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Preparation and microbiological analysis of Tuscan sausage with added propolis extract

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Abstract

The aim of this study was to obtain hydroethanolic extract of propolis by extraction, assisted by focused microwave, and to apply it in Tuscan-style sausage. The extract was used at concentrations of 0.5%, 1.0% and 2.0% (w/v) in the manufacture of the sausage, which was then analyzed in cold storage at 4 °C for 56 days. The following analyses were performed: mesophilic and psychotrophic organisms; coliforms at 35 and 45 °C; positive and negative-coagulase *Staphylococcus*, sulfite-reducing *Clostridium*, and *Salmonella* spp. The results were below the limits established by the Brazilian legislation, with some changes at the end of the study. Consequently, propolis extract prolonged the shelf life of the Tuscan-style sausage for 56 days and it is therefore an ingredient that can be potentially used in the preparation of this product.

Keywords: sausage; propolis; extraction.

Practical Application: The propolis extract provided microbiological stability to the sausage.

1 Introduction

Propolis is a generic term used to describe a complex mixture of resinous, gummy and balsamic substances that are collected by honeybees from shoots, flowers and plant exudates; the bees add salivary secretions, wax and pollen which results in the creation of propolis. The role of propolis in the hive is related to its mechanical properties and it is used to construct, adapt and protect the hive; its antimicrobial activity ensures an aseptic environment (Funari & Ferro, 2006).

The chemical composition of propolis is very complex and it contains more than 180 identified compounds, of which flavonoids are an important feature. Flavonoids, along with phenolic acids and esters, phenolic aldehydes and ketones are considered to be the most important antimicrobial compounds contained in propolis. The other compounds are volatile oils and aromatic acids (5-10%), waxes (30-40%), resins, balms and pollen, which is a rich source of essential elements such as magnesium, nickel, calcium, iron and zinc. The mechanism of antibacterial activity is considered to be complex and it has been attributed to the synergism between flavonoids, hidroxiácidos and terpenes (Fernandes et al., 2006).

The antimicrobial activity of propolis has a wide range of applications in food technology. One particular advantage is that, unlike some conventional preservatives, propolis and its residues generally have a beneficial effect on human health. Given the above, this study aimed to prepare Tuscan-style sausage with propolis extract (PE) and to evaluate the effect of PE on the microbiological stability of the sausages during storage.

2 Materials and methods

2.1 Obtaining the propolis extract

The extractions were carried out using a focused microwave with two cavities and equipped with glass jars with a maximum capacity of 180 mL (Star System 2, 800 W, CEM, Matthews, NC, USA). The ground propolis was initially weighed (6 g) and then transferred to the glass jars. Then 70% grain alcohol solvent (60 mL) (v/v) at a ratio of 1:10 (w/v) was added and submitted to the effect of microwaves for 20 minutes at 70 °C.

After the end of extraction, the extract was filtered on filter paper and centrifuged at 3000 rpm for 20 min. The supernatant was subsequently concentrated in a rotary evaporator (Fisatom 802), packed in amber bottles, and stored in a freezer (–18 °C) until analysis.

2.2 Preparation of the product

The preparation of the Tuscan-style sausages followed the requirements regarding ingredients described by the relevant legislation (Brasil, 2000) as shown in Table 1. The procedures followed were as described by Terra (1998).

The pork and bacon were initially ground using a grinder (Jamar PJ22, Jamar Ltda, São Paulo, Brazil). Then the raw material was carried to a mixing machine (Jamar MJI 35) where the other ingredients were mixed to obtain a bind. The mixture was subsequently divided into four batches of 5 kg, to which were added the pre-defined concentrations of propolis extract. This provided

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Table 1. Formulation of Tuscan-style sausage.

Raw materials and ingredients	Quantity (g/100g)
Pork meat	85
Bacon	15
Water/ice	3
Salt	2.5
Seasoning for Tuscan-style sausage (BREMIL)	0.5*
White pepper powder	0.1
Flavor enhancer	0.05
Garlic	0.2
Seasoning (BREMIL)	0.25
Fixative (BREMIL)	0.25

*According to the manufacturer's recommendation.

the following four treatments: Treatment 1 Control (0% PE) - no added propolis extract; Treatment 2 (0.5% PE) – Tuscan-style sausage with 0.5% added propolis extract; Treatment 3 (1.0% PE) - Tuscan-style sausage with 1% added propolis extract; and Treatment 4 (2.0% PE) - Tuscan-style sausage with 2% added propolis extract. After mixing, the mixture was packed into pig intestine and then washed to remove the salt; it was then immersed in 1% lactic acid for 30 minutes to hydrate. For storage, the sausages were packed in polystyrene trays, wrapped with plastic wrap, identified and immediately taken to a D.B.O oven (ELETROLAB, model EL 101) and stored at 4 °C.

2.3 Microbiological analyses

The analyses were performed regarding counts for psychotrophic and mesophilic microorganisms (American Public Health Association, 2001); positive and negative-coagulase *Staphylococcus*; coliforms at 35 °C and 45 °C; sulfite-reducing *Clostridium* and *Salmonella* spp (Brasil, 2003). The analyses were performed on days 0, 7, 14, 21, 28, 35, 42, 49 and 56 of storage at 4 °C.

2.4 Statistical analysis

The data were evaluated by analysis of variance (ANOVA). The means were compared by Tukey's test, with a significance level of 95% ($p < 0.05$) using SPSS 17.0 statistical software.

3 Results and discussion

Counts of mesophilic aerobic microorganisms are commonly used to indicate the sanitary quality of food (Franco & Landgraf, 2005) and to detect the number of aerobic or facultative mesophilic bacteria, which are present both in vegetative form and also as spores in food. In the present study microbiological analyses was performed in relation to the meat, pork intestine and propolis extract used in the preparation of the Tuscan-style sausages. The results (not shown) were within the tolerance limits set by the relevant legislation, i.e. RDC No. 12 (Brasil, 2001) and according to Terra (1998), indicating that the raw materials were properly treated, in optimal hygienic conditions, and that they were well kept, with microbiological quality that was sufficient to be used safely in developing products.

The results obtained for the counts of total mesophilic aerobic bacteria, psychotrophic bacteria, positive and negative-coagulase *Staphylococcus*, total coliforms at 35 °C, coliforms at 45 °C, sulfite-reducing *Clostridium* and *Salmonella* spp for the different formulations of Tuscan-style sausages are shown in Table 2.

From the results presented in Table 2, it can be seen that in terms of the means of the total aerobic mesophilic counts at zero storage time there was no significant difference between the treatments; they all showed a value lower than 10^{-6} CFU/g, which has been cited by Terra (1998) as an acceptable level of bacterial contamination.

During the final period of storage (days 49-56) the mesophilic aerobic microorganism count rose and the treatments with added propolis extract showed significant difference compared with the standard; these values were lower than 10^{-6} CFU/g. Therefore, it was possible to see the influence of the addition of propolis extract in the sausages because the lowest counts for mesophilic aerobic bacteria occurred in the sausages with added propolis extract. These values were 5.41; 3.84 and 3.73 Log_{10} CFU/g on day 49; and 5.98; 6.11 and 4.85 Log_{10} CFU/g on day 56 for the treatments with 0.5%, 1% and 2% of added extract, respectively. The standard treatment had average values of 5.97 and 6.44 Log_{10} CFU/g for the same period. During this period visual changes such as molds, yeasts and fungi were observed in greater quantity in the standard treatment than in the other treatments. Values for the count of total mesophilic aerobic bacteria in the present study were lower than those reported by Pereira (2009) when assessing the use of propolis extract (0.10%) in mechanically separated chicken meat at the end of 10 days of refrigerated storage. The aforementioned study found a value of 6.74 log_{10} CFU/g, which was even higher than the standard treatment.

Bradford et al. (1993) have argued that the psychotrophic microorganism count is one of the most important criteria for assessing deterioration due to temperature under refrigeration. In the study conducted by the aforementioned authors, the psychotrophic count increased significantly after seven days of storage. The authors attributed these values to the lactic acid-producing bacteria, which were probably responsible for the deterioration of the meat products. The present study also found increased levels of psychotrophic microorganisms between the treatments, but from day 21 of storage. This increase was significantly greater ($p<0.05$) when compared to the standard treatment (0% PE), with counts higher than the treatment with 2% added PE.

The count for coagulase-negative *Staphylococcus* and coagulase-positive *Staphylococcus* (Table 2) was lower than 1.0 Log_{10} $CFU.g^{-1}$ and showed no significant difference between the treatments during the storage period. The RDC No. 12 (Brasil, 2001) approves the Technical Regulation on microbiological standards for food and states that the tolerance in fresh pork sausage for coagulase-positive *Staphylococcus* is 3×10^3 CFU/g; consequently, all the treatments in the present study remained within the legally allowed limit during storage. According to Lee et al. (2007) the ethanol extract of propolis has antimicrobial activity in relation to *Staphylococcus aureus*.

Table 2. Microbiological analysis of Tuscan-style sausages during storage at 4 °C.

Mesophilic aerobic bacteria (Log_{10} CFU.g^{-1})	**0% PE***	**0.5% PE**	**1% PE**	**2% PE**
Day 0	4.72 ± 0.083^{a}	4.78 ± 0.144^{a}	4.79 ± 0.081^{a}	4.73 ± 0.015^{a}
Day 7	4.57 ± 0.063^{a}	4.51 ± 0.133^{a}	4.60 ± 0.048^{a}	4.56 ± 0.058^{a}
Day 14	4.45 ± 0.079^{b}	4.44 ± 0.093^{b}	4.60 ± 0.078^{a}	4.37 ± 0.054^{b}
Day 21	3.97 ± 0.043^{a}	3.98 ± 0.021^{a}	3.97 ± 0.022^{a}	3.85 ± 0.036^{b}
Day 28	3.94 ± 0.038^{a}	3.87 ± 0.048^{ab}	3.84 ± 0.010^{b}	3.59 ± 0.079^{c}
Day 35	3.82 ± 0.042^{b}	3.87 ± 0.035^{b}	3.99 ± 0.020^{a}	3.64 ± 0.082^{c}
Day 42	4.64 ± 0.048^{b}	4.83 ± 0.064^{a}	4.53 ± 0.048^{c}	3.70 ± 0038^{d}
Day 49	5.97 ± 0.022^{a}	5.41 ± 0.091^{b}	3.84 ± 0.130^{c}	3.73 ± 0.043^{c}
Day 56	6.44 ± 0.034^{a}	5.98 ± 0.021^{c}	6.11 ± 0.047^{b}	4.85 ± 0.045^{d}
Psychotrophic bacteria (Log_{10} CFU.g^{-1})	C	T1	T2	T3
Day 0	4.26 ± 0.057^{b}	4.49 ± 0.117^{a}	4.06 ± 0.035^{c}	3.94 ± 0.061^{c}
Day 7	4.55 ± 0.050^{a}	4.58 ± 0.068^{a}	4.54 ± 0.061^{a}	4.45 ± 0.104^{a}
Day 14	4.53 ± 0.052^{a}	4.68 ± 0.076^{a}	4.56 ± 0.103^{a}	4.62 ± 0.053^{a}
Day 21	4.32 ± 0.055^{a}	4.34 ± 0.037^{a}	4.13 ± 0.095^{b}	4.14 ± 0.056^{b}
Day 28	4.47 ± 0.048^{a}	4.28 ± 0.075^{b}	4.20 ± 0.142^{bc}	4.04 ± 0.051^{c}
Day 35	4.23 ± 0.263^{a}	4.26 ± 0.041^{a}	4.03 ± 0.038^{a}	3.39 ± 0.030^{b}
Day 42	4.74 ± 0.050^{a}	4.75 ± 0.053^{a}	4.89 ± 0.092^{a}	3.97 ± 0.260^{b}
Day 49	6.00 ± 0.029^{a}	5.88 ± 0.048^{a}	5.28 ± 0.122^{b}	4.75 ± 0.128^{c}
Day 56	6.73 ± 0.044^{b}	6.68 ± 0.119^{b}	6.96 ± 0.036^{a}	5.39 ± 0.067^{c}
Coagulase-negative *Staphylococcus* (Log_{10} CFU.g)				
Day 0	3.56 ± 0.078^{a}	3.50 ± 0.031^{a}	3.34 ± 0.070^{b}	3.26 ± 0.024^{b}
Day 7	3.56 ± 0.046^{a}	3.33 ± 0.053^{bc}	3.41 ± 0.037^{b}	3.30 ± 0.037^{c}
Day 14	3.79 ± 0.111^{a}	3.71 ± 0.036^{a}	3.59 ± 0.026^{b}	3.43 ± 0.040^{c}
Day 21	3.32 ± 0.145^{ab}	3.41 ± 0.096^{a}	3.47 ± 0.047^{a}	3.21 ± 0.090^{b}
Day 28	3.43 ± 0.133^{a}	3.38 ± 0.059^{ab}	3.26 ± 0.099^{ab}	3.21 ± 0.088^{c}
Day 35	3.29 ± 0.059^{a}	3.27 ± 0.094^{a}	3.48 ± 0.114^{a}	3.11 ± 0.068^{c}
Day 42	3.33 ± 0.085^{a}	3.19 ± 0.073^{bc}	3.31 ± 0.026^{ab}	3.15 ± 0.079^{c}
Day 49	3.57 ± 0.073^{a}	3.22 ± 0.102^{b}	3.06 ± 0.064^{c}	2.81 ± 0.087^{d}
Day 56	3.73 ± 0.029^{a}	3.41 ± 0.087^{b}	3.29 ± 0.070^{bc}	3.14 ± 0.118^{c}
Coagulase-positive *Staphylococcus* (Log_{10} CFU.g^{-1})				
Day 0	< 1.00	< 1.00	< 1.00	< 1.00
Day 7	< 1.00	< 1.00	< 1.00	< 1.00
Day 14	< 1.00	< 1.00	< 1.00	< 1.00
Day 21	< 1.00	< 1.00	< 1.00	< 1.00
Day 28	< 1.00	< 1.00	< 1.00	< 1.00
Day 35	< 1.00	< 1.00	< 1.00	< 1.00
Day 42	< 1.00	< 1.00	< 1.00	< 1.00
Day 49	< 1.00	< 1.00	< 1.00	< 1.00
Day 56	< 1.00	< 1.00	< 1.00	< 1.00
Total coliforms at 35 °C (Log_{10} CFU.g^{-1})				
Day 0	3.28 ± 0.149^{b}	3.50 ± 0.037^{a}	3.16 ± 0.128^{b}	3.21 ± 0.111^{b}
Day 7	3.18 ± 0.107^{a}	3.44 ± 0.452^{a}	3.16 ± 0.054^{a}	2.98 ± 0.059^{a}
Day 14	3.04 ± 0.033^{b}	2.95 ± 0.026^{b}	3.16 ± 0.039^{a}	2.87 ± 0.043^{c}
Day 21	3.92 ± 0.040^{a}	3.21 ± 0.123^{b}	3.25 ± 0.046^{b}	3.04 ± 0.033^{c}
Day 28	3.63 ± 0.145^{a}	2.87 ± 0.066^{b}	2.85 ± 0.101^{b}	2.83 ± 0.047^{b}
Day 35	2.80 ± 0.059^{ab}	2.91 ± 0.030^{a}	2.68 ± 0.070^{b}	2.53 ± 0.116^{c}
Day 42	2.81 ± 0.039^{a}	2.79 ± 0.071^{a}	2.90 ± 0.013^{a}	2.55 ± 0.071^{b}
Day 49	3.71 ± 0.100^{a}	2.66 ± 0.065^{b}	2.50 ± 0.059^{c}	2.33 ± 0.064^{d}
Day 56	2.61 ± 0.040^{b}	2.82 ± 0.022^{a}	2.67 ± 0.063^{b}	2.28 ± 0.078^{c}

*Values presented as mean ± standard deviation; Different small letters in the same line indicate significant difference ($p < 0.05$) by Tukey's test; Different capital letters in the same column indicate significant difference ($p < 0.05$) by Tukey's test; PE: propolis extract.

Table 2. Continued...

Coliforms at 45 °C (Log_{10} CFU.g^{-1})				
Day 0	< 1.00	< 1.00	< 1.00	< 1.00
Day 7	< 1.00	< 1.00	< 1.00	< 1.00
Day 14	< 1.00	< 1.00	< 1.00	< 1.00
Day 21	< 1.00	< 1.00	< 1.00	< 1.00
Day 28	< 1.00	< 1.00	< 1.00	< 1.00
Day 35	< 1.00	< 1.00	< 1.00	< 1.00
Day 42	< 1.00	< 1.00	< 1.00	< 1.00
Day 49	< 1.00	< 1.00	< 1.00	< 1.00
Day 56	< 1.00	< 1.00	< 1.00	< 1.00
***Salmonella* spp./25g of sample**				
Day 0	absent	absent	absent	absent
Sulfite-reducing *Clostridium* 46 °C/ 25g of sample				
Day 0	< 1.00	< 1.00	< 1.00	< 1.00

*Values presented as mean ± standard deviation; Different small letters in the same line indicate significant difference ($p < 0.05$) by Tukey's test; Different capital letters in the same column indicate significant difference ($p < 0.05$) by Tukey's test; PE: propolis extract.

Table 2 shows that in relation to the average count of total coliforms at 35 °C there was significant difference between the treatments. Once again, the action of propolis extract of 2% can be seen in reducing the number of total coliforms compared to the standard treatment. This reduction was very clear in relation to the treatment with the addition of 2% propolis extract compared to the standard treatment during the period from day 14 to day 56 of storage. Similar results were found by Pereira (2009), who noted a significant reduction in the average total coliform at 35 °C count in mechanically separated meat with propolis extract at the end of the storage period. However, during the course of storage the mechanically separated meat with propolis extract showed no significant difference from the negative treatment (BHT), therefore differing from the present study. Borges et al. (2009) studied the antibacterial and antifungal activity of different concentrations of the hydro-alcoholic extract of propolis in fresh pork sausage and they also found significant differences between the treatments regarding the coliform count at 35 °C. The aforementioned authors found that the use of higher concentrations of propolis extract was more effective in controlling these microorganisms. In the present study, no significant differences were observed between the treatments regarding coliforms at 45 °C; the values were less than <1.00 log_{10} CFU/g and were in accordance with the limit established by RDC No. 12 (Brasil, 2001), which is 5×10^3 CFU/g.

The main pathogenic microorganisms which potentially could have been in the products developed in the present study were *Salmonella* spp., *Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes*. The latter can enter slaughterhouses through live animals and also humans working in the premises (Birzele et al., 2005). Castagna et al. (2004) investigated the prevalence of *Salmonella* spp. in a refrigerated pork slaughterhouse and detected the microorganism in 83.33% of the animals. The average prevalence found in the final product (fresh sausage) manufactured using raw materials originating from these animals was 93.94%, with no statistical difference between the prevalence of the carrier animals and that found in the final products. The present study found no presence of *Salmonella* spp and sulfite-reducing *Clostridium* in any of the samples throughout the entire storage period. This result was in line with the regulations defined by RDC No. 12 (Brasil, 2001), which establishes a negative limit of *Salmonella* in 25 g of sample, and a maximum of 3×10^3 CFU/g for sulfite-reducing *Clostridium*.

In recent years, the *in vitro* antimicrobial activity of propolis has been increasingly reported; this activity is due to flavonoids, aromatic acids and esters, which are present in the natural resin (Gebara et al., 2002). Caffeic acid and ferulic acid also contribute to the bactericidal action of propolis. These factors may explain the positive action of propolis in the present study in relation to certain microorganisms, such as total coliforms, aerobic mesophilic bacteria, psychotrophic bacteria and *Staphylococcus aureus*, which provided the lowest average scores during the storage period. Nagai et al. (2006) also reported that propolis has a high inhibitory effect in relation to microbial growth during the storage of meat and muscle.

4 Conclusion

It was concluded that the values found in the microbiological analyses were within the tolerance limits established by Brazilian legislation for all the treatments during the storage period, with changes only at the end of that period. Given these results it is suggested that propolis extract can be used as an ingredient in the preparation of Tuscan-style sausage because it extends the shelf life of the product.

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