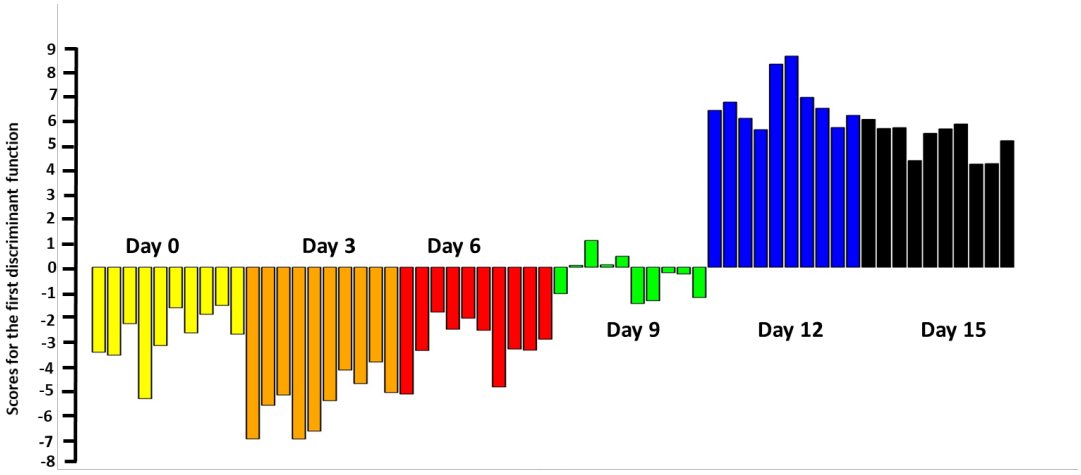

Figure 8: First discriminant scores (LD1) as a function of time (days).

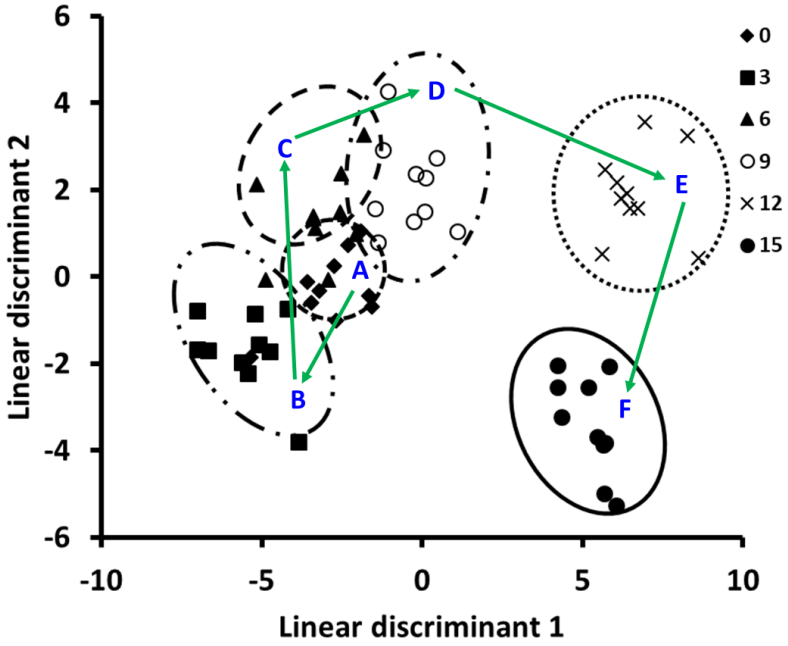

Figure 9: Biplot of Linear Discriminant Analysis of e-nose signals of fresh pasteurized milk stored at 4 ^{o}C as a function of time (15 days). (Circles show the groupings).

Table 1: Quality classes of pasteurized milk during common storage (4 ^{o}C).

Quality Class	Storage (days)	Total Bacterial Count ($\log_{10}$ cfu mL^{-1})
Excellent	0-6	$\leq 2.48 \pm 0.04$
Good	9	4.89 ± 0.21
Poor	12-15	$\geq$ *5.00

* Spoilage onset

Table 2: Quality classes of pasteurized milk during room temperature storage (25 ^{o}C)

Quality Class	Storage (days)	Total Bacterial Count ($\log_{10}$ cfu mL^{-1})
Non-spoiled	0-0.3	$\leq 2.42 \pm 0.20$
Spoiled	1-2.3	$\geq$ *5.00

* Spoilage onset

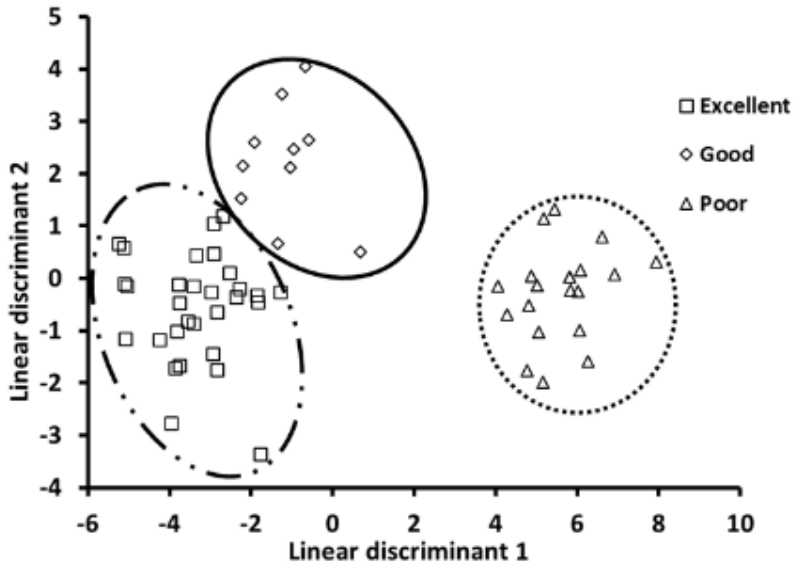

Figure 10: Quality classes of fresh pasteurized milk stored at 4 ^{o}C as a function of time (15 days) (Circle show the groupings).

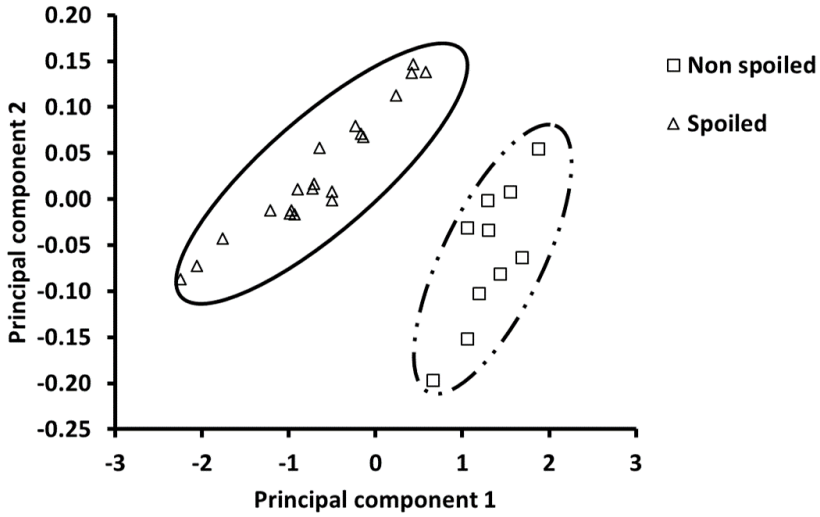

Figure 11: Quality classes of fresh pasteurized milk stored at 25^{o}C as a function of time (2.3 days).

and poor compared to two quality classes (i.e. non-spoiled and spoiled) of milk stored at 25 °C (Table 2 and Fig. 11). In general, the e-nose was a good classifier of the volatile profiles of pasteurized milk stored both at 4 °C and 25 °C. Similarly, Vallejocordoba and Nakai (1994b) classified the quality of pasteurized milk into three classes, good, marginal, and poor using LDA.

4 Conclusions

The current study investigated the possibility of using the e-nose for shelf life determination of milk during storage (i.e. at 25 °C and 4 °C). The electronic nose responses were correlated with the shift in the bacterial count of the tested milk samples. In addition, the e-nose was a good classifier of the aroma print of pasteurized milk at both room temperature and during refrigerated storage. Furthermore, milk quality was successfully classified using e-nose sensor responses and microbial data into three classes namely excellent, good, and poor. Early detection of milk spoilage is very important in reducing economic loss and the health risks of produced milk. Aroma-based detection of milk spoilage was very effective and could express both milk ageing and end of shelf life.

Acknowledgements

The authors would like to acknowledge the financial support from SQU-UAEU collaborative project CL/SQU-UAEU/2016/03.

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International Journal of Food Studies | IJFS October 2021 | Volume 10 | pages 398–410

Effect of Storage on Physico-Chemical, Microbiological and Sensory Characteristics of Goat Milk Fermented by *Lactobacillus* Strains Isolated from Minas Artisanal Cheeses

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Received: 2 September 2020; Published online: 18 October 2021

Abstract

Lactobacillus spp. are lactic acid bacteria which have important implications for the food industry due to their fermentation capacities. The aims of this research were to produce fermented goat milks with *Lactobacillus plantarum* B7 and *Lactobacillus rhamnosus* D1, isolated from Brazilian artisanal cheeses, and to evaluate their physico-chemical, microbiological and sensorial qualities during 30 days of storage at 7 °C. The goat milks, fermented by B7, D1, co-culture and a *Lactobacillus casei* Shirota control, possessed acceptable physico-chemical characteristics to meet fermented milk standards established by Brazilian legislation and maintain the viability of *Lactobacillus* spp. throughout the shelf life of the products. The products were microbiologically safe. D1 fermented goat milk gave higher consumer sensory quality acceptance and purchase intention ($p<0.05$) than other treatments, thus *Lactobacillus rhamnosus* D1 is recommended for fermented goat milk production.

Keywords: Lactic acid bacteria; Probiotics; Food quality; Milk and dairy products; Fermentation

1 Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, facultative anaerobic or microaerophilic, rod or cocci-shaped microorganisms. LAB may be naturally found in some foods such as animal products and vegetables, soil, water and mucosa of animals. The production of lactic acid by carbohydrate fermentation represents an important biochemical pathway of these bacteria (Gaenzle, 2015). Among the 13 genera of LAB, *Lactobacillus* spp. are the most commom and are generally recognized as safe by agencies such as Food and Agriculture Organization of the United Nations (FAO), Food and Drug Administration (FDA), Qualified Presumption of Safety (QPS) and European Food Safety Authority (EFSA) (Bermudez-Humaran et al., 2013). In addition, studies have shown that some *Lactobacillus* strains can be considered probiotics, that is, they may promote health benefits when consumed in adequate concentrations (Food and Ag-

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riculture Organization, 2002). For these reasons, *Lactobacillus* spp. are widely used in fermented foods (Ranadheera et al., 2016).

Goat milk is considered a functional food due to its health-beneficial properties, mainly the easier digestion and absorption of its components compared to the milk of other species (Slacanac et al., 2010). Because of its functional characteristics, goat milk is recommeded to individuals with malabsorption syndromes, neoplasms and allergies to cow milk (Yadav et al., 2016). Goat milk also has higher levels of macro and micronutrients than milks from other mammals (El-Hatmi et al., 2015). However, goat dairy products are rejected by many consumers because of their sensory characteristics. They have high concentrations of short and medium-chain fatty acids, such as butyric, caproic, caprylic, capric, lauric and myristic acids, which are responsible for the development of a "goaty" flavour (Slacanac et al., 2010).

In this regard, the fermentation of goat milk presents itself as a mechanism for reducing the perception of the "goaty" flavour. Some studies have shown that goat milk fermented by *Lactobacillus* strains had high acceptance by consumers (Ranadheera et al., 2016). This suggests that fermented goat milk may be an interesting option for the introduction of goat dairy products to consumers, while combining the health benefits provided by goat milk and probiotics. Based on this, the aims of this study were to produce fermented goat milks with *Lactobacillus plantarum* B7 and *Lactobacillus rhamnosus* D1, isolated from Brazilian artisanal cheeses, and to evaluate their physico-chemical, microbiological and sensorial qualities during 30 days of storage at 7 ^{o}C.

2 Materials and Methods

2.1 Lactic acid bacteria strains

The cultures of *L. plantarum* B7 and *L. rhamnosus* D1 were previously isolated from Minas artisanal cheeses produced in the Serra da Canastra region, Brazil. Both strains had shown probiotic properties in previous *in vitro* assays (Costa et al., 2013). *L. casei* Shirota (LC), used as control, was donated by the Departamento de Microbiologia at the Universidade Federal de Minas Gerais, Brazil.

2.2 Milk and inoculum preparation

8 % (w/v) sucrose was added to raw goat milk and the solution sterilized by autoclaving at 110 ^{o}C for 10 minutes (Autoclave CS, Generalmed, São Paulo, Brazil). Each strain was aerobically cultured in MRS broth (Difco, Detroit, Michigan, United States) at 37 ^{o}C from 24 to 48 hours to prepare the inocula. Then, 5 mL of the broths were transferred to glass bottles containing 200 mL of sterilized goat milk solution, followed by incubation at 37 ^{o}C until coagulation. The inocula of B7 and D1 reached a count of 6.1 x 10^{8} and 2.4 x 10^{8} cfu/mL, respectively. Next, the inocula were added to the sterilized goat milk, in the concentration of 2.5 % (v/v), to start the fermentation process. In the co-culture of B7 and D1, the concentration of each inoculum was 1.25 % (v/v) in the sterilized goat milk.

2.3 Determination of fermentation kinetics

Initially, a study was conducted to determine the fermentation kinetics of B7, D1 and co-culture in goat milk. For this, the strains were inoculated in a liter of sterilized goat milk followed by incubation at 37 ^{o}C for 24 hours. Milk samples were collected at time 0 (immediately after inoculation of the strains) and every two hours until 24 hours of fermentation. The samples were analyzed for pH (Gehaka PG1800 digital pH meter, São Paulo, Brazil), titrable acidity (0.1N NaOH) and *Lactobacillus* counts on MRS agar (Difco, Detroit, Michigan, United States) (International Dairy Federation, 1988). Each assay was performed in triplicate.

2.4 Production of fermented goat milk

Goat milks, fermented by B7, D1, co-culture and LC, were produced for micobiological, physico-

chemical and sensory analyses. Each inoculum was added into erlenmeyers containing two liters of sterilized goat milk solution, with 8 % (w/v) sucrose. Then, the erlenmeyers were incubated at 37 ^{o}C and the fermentation time was determined by the results of previously performed fermentation kinetics. Soon after the end of the fermentation process, the erlenmeyers were stored in a BOD incubator (Eletrolab, São Paulo, Brazil), at 7 ^{o}C for 30 days, simulating the conditions of a domestic refrigerator. Each assay was also performed in triplicate.

2.5 Fermented goat milk analyses

Microbiological analyses

Fermented goat milks were analyzed after 0, 15 and 30 days of storage for the presence of *Salmonella* spp., the most probable number of coliforms at 30 ^{o}C and 45 ^{o}C, counts of coagulase-positive *Staphylococcus* and counts of molds and yeasts (Downes & Ito, 2001; Tournas et al., 2001). The count of *Lactobacillus* was also performed in order to evaluate the bacterial viability during storage (International Dairy Federation, 1988). The microbiological analyses were carried out in triplicate.

Physico-chemical analyses

Fermented goat milks at 0, 15 and 30 days of storage were submitted to the following physico-chemical analyses, according to Association of Official Analytical Chemists (2019): titratable acidity by titration with 0.1N NaOH (Official Methods of Analysis, 2019a); pH (Gehaka PG1800 digital pH meter, São Paulo, Brazil); and contents of fat by Roese-Gottlieb method (Official Methods of Analysis, 2019d), protein by micro-Kjeldahl method (Official Methods of Analysis, 2019c) solids and ash by gravimetric method (Official Methods of Analysis, 2019b). The free fatty acids concentration of the samples was also measured following the adapted methodology described by Deeth et al. (1975). The physico-chemical analyses were also performed in triplicate.

Sensory analysis

The evaluation of the sensory characteristics and purchase intention of fermented goat milks after 15 and 30 days of storage were also performed in three repetitions. Seventy non-trained panelists were served with approximately 30 mL of each sample, kept at 10 ^{o}C. Sensory evaluations were recorded on a 5-point hedonic scale, with 1 representing 'dislike very much' and 5 'like very much'. The purchase intention of each product was also determined by panelists, who had to mark "yes" or "no", if they would buy the product. Lastly, panelists were given the option to make general comments about the samples.
The consumer acceptability was determined by equation (1), proposed by Emediato et al. (2009). According to the authors, an acceptable outcome in sensory tests would be obtained when the sample acceptability index was equal to or greater than 70 %.

$$\text{Acceptability index} = \frac{\text{Average score}}{\text{Maximum value}} \times 100 \quad (1)$$

The sensory analyses were approved by the Ethics Committee on Research with Human Beings at the UFMG under protocol number: CAAE-48320015.1.0000.5149.

2.6 Statistical analysis

Data analyses were performed using GraphPad Prism 7.0 software (GraphPad software, San Diego, CA, USA). The means of physico-chemical parameters of fermented goat milks were submitted to Two-way ANOVA and compared using the Tukey test at a 5 % significance level. The same statistical test was used to compare *Lactobacillus* spp. counts during the storage period, after log-transformation of the data. The means of acceptance scores in sensory analyses were compared by the Tukey (between treatments) and t tests (between days of storage), at a significance level of 5 %. Finally, the purchase intention was analyzed by the Fisher test, at a significance level of 5 %.

3 Results and Discussion

3.1 Fermentation kinetics profiles

The changes of pH, titratable acidity and *Lactobacillus* count during goat milk fermentation by B7, D1 and co-culture are shown in Figures 1, 2 and 3. The curves are representative of the fermentation process because they illustrate the bacterial growth in milk and acid production by the strains over time (decreasing pH and increasing titratable acidity). The pH values between treatments did not show significant differences in the first ten hours of fermentation. However, after 12 hours, goat milk fermented by B7 had a significantly lower pH than the others. Statistical differences between the three treatments were verified after 18 hours of fermentation and the mean of the pH values were found to be 4.18 (B7), 4.80 (D1) and 4.53 (co-culture) at the end of the fermentation process. The titratable acidity curves presented the same trend: the same lactic acid concentrations in the goat milks fermented by B7, D1 and co-culture up to ten hours of fermentation and differences between them after 20 hours. The lactic acid concentrations in the goat milks fermented by B7, D1 and co-culture were, respectively, 0.90, 0.61 and 0.77 g/100 g at 24 hours.

Minervini et al. (2009) observed a more pronounced reduction in pH during fermentation of goat milk by *L. plantarum*. They used 1 % (v/v) of inoculum with 10^7 cfu/g. After eight hours of incubation at 30 ^{o}C, the pH value was approximately 4.60. Despite differences in inoculum and incubation conditions, this result highlights the variation in the fermentative pattern that may exist between different strains. Salva et al. (2011) also showed that co-cultures of *L. rhamnosus* and *Streptococcus thermophilus* with different ratios caused a faster pH decrease in goat milk, when incubated at 42 ^{o}C.

B7 showed higher growth in the inoculm preparation stage, according to the highest cell concentration at the beginning of the fermentation (time 0). The differences between B7 and D1 and co-culture counts remained throughout the whole process, resulting in a higher fermentation rate for the strain B7, observed in the pH and titratable acidity curves. On the other hand, among the three treatments, D1 showed the lowest initial counts. This profile resulted in the lowest pronounced increase of acidity and decrease of pH in goat milk during fermentation. In the study of (Zalan et al., 2010), *L. rhamnosus* also produced less organic acid than other bacteria when used to ferment skim milk. Among the *Lactobacillus* strains used for fermentation of different substrates - such as milk and MRS broth - for 18 hours, the products with *L. rhamnosus* showed the lowest titratable acidity. In addition, Gaudreau et al. (2005) suggest that the growth and consequently the acid production of *L. rhamnosus* in cow milk are slower than that of different bacteria.

The curves of pH, titratable acidity and bacterial counts in the goat milk fermented by co-culture were intermediate to the B7 and D1 curves, that is, while B7 and D1 showed the highest and the lowest values, respectively, the co-culture results were within the range of the values found for the isolate cultures. Ranadheera et al. (2016) also observed the same behavior comparing the fermentation of goat milk by co-culture and pure cultures of *Lactobacillus*. The fermented milk standards established by the Brazilian legislation are titratable acidity ranging from 0.6 to 2 g/100 g and *Lactobacillus* count higher than 10^6 cfu/g (Brasil, 2007). In addition, studies have shown that the final pH to maintain the viability of *Lactobacillus* spp. throughout the shelf life of the product should be from 4.5 to 5.0 (Lee & Salminen, 1995). Based on this information and the fermentation kinetics' profiles, it was possible to determine the fermentation time for each treatment as 16, 24 and 20 hours, respectively for B7, D1 and co-culture. At these times, it is possible to associate approximate values of pH (4.89 $\pm$ 0.02, 4.80 $\pm$ 0.24 and 4.83 $\pm$ 0.19, respectively), titratable acidity (0.55 $\pm$ 0.01, 0.61 $\pm$ 0.10 and 0.61 $\pm$ 0.02 g/100 g) and bacterial counts (9.05 $\pm$ 0.13, 8.63 $\pm$ 0.05 and 8.62 $\pm$ 0.32 log cfu/g) to meet the requirements of Brazilian legislation.

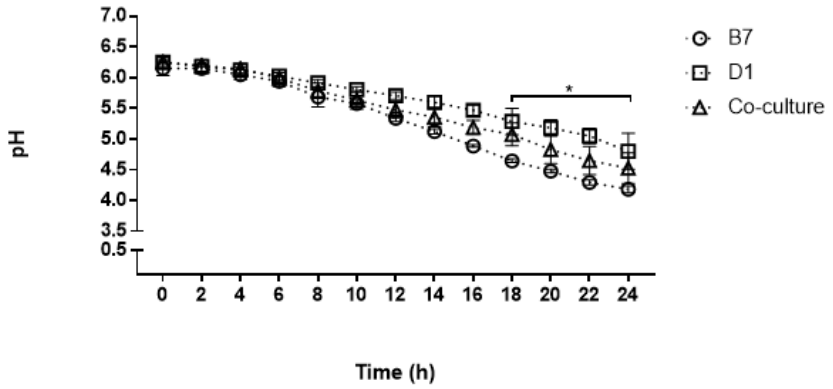

Figure 1: Means and standard deviations of pH during goat milk fermentation by *Lactobacillus* plantarum B7, *L. rhamnosus* D1 and co-culture for 24 hours. Data are plotted as means ± SD. The results shown are the average of triplicate experiments. *($p < 0.05$) Two-way ANOVA, followed by Tukey's test.

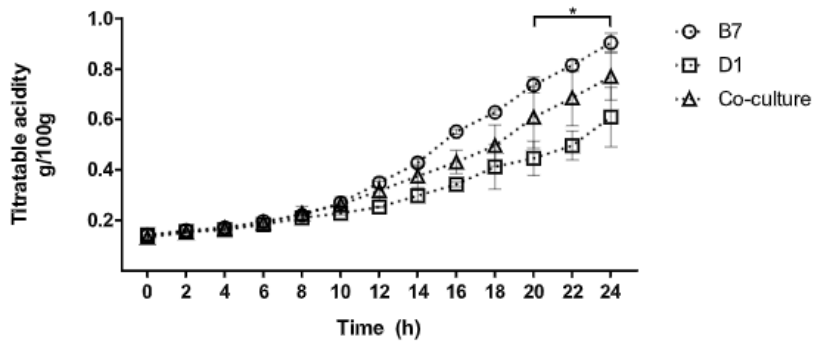

Figure 2: Means and standard deviations of titratable acidity during goat milk fermentation by *Lactobacillus plantarum* B7, *L. rhamnosus* D1 and co-culture for 24 hours. Data are plotted as means ± SD. The results shown are the average of triplicate experiments. *($p < 0.05$) Two-way ANOVA, followed by Tukey's test.

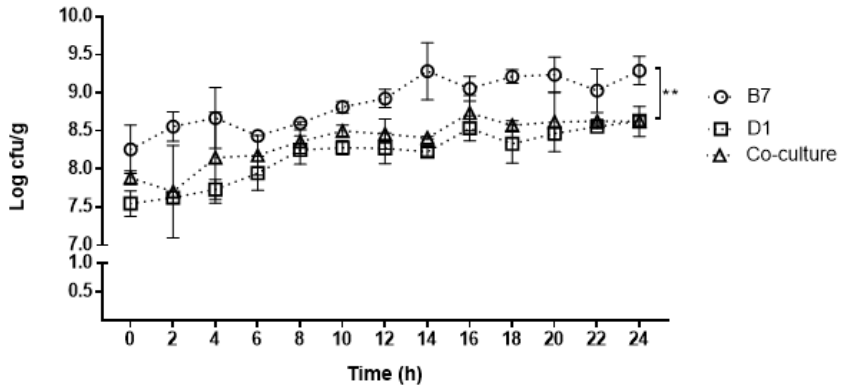

Figure 3: Means and standard deviations of *Lactobacillus* count during goat milk fermentation by *Lactobacillus plantarum* B7, *L. rhamnosus* D1 and co-culture for 24 hours. Data are plotted as means ± SD. The results shown are the average of triplicate experiments. *($p < 0.05$) Two-way ANOVA, followed by Tukey's test.

3.2 Physico-chemical parameters of fermented goat milk during storage

Titratable acidity

Only the sample D1 on 0 day of storage had titratable acidity outside the range established by the Brazilian legislation (from 0.6 to 2.0 g/100 g). However, it can be seen that the results of this parameter were adequate in the subsequent analyses (Brasil, 2007).

During the storage period, an increase in the acidity of the fermented milks with B7 and D1 was observed, while the milks inoculated with co-culture and LC showed a decrease in acidity from 15 to 30 days. The increase in acid concentrations during refrigeration is an expected phenomenon. Lactic acid bacteria can maintain their metabolism even at temperatures ranging from 0 to 5 ^{o}C. The decrease in the titratable acidity of fermented milks stored under refrigeration was also observed by Amani et al. (2017). Bacterial cultures with high proteolytic activity may cause a decrease of acidity in products under these conditions, due to the production of alkaline compounds (Lim et al., 2019). As noted in Table 1, this event may not directly influence pH changes since the behavior of this parameter is determined by other acidic components besides lactic acid, such as fatty acids.

Approximate values of titratable acidity were found in other studies on physico-chemical parameters of fermented goat milk under refrigeration. Salva et al. (2011) found results ranging from 0.60 to 0.95 g/100 g in products stored at 4 ^{o}C for 15 days, varying according to the storage time and the concentration and proportion of inoculum added. Using *L. rhamnosus* as a starter culture, dos Santos et al. (2017) found titratable acidity of 0.52, 0.69 and 0.87 g/100 g in products with 1, 14 and 28 days of refrigeration, respectively.

pH

The lower pH values were observed in goat milks fermented by B7, coinciding with the higher concentration of lactic acid obtained in the titratable acidity analysis. There was no significant difference (p>0.05) between them and the pH of the products with co-culture and LC, while D1 fermented milks had significantly (p<0.05) higher pH than the others treatments, independently of the storage period. Regardless of the starter culture, there was a tendency to decrease pH throughout the refrigeration. As discussed, post-acidification is an expected process, but if

Table 1: Results of physico-chemical analyses of goat milk fermented by *Lactobacillus plantarum* B7, *L. rhamnosus* D1, co-culture and *L. casei* Shirota (LC) during 30 days of storage at 7 ^{o}C

Parameter	Storage days	*Lactobacillus* strain			
		B7	D1	Co-culture	LC
Titratable acidity	0	$0.68^{Bac} \pm 0.10$	$0.49^{Bb} \pm 0.07$	$0.65^{Ca} \pm 0.12$	$0.78^{Bc} \pm 0.11$
(g/100 g)	15	$0.92^{Aa} \pm 0.02$	$0.73^{Ab} \pm 0.04$	$0.90^{Aa} \pm 0.09$	$0.91^{Aa} \pm 0.03$
	30	$0.94^{Aa} \pm 0.14$	$0.73^{Ab} \pm 0.04$	$0.80^{Bb} \pm 0.09$	$0.76^{Bb} \pm 0.03$
	0	$4.53^{Abc} \pm 0.30$	$5.20^{Aa} \pm 0.19$	$4.72^{Ab} \pm 0.29$	$4.36^{Ac} \pm 0.20$
pH	15	$3.99^{Bb} \pm 0.09$	$4.42^{Ba} \pm 0.27$	$4.14^{Bb} \pm 0.07$	$4.08^{Bb} \pm 0.13$
	30	$3.93^{Bb} \pm 0.10$	$4.21^{Ca} \pm 0.18$	$4.02^{Bb} \pm 0.10$	$4.01^{Bb} \pm 0.13$
Free fatty acids	0	$1.2^{Ba} \pm 0.12$	$1.09^{Ba} \pm 0.11$	$1.2^{Ba} \pm 0.21$	$1.09^{Ba} \pm 0.20$
(μ equiv./mL)	15	$1.09^{Ba} \pm 0.10$	$1.07^{Ba} \pm 0.18$	$1.07^{Ba} \pm 0.10$	$1.07^{Ba} \pm 0.16$
	30	$1.55^{Aa} \pm 0.24$	$1.41^{Aa} \pm 0.22$	$1.59^{Aa} \pm 0.28$	$1.8^{Aa} \pm 1.05$
Fat	0	$3.04^{Aa} \pm 0.14$	$3.34^{Ab} \pm 0.20$	$3.23^{Aab} \pm 0.14$	$3.32^{Ab} \pm 0.36$
(g/100 g)	15	$3.01^{Aa} \pm 0.27$	$3.25^{Aa} \pm 0.31$	$3.13^{ABa} \pm 0.11$	$3.03^{Ba} \pm 0.11$
	30	$2.9^{Aa} \pm 0.23$	$3.07^{Ba} \pm 0.15$	$3.04^{Ba} \pm 0.05$	$3.00^{Ba} \pm 0.09$
Protein	0	$2.73^{Aa} \pm 0.16$	$2.85^{Aa} \pm 0.10$	$2.83^{Aa} \pm 0.04$	$2.78^{Aa} \pm 0.13$
(g/100 g)	15	$2.64^{Aa} \pm 0.14$	$2.60^{Ba} \pm 0.11$	$2.69^{Ba} \pm 0.10$	$2.64^{Ba} \pm 0.17$
	30	$2.50^{Ba} \pm 0.17$	$2.57^{Ba} \pm 0.09$	$2.55^{Ca} \pm 0.06$	$2.55^{Ba} \pm 0.11$
Total solids	0	$17.93^{Aa} \pm 0.45$	$17.88^{ABa} \pm 0.19$	$18.05^{Aa} \pm 0.35$	$18.02^{Aa} \pm 0.41$
(g/100 g)	15	$18.17^{Aa} \pm 0.77$	$18.31^{Aa} \pm 0.63$	$18.30^{Aa} \pm 0.32$	$18.22^{Aa} \pm 0.56$
	30	$17.05^{Ba} \pm 0.20$	$17.39^{Ba} \pm 0.36$	$17.29^{Ba} \pm 0.21$	$17.33^{Ba} \pm 0.28$
Ash	0	$0.69^{Ba} \pm 0.02$	$0.70^{Ba} \pm 0.02$	$0.68^{Ba} \pm 0.03$	$0.68^{Ba} \pm 0.02$
(g/100 g)	15	$0.73^{Aa} \pm 0.03$	$0.73^{Aa} \pm 0.03$	$0.72^{Aa} \pm 0.03$	$0.72^{Aa} \pm 0.02$
	30	$0.73^{Aa} \pm 0.03$	$0.72^{ABa} \pm 0.03$	$0.71^{Aa} \pm 0.03$	$0.71^{ABa} \pm 0.04$

$^{a-c}$Means within rows with distinct superscripts differ significantly ($p < 0.05$); $^{A-C}$Means within columns with distinct superscripts differ significantly ($p < 0.05$).

exacerbated, it can lead to undesirable changes in the products, such as bacterial death, syneresis and rejection by consumers (Coggins et al., 2010). In another study, fermented goat milks showed pH oscillating from 3.83 to 4.60 during 28 days of storage (dos Santos et al., 2017).

Free fatty acids

A significant difference (p<0.05) between the concentration of free fatty acids of the treatments was not observed. However, significant increases (p<0.05) in the content of these substances were observed in the products analyzed after 30 days of refrigeration.

Lipolysis may occur in dairy products from the activity of enzymes, such as lipases, produced by psychrotrophic or natural lipases of milk. However, sterilization of milk immediately after milking is capable of denaturing endogenous lipases and eliminating the producing bacteria, although it has no effect on enzymes that have previously been produced by psychrotrophs. In this way, it was concluded that the lipolytic activity of B7, D1, co-culture and LC in the fermented goat milks presented the same intensity during the storage.

Fat

In general, practically no significant differences (p>0.05) in fat content were observed between the treatments. However, goat milks fermented by D1, co-culture and LC showed a decrease in the fat percent over the storage period. The development of lipolytic microorganisms in products is attributed as the main cause of this reduction. As observed in the analysis of free

fatty acids, all strains studied were able to cause lipolysis which may have resulted in this decrease.
Most of the similar studies showed products with higher fat contents, reaching a concentration of 5.37 g/100 g (Ranadheera et al., 2016; Salva et al., 2011). These differences may occur due to variations in the composition of the raw material, since fat is the component most subjected to oscillations in milk. In addition, this study used fresh milk, without addition of cream but with addition of 8 % (w/v) sucrose, which exerted a dilutive effect on the other dairy components.

Protein

No sample, regardless of the treatment or refrigeration period, presented the minimum value of 2.9 g/100 g established by the Brazilian legislation. The same regulation provides that fermented milks may have lower fat and protein concentrations than those recommended where other substances are added, such as sucrose (Brasil, 2007).
No significant differences ($p>0.05$) were observed between the treatments as to the protein content but during the storage period there were significant decreases ($p<0.05$). Ahmed and Razig (2017) reported the influence of the proteolytic activity of cultures and the casein hydrolysis process on the decrease of protein concentration in yogurts during storage. In other studies on fermented goat milk, the protein contents were higher and ranged from 3.51 to 5.39 g/100 g (Ranadheera et al., 2016; Salva et al., 2011). These differences may occur due to the quality of the raw material, as well as the addition of protein components to milk, such as milk powder and whey (Martin-Diana et al., 2003).

Solids

As observed in the analysis of fat and protein contents, the solids concentration also showed a significant decrease ($p<0.05$) during the 30 days of refrigeration. Reports in the scientific literature indicated variations in solids contents of fermented goat milk, ranging from 11.5 to 18.9 g/100 g (Martin-Diana et al., 2003; Ranadheera et al., 2016). As already discussed, these differences are due to the composition of raw milk used and the addition of solid components during production.

Ash

Although the statistical analysis has shown differences in ash content over the storage period, numerically the variation observed between the means is practically inexpressive. In other studies, the ash content ranged from 0.75 to 1.39 g/100 g (Martin-Diana et al., 2003; Ranadheera et al., 2016; Salva et al., 2011). The addition of compounds to milk is the main cause of variation of ash concentration in fermented milks. The ash content in the fermented goat milks produced did not reach higher values since the addition of sucrose exerted a dilutive effect. On the other hand, Martin-Diana et al. (2003) added whey protein concentrate, which has a high mineral content, to the products.

3.3 Microbiological parameters of fermented goat milk during storage

There was no detection of potentially pathogenic microorganisms in the fermented goat milks analyzed, irrespective of the storage period. Cisse et al. (2019) highlighted that fermented goat milks produced under uncontrolled hygienic conditions may have an undesirable microbial population. On the other hand, the enumeration of *Lactobacillus* in the products indicated that these bacteria remained viable throughout the storage period, as shown in Figure 4. While B7, D1 and LC showed constant counts during refrigeration, the co-culture counts showed a significant decrease ($p<0.05$), from days 0 to 15, and from days 0 to 30. However, B7, D1, co-culture and LC counts were greater than 108 cfu/g in fermented goat milks during the 30 days. Likewise, Moreno-Montoro et al. (2018) observed that the counts of *L. plantarum* C4 were maintained above 108 cfu/mL in fermented goat milk stored for six weeks. *Lactobacillus* count in the analyzed products met the requirements established by the Brazilian legislation for inspection of fermented milks and products containing pro-

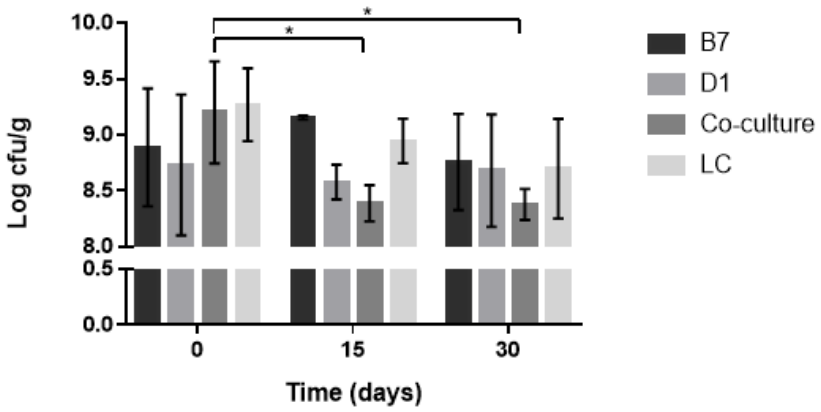

Figure 4: Enumeration of *Lactobacillus plantarum* B7, *L. rhamnosus* D1, co-culture and *L. casei* Shirota (LC) in fermented goat milk during 30 days of storage at 7 ^{o}C. Data are plotted as means ± SD. The results shown are the average of triplicate experiments. *($p < 0.05$) Two-way ANOVA, followed by Tukey's test.

biotics (Brasil, 2007; Ranadheera et al., 2016). The strains analyzed in this research presented a better stability over the storage period compared to other bacteria described in previous studies, in which the lactic acid bacteria count in fermented goat milks showed a reduction of up to 1 log during the storage (Martin-Diana et al., 2003; Ranadheera et al., 2016). On the other hand, dos Santos et al. (2017) observed that *L. rhamnosus* counts remained equal during the first seven days of storage of fermented product made from goat milk (7.38 log cfu/g) and presented a considerable increase until day 28 (7.78 log cfu/g).

3.4 Sensory analyses of fermented goat milk during storage

The overall acceptability, the purchase intention and the acceptability indexes of fermented goat milks attributed by the panelists are shown in Table 2. It can be noticed that the goat milks fermented by D1 excelled over the others in the three evaluated items, both after 15 and 30 days of storage. On the other hand, the goat milks fermented by co-culture tended to present the worst results of sensorial attributes.

The scores attributed to goat milks fermented by B7, D1 and LC were significantly higher ($p<0.05$) than the product inoculated with co-culture after 15 days of refrigeration. At 30 days of storage, there was a considerable decrease ($p<0.05$) in the acceptability of B7 and LC products, while D1 maintained the same score. Although the goat milks fermented by co-culture did not show any variation ($p>0.05$) in acceptability between the analyzed periods, it remained with a lower score ($p<0.05$) than fermented milk D1 at the end of the storage period.

According to the observations made by the panelists, the goat milks fermented by co-culture with 15 days of storage had a "goaty" flavour, being a factor that may have contributed to the lower acceptance. Lipolysis could explain such perception by the panelists. However, there was no significant difference ($p>0.05$) between the concentration of free fatty acids in the products. In addition, the texture was another point highlighted by the panelists. Reports indicated that the product containing co-culture showed greater fluidity. However, as the evaluation was done by untrained panelists, the comparative standards used by them were commercial fermented milks, which are added with additives that positively affect texture perception. Martin-Diana et al. (2003) also observed lower acceptance scores of

Table 2: Overall acceptability, purchase intention and acceptability index of goat milk fermented by *Lactobacillus plantarum* B7, *L. rhamnosus* D1, co-culture and *L. casei* Shirota (LC) after storage at 7 ^{o}C for 15 and 30 days

Parameter	Storage days	*Lactobacillus* strain			
		B7	D1	Co-culture	LC
Overall acceptability	15	$3.28A^{Ab} \pm 1.09$	$3.54^{Aa} \pm 1.08$	$3.14^{Ab} \pm 1.08$	$3.54^{Aa} \pm 1.12$
	30	$2.91^{Bb} \pm 1.23$	$3.53^{Aa} \pm 1.06$	$2.99^{Ab} \pm 1.14$	$3.13^{Bb} \pm 1.14$
Purchase intention (%)	15	49.05^{Ab}	60.00^{Aa}	45.71^{Ab}	58.10^{Ab}
	30	39.52^{Bb}	60.66^{Aa}	38.10^{Ab}	44.29^{Bb}
Acceptability index (%)	15	65.60	70.80	62.80	70.80
	30	58.20	70.60	59.80	62.60

$^{a-c}$Means within rows with distinct superscripts differ significantly ($p < 0.05$); $^{A-C}$Means within columns with distinct superscripts differ significantly ($p < 0.05$).

fermented goat milk when they had lower viscosity and firmness values. Similarly, Ranadheera et al. (2016) observed that the texture is one of the main determinants of the differences observed during the evaluation of yogurts made from goat milk.

At 30 days of storage, the panelists indicated changes in the texture and flavor of goat milks fermented by B7 and LC. Descriptions of flavors that refer to whey and cheese may have contributed to the reduction of acceptance of these products. Whey flavor may be indicative of the occurrence of syneresis throughout storage. Ranadheera et al. (2016) showed that the period of higher syneresis coincided with the lower scores of the products. In addition, the cheese flavor suggests the occurrence of proteolysis in these products, since this is one of the main biochemical reactions that occur during cheese ripening. Proteolysis can be triggered by several factors, such as bacterial enzyme activity, including those produced by lactic acid bacteria (Salva et al., 2011). The reduction in the acceptability of both products with 30 days of storage coincides with the period of higher concentration of free fatty acids. Although this factor did not cause an apparent effect on the acceptance of goat milks fermented by D1, it may have influenced the results of the other treatments.

The goat milks fermented by D1 was also superior to the other products regarding the purchase intention throughout the whole storage period ($p<0.05$). In addition, these products were able to maintain the same standard during refrigeration, since the purchase intention was statistically similar ($p>0.05$) in the two evaluated periods. On the other hand, a significant decrease ($p<0.05$) in purchase intention of products B7 and LC was observed from days 15 to 30 of storage. Garcia and Travassos (2012) observed values varying from 30.95 % to 42.86 % for the purchase intention of fermented goat milk. Alves et al. (2009) observed a purchase intention of approximately 61 % for frozen yogurt made from goat milk added probiotics.

Corroborating with the results already presented, the acceptability index of products inoculated with D1 and LC exceeded 70 %, after 15 days of storage, which are considered satisfactory values. At 30 days of refrigeration, only the D1 fermented milks continued to present an acceptability index in this range, proving their sensory superiority compared to the other treatments.

4 Conclusions

According to Brazilian legislation and scientific reference studies, goat milk fermented by B7, D1, co-culture and LC satisfied microbiological and physico-chemical standards during storage at 7 ^{o}C for 30 days. In addition, the samples were free of pathogenic microorganisms throughout the period and maintained the lactic bacteria counts at a desirable concentration ($>10^8$ cfu/g).

In the sensory analyses, goat milk fermented by D1 showed greater overall acceptability and purchase intention (p>0.05) than the other treatments. Therefore, it is suggested that *L. rhamnosus* D1 is the most promising microorganism to be used as a starter culture for fermentation of goat milk.

Acknowledgements

This research was funded by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Pró-Reitoria de Pesquisa da UFMG.

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