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Physicochemical and sensory characteristics of *Coppa* with *Bifidobacterium animalis ssp. Lactis* (BB12) probiotic

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ABSTRACT. Motivated by the growing demand for functional foods, probiotics added to food products is a reality in the market. Its application in fermented sausages is considered promising, as its processing does not use heat treatment and with that there is no considerable loss of these microorganisms. In this study, the application of microencapsulated Bifidobacterium animalis ssp. lactis (BB12) was carried out in coppa. Three treatments were developed, consisting of control (C) without probiotic, BB1 with the addition of probiotic and 0.02% curing salt, and BB2 with probiotic and 50% reduction in curing salt (0.01%). Subsequently, possible changes in the physicochemical and sensory characteristics were analyzed, as well as the viability of the culture in the fermented product. All samples were presented according to the Brazilian legislation for the attributes of moisture, protein, and lipids. Probiotics showed a positive influence on the stabilization of lipid oxidation and microencapsulated probiotics proved to be viable after the ripening period of 30 days in treatment BB2. There was no significant difference between the samples for sensory analysis, so it is possible to state that the addition of BB12 is an alternative to obtain a product with all the benefits of functional foods.

Keywords: Functional food; meat products; fermented products.

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Introduction

Human body is exposed and linked to several types of microorganisms, which are present, for example, in skin, oral cavity, and intestinal tract (Tang & Lu, 2019). Currently, intestinal microbiota is the main target of studies, and current results suggest that intestinal dysbiosis is related to several diseases (Novik & Savich, 2020).

Thus, the search for functional foods grows, and among them are those that contain the application of probiotic microorganisms. Probiotics are defined as microorganisms that in adequate quantities benefit their hosts (Ministério da Saúde - Agência Nacional de Vigilância Sanitária [Anvisa], 2018). Studies present the beneficial effects promoted by probiotics, mainly the improvement of intestinal transit and the relief of lactose indigestion (De Vuyst, Falony, & Leroy, 2008; Nath et al., 2018; Cao et al., 2020; Liz et al., 2020) and their action can occur according to several mechanisms. Cavalheiro et al. (2015) showed that the most known and used microorganisms are *Lactobacillus* and *Bifidobacterias.*

Probiotic bacteria have been successfully used for some time, mainly in dairy products, since their application in fermented meat sausages is considered a promising market because processing does not use heat treatment and with that, there is no considerable loss of these microorganisms. Besides that, the meat matrix works as a protector during digestion of the human body, so that in this way probiotics have a bioprotective role in the organism (Ruiz-Moyano et al., 2011; Cavalheiro et al., 2015).

Fermented meat has its own characteristics and sensorially its attributes are distinct, thus being appreciated by consumers (Cid, Belletti, Aymerich, & Garriga, 2017). According to the Ministry of Agriculture, Livestock and Supply (Brasil, 2020), *coppa* is an industrialized meat product, obtained from the complete cut of the pork carcass, called neck or pallet, added with ingredients, matured, dried, smoked or not, with the addition of sugars, salts, nitrite and/or nitrate, in addition to starter cultures.

The curing salts nitrite and sodium nitrate are food additives widely used in meat products, to contribute to the fixation of the redness color in the curing process, which is desirable for sensory aspects, in addition to

presenting antimicrobial action collaborating with the preservation of the product (Majou & Christieans, 2018).

The challenge for the proper incorporation of probiotics in the manufacture of meat products is that they should be able to survive the various conditions during the process, such as fermentation; moreover, they should have a dominant position concerning the other microorganisms found in the final product (Song et al., 2018), since to provide benefits to the health of the host the product should contain 10^6-10^7 CFU g^{-1} viable probiotic bacteria (Sidira, Kandylis, Kanellaki, & Kourkoutas, 2015). One possibility to ensure the necessary number of viable cells is the encapsulation of these microorganisms, which is configured to protect them, maintaining their viability, and the encapsulation process consists of placing solids, liquids, or gas in an inert casing (Cavalheiro et al., 2019).

Thus, the objective of this research was to evaluate the viability of the probiotic *Bifidobacterium animalis ssp. Lactis* (BB-12) applied in *coppa* and check for possible changes in the physicochemical characteristics of this food, in addition to the number of viable probiotics under different storage periods and the sensory acceptability of these products.

Material and methods

Probiotic pre-activation

For the preparation of probiotic culture inoculants, the pure strains of *Bifidobacterium animalis ssp. Lactis* (BB12), provided by the company Chs Hansen (Hoersholm, Denmark), were pre-activated on BSM agar (Sigma-Aldrich, Darmstadt, Alemanha) with incubation at 37ºC for 48h. After activation, 23 mL aliquots of the culture were seeded in 77 mL BSM agar and incubated at 37ºC for 24h (Macedo, Pflanzer, Terra, & Freitas, 2008).

After incubation, the total volume of agar containing the activated culture was centrifuged in a refrigerated centrifuge (Nova Analítica Imp. Ltda.), at 15ºC, 3,500 rpm, for 10 min. The supernatant was discarded and the precipitate was microencapsulated.

Bifidobacterium bifidum microencapsulation

Microcapsules were prepared with 2% sodium alginate solution (Vetec, Rio de Janeiro, Brazil) and 0.5 mol L^{-1} calcium chloride solution (Vetec, Rio de Janeiro, Brazil), both solubilized in distilled water and autoclaved at 121ºC for 15 min. The concentrate *Bifidobacterium bifidum* suspension was mixed with a sterile alginate solution that was dripped with a 5 mL syringe into the calcium chloride solution under slight agitation (Shi et al., 2013). The formed microcapsules were kept in a Becker for 40 min. and then separated and washed by filtration with sterile material and distilled water.

Probiotic *coppa* manufacturing

For the preparation of the *coppa*, pork shoulder and condiments were purchased at the local market in the city of Maringá, state of Paraná, Brazil. Meat was boned and cut into portions of approximately 750 g.

Three formulations were made with different concentrations of curing salt (nitrite and nitrate) as follows: control formulation (C) with 0.02% curing salt and no probiotic, BB1 with probiotic, and 0.02% curing salt, and BB2 with probiotic and 0.01% curing salt. For each treatment, we prepared a brine using cold water, sodium chloride (3.00%), sugar (0.50%), black pepper (0.20%), garlic (0.30%) nutmeg (0.30%), 0.01 or 0.02% curing salt and probiotic microencapsulated. Important, the initial cell counts of BB12 in probiotic products were > 6 log CFU g^{-1}. Brine was introduced into the pieces of meat with a syringe. The prepared samples were packed in a natural casing and placed in the maturation chamber for 30 days of ripening with a temperature between 15 and 18°C.

Physicochemical characterization

Moisture, ash, and protein contents were determined according to Association of Official Analytical Chemists (AOAC, 2012) and lipid content according to Bligh and Dyer (1959).

Weight loss and pH

Weight loss was determined by weighing the meat product right after the injection of the brine until the end of ripening on days 0, 10, 20, and 30, expressed as a percentage concerning initial sample weight.

The pH was measured with a portable pH meter (Hanna, HI-99163, Romania) equipped with a probe for the surface in direct contact with the sample. The instrument was calibrated before and immediately after

each session using pH 4.0, 7.0, and 10.0 standards. The measurement was performed at three different points, at the beginning, in the middle, and at the end, with three readings for each treatment on days 0, 15, and 30 (Souza et al., 2020).

Instrumental color

Color parameters L* (lightness), a* (-a = green; + a = red) and b* (- b = blue; + b = yellow) were obtained using a CR-400 Minolta Colorimeter (Osaka, Japan), calibrated according to the manual instructions. All evaluations were made on the surface of the meat product and in triplicate on days 0, 15, and 30.

Lipid oxidation

The TBARS method consists of the analysis of substances reactive to 2-thiobarbituric acid (TBA) (J.T.Baker, Inglaterra), carried out according to the methodology described by Raharjo, Sofos, and Schmidt (1992) modified by Wang, Pace, Dessai, Benjamin, and Phillips (2002), and following the recommendations described by Shahidi and Synowiecki (1997) referring to the addition of sulfanilamide on the samples that contain nitrite. First, 0.5 mL 0.15% BHT (di-tertbutyl methyl phenol) (Synth, São Paulo, Brazil) was added to a tube containing 5 grams of sample. Then, 4 mL 0.5% sulfanilamide solution and 36 mL of 5% TCA (trichloroacetic acid) (Synth, São Paulo, Brazil) as was added and left to stand for 10 minutes. After, the solution was filtered. In a test tube, 2 mL filtrate and 2 mL 0.08 mol L^{-1} TBA were placed. Tubes were maintained at 80°C for 40 min. and then, the absorbance was read on a spectrophotometer (Agilent UV-8553, Santa Clara, EUA) at 532 nm. The quantification was performed using a standard curve (1×10^{-8} to 10×10^{-8} moL mL^{-1}) of the diethylacetal solution (TEP) (Sigma-Aldrich, Darmstadt, Alemanha), and the results were expressed in mg malondialdehyde kg^{-1} sample. Analyses were performed, in triplicate, during the ripening period on days 0, 15, and 30.

Probiotic viability

Triplicate analyses of probiotic viability were performed on days 0 and 30. Samples were evaluated by serial decimal dilution, in which 10 g sample was transferred to an Erlenmeyer with 90 mL 0.1% sterile peptone water and stirred, afterwards, the subsequent dilutions were made in serial tubes with the same diluent and then the inoculation was done by depth in plates with selective BSM agar medium (Sigma-Aldrich, Darmstadt, Alemanha). Plates were incubated at 37°C for 48h using anaerobic conditions, and afterwards, the results were expressed in log $CFUg^{-1}$ (colony forming units per gram).

At the end of the ripening period, for sensory analysis, the samples were evaluated for the presence of fecal coliforms. Analyses were carried out as described and the results are expressed as MPN g-1 (Downes & Ito, 2001).

Sensory acceptance

The current investigation was approved by the Ethics and Research Committee of the State University of Maringá (Protocol 21879413.9.0000.0104). Participants signed a consent form on their participation in consumer analysis. Sensory acceptance and purchase intent tests were carried out with 100 untrained panelists (40% female and 60% male), predominantly young (between 18 and 35 years old). The evaluated attributes were color, aroma, flavor, texture, and overall appearance, by affective test on a 9-point hedonic scale, following the methodology of Meilgaard, Civille, and Carr (1988). Samples were served in slices and coded with three random numbers and the presentation order was varied. Panelists received the sensory evaluation form in a single session and evaluated the samples in a monadic sequence using a 9-point hedonic scale ("1 - disliked it very much" to "9 - liked it very much") for each attribute. The purchase intent for each of the treatments was also evaluated on a 5-point hedonic scale ("1 - definitely I would not buy" to "5 - definitely I would buy").

Statistical analysis

The experiment was repeated three times on three different days. Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's test, using software Statistica® 7.0 with a significance level of 5%.

Results and discussion

Physicochemical characterization

The physicochemical composition of the probiotic *coppa* was in the following range (%, w w^{-1}, Table 1): moisture (18.53–26.65), proteins (20.00–20.52), lipids (26.40–34.89), and ash (7.34–8.96). The addition of

probiotic components did not change ($p > 0.05$) the protein and ash contents of the *coppas,* but increased the moisture content ($p > 0.05$) and decreased the lipids content ($p < 0.05$).

Table 1. Physicochemical composition of *coppas* with BB12.

Parameter	C	BB1	BB2
Moisture (%)	18.53 ± 0.46^{c}	26.65 ± 0.37^{a}	24.39 ± 0.14^{b}
Protein (%)	20.25 ± 2.35^{a}	20.52 ± 1.44^{a}	20.00 ± 1.17^{a}
Lipid (%)	34.89 ± 1.42^{a}	33.16 ± 1.02^{a}	26.40 ± 1.32^{b}
Ash (%)	8.96 ± 2.53^{a}	7.57 ± 0.20^{a}	7.34 ± 1.94^{a}

Means in the same row followed by different letters are significantly different ($p < 0.05$). C - no probiotic; BB1- probiotic + 0.02% curing salts; BB2 - probiotic + 0.01% curing salts.

According to the Brazilian Standard, the *coppa* should have the following characteristics: moisture (max.) of 40%; lipid (max.) of 35%, and protein (min.) of 20% (Brazil, 2000). From data in Table 1, there was a significant difference between the moisture content of the samples with and without probiotic, in which sample C had a lower moisture content. Values of moisture content were below 40%, as provided by Brazilian legislation.

Regarding the protein content, there was no statistical difference between treatments and all met the standards of the Brazilian legislation of at least 20%. Slima et al. (2017) showed similar effects, reporting that the inoculation of the probiotic *Lactobacillus plantarum* in salami had no significant effect on the average protein content during 10 days of ripening. The contents of lipids and ash meet the legislation.

pH analysis

Table 2 lists the pH values for the treatments during the ripening. There was a significant difference between treatments ($p < 0.05$) and between the ripening period. The *coppas* at the beginning showed values between 6.19 and 6.30 and during the ripening time, the pH values decreased due to the lactic acid production process. After day zero, it is possible to notice that the pH values decreased for the three treatments, and after day 15, the pH values increased. According to Trzaskowska, Krajewska, Wójciak, and Dolatowski (2014), lactic acid bacteria degrade sugars with consequent lactic acid production and pH drop. Otherwise, the increase in pH after a few days is caused by reactions of decarboxylation and deamination of amino acids, releasing ammonia, making the medium more alkaline.

The mean pH value on day 30 was 6.25, close to Di Cagno et al. (2008), who also showed pH values above 6.30 for Italian salami. As all treatments showed the same behavior, it is not possible to say that the addition of the probiotic and that the reduction of curing salts influenced the behavior of pH.

Table 2. pH during ripening of *coppas* added with BB12.

Treatment	0d	15d	30d
C	6.19 ± 0.04^{bA}	5.76 ± 0.11^{bB}	5.90 ± 0.09^{bB}
BB1	6.25 ± 0.03^{bA}	6.15 ± 0.04^{aB}	6.29 ± 0.03^{aA}
BB2	6.30 ± 0.02^{aA}	6.02 ± 0.05^{aB}	5.97 ± 0.02^{bB}

Means in the same row followed by different uppercase letters are significantly different ($p < 0.05$). Means in the same column followed by different lowercase letters are significantly different ($p < 0.05$). C - no probiotic; BB1- probiotic + 0.02% curing salts; BB2 - probiotic + 0.01% curing salts.

Weight loss

Weight loss is directly related to moisture reduction, as it is due to water loss through evaporation or dripping that occurs and its determination depends on temperature, relative humidity, and processing time (Coelho et al., 2019). At the end of the ripening period, weight loss (%) of the *coppas*, compared to the initial day, were 40.48, 44.60, and 55.77% for samples C, BB1, and BB2, respectively (Figure 1). Therefore, it was possible to observe the greatest weight loss ($p < 0.05$) for *coppas* inoculated with BB12. Possibly, the probiotics, through changes in pH of these samples, allowed a greater weight loss.

Instrumental color

Color is the most important characteristic of the appearance of cured products (Ruiz, Garcia, Muriel, Andrés, & Ventanas, 2002), it is an attribute that directly influences sensory acceptance. Results of instrumental color analyzed for *coppa* samples are listed in Table 3. There was a significant difference between treatments ($p < 0.05$) for L*, and after 30 days, all samples suffered a reduction in this parameter. This

reduction may be the result of the concentration of solids in the product due to the loss of water, that is, dehydration of the product causes browning, as observed in other dry curing products (Sayas-Barberá, Viuda-Martos, Fernández-López, Pérez-Alvarez, & Sendra, 2012). Cavalheiro et al. (2019) and Ge et al. (2019) also observed a reduction in lightness in fermented meat products.

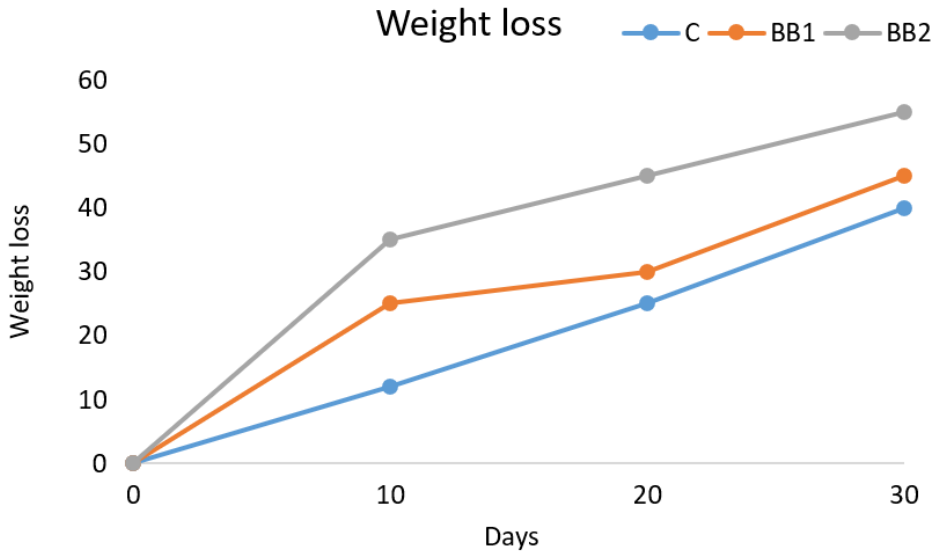

Figure 1. Weight loss (%) along the days of ripening.

Table 3. Color parameters during the ripening of *coppas* with BB12.

Day	0 d	15 d	30 d
L*			
C	39.01±2.01^{aA}	21.62±1.61^{bC}	30.05±2.82^{aB}
BB1	34.58±2.48^{aAB}	37.21±0.84^{aA}	30.13±1.88^{aB}
BB2	37.34±1.56^{aA}	25.31±1.86^{bB}	25.35±1.10^{bB}
a*			
C	6.41±1.34^{aB}	6.66±0.12^{aB}	7.08±1.17^{aA}
BB1	6.05±0.89^{aA}	4.19±1.41^{aB}	6.38±1.98^{aA}
BB2	6.44±0.78^{aB}	9.76±0.62^{aA}	10.00±1.22^{aA}
b*			
C	13.67±1.14^{aA}	5.17±0.22^{bB}	5.28±1.08^{aB}
BB1	10.33±1.33^{bAB}	13.67±2.87^{aA}	5.62±0.84^{aC}
BB2	10.28±0.40^{bAB}	4.90±0.35^{bC}	7.37±1.59^{aBC}

Means in the same row followed by different uppercase letters are significantly different ($p < 0.05$). Means in the same column followed by different lowercase letters are significantly different ($p < 0.05$). C - no probiotic; BB1- probiotic + 0.02% curing salts; BB2 - probiotic + 0.01% curing salts.

Values of a * showed a significant difference between treatments. Significantly higher ($p < 0.05$) redness was found after 30 days in all samples. BB2 with a 50% reduction in curing salts showed higher values of a* on day 30. Skwarek, Dolatowski, and Krajewska (2014) reported values of a* of 9.7 for raw-ripening probiotic ham (a* 5.38 and 5.87, respectively). On the other hand, Kaya and Aksu (2005) in *Sucuk* with starter culture included BB12 and observed a* values of 11.8, values greater than in this study. Values of b* showed a decrease during ripening time. Values ranged from 10.22 to 13.67 at the beginning of the ripening to values between 5.28 and 7.37, at the end of the process. This reduction was likely due to oxygen consumption by microorganisms in the starter culture during their exponential growth phase, producing a decrease in oxymyoglobin and due to the reaction of nitric oxide with myoglobin to form nitrosomyoglobin that contributed to the decrease in the concentrations of myoglobin and oxymyoglobin, allowing a reduction in b* (Pérez-Alvarez, Sayas-Barberá, Fernández-López, & Aranda-Catala, 1999).

Lipid oxidation

Among the factors limiting shelf life and the stability of meat products, the most important is lipid oxidation. This process is related to the composition of natural antioxidants and the degree of polyunsaturation of fatty acids (Boselli et al., 2005). Table 4 lists the values of TBARS.

Table 4. Effect on TBARS assay (mg malondialdehyde kg^{-1} sample) in probiotic *coppa*.

Treatment	0 d	15 d	30 d
C	0.331 ± 0.05^{aA}	0.391 ± 0.08^{aA}	0.371 ± 0.23^{aA}
BB1	0.202 ± 0.01^{bA}	0.252 ± 0.03^{cA}	0.236 ± 0.04^{bA}
BB2	0.207 ± 0.03^{bB}	0.315 ± 0.04^{bA}	0.270 ± 0.01^{bAB}

Means in the same row followed by different uppercase letters are significantly different ($p < 0.05$). Means in the same column followed by different lowercase letters are significantly different ($p < 0.05$). C - no probiotic; BB1- probiotic + 0.02% curing salts; BB2 - probiotic + 0.01% curing salts.

The addition of probiotics affected the values of TBARS. There was a significant difference between the control sample and the addition of BB12 during the ripening period. The control sample showed higher values of TBARS on days 0, 15, and 30 compared to samples with the addition of BB12. Song et al. (2018) and Arief, Afiyah, Wulandari, and Budiman (2016) observed that probiotic microorganisms had a positive influence on the stabilization of lipid oxidation, a similar result was also reported by Slima et al. (2017). These results showed that inoculation of the probiotic *Lactobacillus plantarum TN8* in salami has the same antioxidant power as that of nitrite in terms of retarding oxidative rancidity.

The BB2 treatment inoculated with probiotic and reduction of curing salt showed a lower amount of malonic aldehyde compared to the control. This shows that it is possible to reduce the curing salt in the production of probiotic *coppa,* offering a safe product for the consumer health. Some authors have reported that oxidation can be perceived by consumers in fermented sausages at TBARS values of 2 mg MDA kg^{-1} (Ahmad & Srivastava, 2007), and in the present study, the results of all treatments were below 0.5 mg MDA kg^{-1} indicating that the products do not have these unpleasant properties.

Probiotic viability

The probiotic viability analysis was carried out on days 0 and 30, to enumerate and ensure the probiotic properties until the end of the 30-day ripening period. On day 0, samples showed viability of 10.60 log CFU g^{-1} for both treatments, BB1 and BB2. After a 30-day ripening period, the results were 5.8 log CFU g^{-1} and 7.3 log CFU g^{-1} for treatments BB1 and BB2, respectively.

According to Sidira et al. (2015), it is necessary to have at least 6 CFU log probiotics g^{-1} of the product to achieve the physiological benefits related to the consumption of probiotics. Thus, with the values obtained, only the BB2 treatment, with a 50% reduction in curing salt, can be considered a potentially probiotic product. According to Macedo et al. (2008), resistance of the probiotic bacteria *Lactobacillus* ssp was tested up to 200 ppm curing salt in meat product, and it was observed that 150 ppm is the optimum point of growth and then there is a decrease. Thus, the amount of curing salt can influence probiotic survival.

Ayyash et al. (2019) found positive results in the application of *Lactobacillus plantarum* in a meat product fermented with camel meat, reaching the necessary and recommended number of viable probiotic cells and obtaining products with probiotic potential. Coelho et al. (2019) also obtained good results concluding that "salaminhos" with *L. paracasei LPC02* developed in that research can be considered promising vehicles of this probiotic.

Sensory acceptance

For sensory analysis, microbiological analysis of total coliforms was performed, as this is an indicator of hygienic-sanitary conditions. The result was <3 MPN g^{-1} of product, with all samples meeting the standards allowed by law.

The addition of probiotic and reduction of curing salt did not affect ($p > 0.05$) the sensory acceptance of probiotic *coppa* by potential consumers (Table 5). The mean scores obtained were: 6.65 ± 1.64 color; 6.28 ± 1.56 taste; 6.11 ± 1.60 flavor; 6.34 ± 1.61 texture; and 6.45 ± 1.58 overall acceptance. In general, samples were well accepted, with the perceptions between "I liked slightly" and "I liked regularly". There was no significant difference between treatments, thus showing that BB12 is a good alternative for meat products for consumers who want to consume food with probiotic characteristics.

Table 5. Sensory acceptance of probiotic *coppa*.

Attribute	C	BB1	BB2
Color	6.89 ± 1.53^{a}	6.89 ± 1.38^{a}	6.17 ± 1.68^{a}
Flavor	6.01 ± 1.78^{a}	6.14 ± 1.66^{a}	6.18 ± 1.86^{a}
Texture	6.54 ± 1.64^{a}	6.13 ± 1.90^{a}	6.36 ± 1.72^{a}
Taste	6.23 ± 1.79^{a}	6.20 ± 1.81^{a}	6.42 ± 1.67^{a}
Overall Acepptance	6.49 ± 1.50^{a}	6.50 ± 1.40^{a}	6.36 ± 1.38^{a}

Means in the same row followed by different letters are significantly different ($p < 0.05$). C - no probiotic; BB1- probiotic + 0.02% curing salts; BB2 - probiotic + 0.01% curing salts.

A similar result was previously reported by Ruiz, Villanueva, Favaro-Trindade, and Contreras-Castillo (2014). According to those authors, there was no difference in the acceptance of Italian salami control without probiotics from samples with *L. acidophilus*. Different results from Holko, Hrabe, Salaková, and Rada (2013) showed that consumers preferred mutton fermented sausage with *L. acidophilus* and *B. animalis* over control without probiotics.

As for the purchase intent, Figure 2 illustrates that both the control sample and the BB2 sample containing the highest probiotic content achieved the best scores, where 12% "would buy" the product, and this is proven in the responses of "possibly buy it" because the scores are higher for the BB2 sample. This demonstrates that, within the studied period, BB2 samples can be consumed, as they did not have negative characteristics and can serve as a vehicle for probiotic microorganisms.

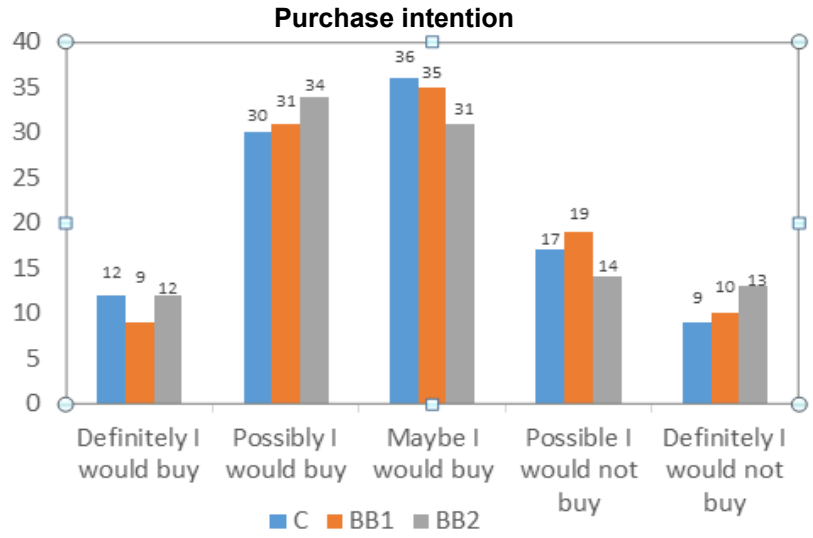

Figure 2. Representation of the intent to purchase the samples. C - no probiotic; BB1- probiotic + 0.02% curing salts; BB2 - probiotic + 0.01% curing salts.

Conclusion

The microencapsulated probiotic *Bifidobacterium animalis* spp. *lactis* (BB12) had no negative influence on the physicochemical and sensory attributes of the *coppas* and inhibited lipid oxidation. Probiotic cells were shown to be viable in the BB2 sample after 30 days of ripening. Therefore, the final results were favorable, showing that it is possible to reconcile the use of probiotics with a 50% reduction in curing salts in *coppas*, thus obtaining a product with all the benefits of functional foods.

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