Original paper

OPTIMIZATION OF PROTEIN FEED FERMENTATION PROCESS FOR SUPPLEMENTATION OF *APIS MELLIFERA* HONEYBEES

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Abstract

An alternative to natural food shortage for honeybees is the provision of supplements. The objective of this research was to develop a method with ideal conditions for the fermentation of a protein supplement for honeybees. The microorganisms used were *Lactobacilli*, *Bifidobacteria* and *Saccharomyces* and the inulin prebiotic. There was an increase in lactic acid and a decrease in pH over a five-day fermentation period. The amount of acid bacteria present was 108-109 CFU/g of supplement. In the first phase the temperature and the humidity had significantly affected the fermentation but the variables inulin and probiotic didn't. In the second phase, the lower temperatures and higher humidity produced the best effects on the fermentative responses. It was concluded that an ideal environment with temperature, humidity, inulin and probiotics could be created which allowed the fermentation of the protein supplement in five days obtaining final product fermentative characteristics close to bee bread.

Keywords: *Bifidobacteria*, inulin, lactic acid, *Lactobacilli*, probiotic, *Saccharomyces*

INTRODUCTION

The assimilation of nutrients is fundamental for the growth and development of a honeybee colony (Brodschneider & Crailsheim, 2010). The main nutrients for honeybees present in pollen and nectar are carbohydrates, proteins, lipids, vitamins, minerals and water (Manning et al., 2007). Honeybees convert nectar into honey and pollen into bee bread, allowing the availability of nutritional content (Altaye et al., 2010). Bee bread is the most nutritious food of honeybees and the main source of protein. The pollen collected by the foragers is stored in cells and mixed with honey. This mixture undergoes physical, chemical and microbiological processes with the action of enzymes, microorganisms,

humidity and temperature. In about two weeks the pollen is fermented (Nagai et al., 2005). In this process, the yeast *Saccharomyces cerevisiae* consumes oxygen promoting an anaerobic environment, favoring bacteria growth (Hu et al., 2008). Bacteria cause reduction in pH and acidification of medium, mainly through the production of lactic acid (Leroy & Vuyst, 2004). An antimicrobial action is observed in bee bread's upper layer and higher values of total phenolics in the lower layer (Podrižnik & Božič, 2015). Microorganisms are not only involved in the production and preservation of food, honey and bee bread, but also important for honeybee health (Vásquez et al., 2012; Raymann, Shaffer, & Moran, 2017). To understand these benefits, such factors as microorganism composition

and quantification, honeybee genetics and physiology and environmental factors must be known (Engel et al., 2016).

The intense transformation of agriculture, degradation of natural habitats and creation of monocultures alter the abundance, transmission rate and survival of the beneficial microorganisms that inhabit a place and affect the adequate supply of nutrients required by honeybees (Nicholls & Altieri, 2012; Anderson et al., 2013). As a result, the nutrition and immunity of a colony is compromised because even it is not sick does not mean that it is healthy. Healthy colony should be disease-free and above all, well-nourished and capable of perpetuation (Brodschneider & Crailsheim, 2010).

An alternative to natural food shortage for honeybees is the provision of supplements. Research related to this supply shows good results in the development and colony maintenance, increased royal jelly production and honeybee's longevity, decreased mortality rate, increased protein quantification in hemolymph and financial viability (Pereira et al., 2006; Cappelari et al., 2009; De Jong et al., 2009; Sereia et al., 2010a,b; 2013; Morais et al., 2013a, b). Usually the substitutes are not fermented and do not contain specific microflora. The administration of probiotic preparations with or without prebiotics has a positive effect on honeybees (Evans & Lopez, 2004; Kaznowski et al., 2005; Kazimierczak-Baryczko & Szymaś, 2006; Szymaś, Łangowska, & Kazimierczak-Baryczko, 2012; Pătruică, & Mot, 2012; Pătruică et al., 2012; Pătruică & Hutu, 2013). Their social lifestyle facilitates the inoculation and dissemination of probiotics in the colonies (Engel & Moran, 2013). The maintenance of these microorganisms, mainly *Bifidobacteria* and *Lactobacilli*, can be maintained with the supply of such prebiotics as inulin (Gallina et al., 2011).

Araneda et al. (2014) stated that it is possible to develop a supplement in the laboratory, provided that it has constituent elements similar to bee bread. To produce a fermented supplement, one should think not only of the ingredients of the formulation, but also of the microorganisms used and the method to be employed. Microor-

ganism activity is affected by such factors as available water, temperature, oxygen, pH and available nutrients (Siqueira, 1995). Therefore, the development method must encompass these factors to get an ideal end product. Due to the necessity and the possibility of creating an improved supplement this experiment's objective was to develop a method with optimal conditions to ferment a protein supplement for honeybees with ideal fermentative characteristics similar to bee bread.

MATERIAL AND METHODS

The analysis was carried out at the Laboratory of Service Provision of the Federal Technological University of Parana at Campo Mourão in the State of Parana (24°02'44"S, 52°22'09"W) between June and December 2016.

Solutions

Himedia® Malt Extract Powder Refined Broth; De Man broth, Rogosa and Sharpe *Lactobacillus* from Acumedia® (MRS); De Man agar, Rogosa and Sharpe *Lactobacillus* agar from MicroMed® (MRS); *Streptococcus thermophilus* agar (ST); Acumedia® Dextrose (BDA) potato agar and peptone water.

Protein supplement

The supplement used was developed by Sereia et al. (2013) and composed of isolated soy protein, linseed oil, palm oil, brewing yeast, refined sugar, honey, pollen, soy lecithin and vitamins. It was modified with the elimination of the microbial load present in the pollen following the method described by Fuenmayor, Quicazán, & Figueroa (2011). In sterilized 200 mL glass vials, a mixture of pollen: distilled water (2:1) autoclaved at 121°C for fifteen minutes was added. After sterilization, the supplement ingredients were weighed, homogenized and sieved. The percentage moisture content of the supplement was determined following the method described by the Adolfo Lutz Institute (2008). It was used for the analysis of humidity, scale and sterilization oven and drying.

Variables analyzed

Analyzes prior to the experiment were performed to verify which variables could contribute positively to the fermentation of the protein supplement. It was observed that temperature, moisture, possible probiotics (Fuenmayor, Quicazán, & Figueroa, 2011; Ríos et al., 2012; Araneda et al., 2014; Ríos et al., 2014) and inulin (Vamanu et al., 2010) would be important variables. Possible probiotics had only efficient activity when used together. Probably the presence of one species favors the development and activity of other species. This was due to symbiosis between the different microorganisms and when separated showed no efficient activity (Gandra & Gandra, 2007; Ellis & Hayes, 2009).

So, the variables analyzed to verify their effects in the fermentation were:

- a) Temperature: controlled in a bacteriological stove.
- b) Moisture: determined according to the method described by the Adolfo Lutz Institute (2008). Subsequently, the global equation for mass balance was performed.
- c) Prebiotic: Orafiti® GR inulin was the prebiotic used. The weighing was performed in a precision analytical scale. After the inulin was added to the supplement, the tests underwent a final sterilization remaining for fifteen minutes in a sterilization oven and drying at 90°C.
- d) Microorganisms potentially probiotic: *Streptococcus thermophilus* (strain BV-YOG 03/2 Bela Vista®), *Lactobacillus delbrueckii* spp. *Bulgaricus* (strain YF-L811 Chr. Hansen®), *Bifidobacterium animalis* spp. *lactis* (strain BLC 1 SACCO®), and *Lactobacillus casei shirota* isolated from commercial fermented beverages and *Saccharomyces cerevisiae* (Fleischmann®).

All the microorganisms used in this study are naturally found in bee bread, and the amount depends on the floral origin. Pollen fermentation begins with the activity of yeast. Subsequently the fermentation process continues with the aerobic lactic acid bacteria and ends with the activity of the anaerobic lactic acid bacteria (Gilliam, 1979). These microorganisms were inoculated on average at 2% in the activation

broth (Ríos et al., 2012). The percentage in each treatment was defined by the statistical program.

Activation of microorganisms

The microorganisms used were *Streptococcus thermophilus* (strain BV-YOG 03/2 Bela Vista®): 2 g / 100 mL of broth; *Lactobacillus delbrueckii* spp. *Bulgaricus* (strain YF-L811 Chr. Hansen®): 2 g / 100 mL of broth; *Bifidobacterium animalis* spp. *lactis* (strain BLC 1 SACCO®): 2 g / 100 mL of broth; *Lactobacillus casei shirota* isolated from commercial fermented beverages: 10 mL / 100 mL; and *Saccharomyces cerevisiae* (Fleischmann®): 2 g / 100 mL of broth.

Activation was performed in specific broths previously sterilized in an autoclave at 121°C for fifteen minutes. The yeast in Broth Malt Extract Powder was refined for thirty minutes at $30 \pm 2^\circ\text{C}$ without being stirred in a metabolic bath. The bacteria producing lactic acid were kept in MRS broth for 72 hours at $37 \pm 2^\circ\text{C}$ in a bacteriological oven. When all probiotic cultures were active, the microorganisms were counted.

Counting of microorganisms

The method used was inoculation in triplicate. Cultures active in a specific broth formed the 10^{-1} dilution. After the initial dilution, consecutive serial dilutions were made up to 10^{-10} , withdrawing 1 mL of the initial dilution to 9 mL of 0.1% peptone water. Of the desired dilutions, 1 mL was pipetted to sterile Petri dishes. To each plate, 15 mL of the MRS agar medium was added for the growth of *Lactobacilli* and *Bifidobacteria*, ST agar was added for *Streptococci*. The acidified agar BDA was used for yeast *Saccharomyces cerevisiae*.

All media were pre-melted and cooled to $44 \pm 2^\circ\text{C}$. The plates were manually moved in an eight-fold format for the uniform mixture of the inoculum in the agar. After solidification, they were incubated in a bacteriological oven. The plates with the MRS agar medium were kept in anaerobic atmosphere generator with an oxygen-absence indicator for 72 hours at $37 \pm 2^\circ\text{C}$. The plates with ST agar medium were kept in aerobiosis wrapped in 24-hour film paper at 37

$\pm 2^{\circ}\text{C}$. The plates with BDA medium were kept in aerobiosis wrapped with film paper for 72 hours at $25 \pm 2^{\circ}\text{C}$. After incubation, the number of colony-forming units (CFU/g) was counted. After the bacteria were counted, they were pipetted into the supplement according to the needs of each trial. The amount of probiotic used in Trial 1, 2, 3 and 4 was 7.5%, 12.5% in Trial 5, 6, 7, and 8 while in Trial 9, 10, 11 and 12 was 10%. With 7.5% of probiotic only 1.5% of each species was inoculated, 2.5% of each species was inoculated when the amount of probiotic was 12.5% and when the probiotic was 10%, 2.0% of each species was inoculated.

Fermentation of the protein supplement

Inside the laminar flow hood the probiotic mix was inoculated into 100 g of the supplement with values according to the experimental design. The probiotic supplement was kept in a 200 mL glass and kept in anaerobic atmosphere generator with an oxygen-absence indicator for five days in a bacteriological oven.

Fermentation Responses

In the first experimental planning, the variables' effects on pH and lactic acid were verified on the first and fifth days of fermentation. In the second experimental planning, the variables' effects on pH, lactic acid production and viable lactic acid bacteria on the last day of fermentation were verified.

a) pH: followed the method described by the Adolfo Lutz Institute (2008). Samples were weighed on an analytical scale. The pH meter was calibrated and operated according to the manufacturer.

b) Lactic acid: followed by the method described by the Adolfo Lutz Institute (2008). An analytical scale was used for the weighing. The contents were shaken on a magnetic stirrer with controlled shaking at 20 rpm. Colors of the samples interfered with the visualization of the phenolphthalein turning point, so a potentiometric titration was used. For this, the pH meter was immersed in the samples. The lactic acid produced was calculated following the equation: Lactic acid produced = final lactic acid - initial

lactic acid.

c) Counting of viable acid bacteria: The method described by Siqueira (1995) was followed. In the laminar flow hood, 25 g of the fermented supplement was added to 225 mL of 0.1% peptone water, forming the dilution 10^{-1} . From this dilution, successive dilutions up to 10^{-10} were carried out. The MRS agar was used and kept in anaerobic atmosphere generator with an oxygen-absence indicator. After the bacteria were counted, all presented values were above 1×10^8 (CFU/g), an ideal concentration to be considered probiotic (Gallina et al., 2011).

Lyophilization

The fermented protein supplement was placed in Petri dishes covered with perforated aluminum foil and frozen in a freezer at -20°C by 24 hours. Drying took place in a freeze dryer at -57°C for 96 hours. After lyophilization, the supplement was packed in 100% polyethylene plastic bags with hermetic closure, hand crushed, sieved and stored in laminated packaging. A sealing machine was used for the closure.

Statistical analysis

The statistic used was according to Rodrigues & Iemma (2009) using the Software Protimiza Experimental Design. Twenty-four trials were performed in total to identify the optimized fermented protein supplement.

In the first stage of the experiment, twelve Fractional Factorial Planning tests were used for five days to identify which of the four independent variables with four central points ($2^{4-1} + 4\text{pc}$) influence the fermentation and thus define the next factorial to be adopted. The samples were temperature (35.00°C , 40.00°C and 45.00°C), moisture (36.00%, 56.00%, 76.00%), probiotic (7.50%, 10.00%, and 12.50%) and inulin (1.00%, 2.00%, 3.00%).

To identify the main variable responsible for fermentation, Central Compound Rotational Delineation was performed for two independent variables with four extremes and four central points ($2^2 + 4\text{ axials} + 4\text{ pc}$), totaling 12 trials. Temperature (38.96°C , 40.00°C , 42.50°C , 45.00°C and 46.04°C), moisture (51.86%,

56.00%, 66.00%, 76.00% and 80.14%) were used. The level of significance was set at 5% ($p < 0.05$). The results were evaluated by analysis of variance and regression analysis.

RESULTS

In the treatments after fermentation, there was an increase in lactic acid and, consequently, a reduction in pH (Tab. 1). pH values after fermentation ranged from 3.58 ± 0.01 to 5.35 ± 0.02 . The highest pH after fermentation of the product was observed in the Trial 1. The effects of the variables on pH can be observed in Fig. 1. A significant negative effect ($p < 0.10$) of curvature (-1.68), moisture (0.18), and temperature (1.56) was observed. Probiotic and inulin showed no significant effects.

Fig. 1. Standardized effects (tcalc) of the variables: mean, moisture, curvature, temperature, probiotic and inulin on pH.

Fig. 2. Standardized effects (tcalc) of the variables: mean, moisture, curvature, temperature, probiotic and inulin on lactic acid.

The percentage of lactic acid after fermentation ranged from $1.40 \pm 0.05\%$ to $3.18 \pm 0.03\%$. The lowest lactic acid value occurred under the conditions of Trial 1 and 2 (Tab. 1). Curvature and moisture presented significant effects ($p < 0.10$) with values of 2.85 and 0.28, respectively (Fig. 2). Because the amount of moisture was significantly greater, the percentage of lactic acid in the product also increased. Temperature, probiotic and inulin were not significantly affected.

From these results the next planning was determined to eliminate the lower temperature (35°C) and the lower moisture (36%), and fix the probiotic and inulin variables in the central values, 10% and 2%, respectively.

pH ranged from 3.49 ± 0.01 to 3.96 ± 0.01 . The lowest pH value was observed under the

Fig. 3. Standardized effects (tcalc) of the variables: mean, temperature and moisture on pH.

Fig. 4. pH behavior with experimental values x predicted by the model.

conditions of Trial 3 (Tab. 2). In equation 1, the pH predicted by the model is presented as a function of the coded variables. The temperature and moisture had potentially important

effects on pH (Fig. 3), while pH behavior was observed in Fig. 4.

$$(Eq. 1): Y_1 = 3.75 + 0.07 X_1 - 0.12 X_2$$

The adjusted model of the line was significant

Table 1.

Experimental design matrix with the variables temperature (X1), moisture (X2), probiotic (X3) and inulin (X4) for protein supplement fermentation and mean values (standard deviation) of the responses (n = 3) pH (Y1) and lactic acid (Y2) in the first (1st day) and fifth day (5th day) of fermentation

Variables					Responses			
Trial	Temperature (°C)	Moisture (%)	Probiotic (%)	Inulin (%)	pH	pH	Lactic acid (%)	Lactic acid (%)
					1 st day	5 th day	1 st day	5 th day
1	35.00	36.00	7.50	1.00	5.48 (0.03)	5.35 (0.02)	1.33 (0.02)	1.40 (0.05)
2	45.00	36.00	7.50	3.00	5.31 (0.03)	5.28 (0.05)	1.39 (0.02)	1.40 (0.05)
3	35.00	76.00	7.50	3.00	5.62 (0.01)	3.88 (0.01)	0.47 (0.01)	1.54 (0.01)
4	45.00	76.00	7.50	1.00	5.67 (0.01)	3.58 (0.01)	0.43 (0.01)	2.00 (0.03)
5	35.00	36.00	12.50	3.00	5.48 (0.01)	5.34 (0.01)	1.35 (0.01)	1.49 (0.02)
6	45.00	36.00	12.50	1.00	5.37 (0.02)	5.34 (0.01)	1.35 (0.01)	1.45 (0.01)
7	35.00	76.00	12.50	1.00	5.38 (0.02)	3.98 (0.01)	0.63 (0.01)	1.48 (0.03)
8	45.00	76.00	12.50	3.00	5.28 (0.01)	3.62 (0.01)	0.51 (0.01)	1.83 (0.01)
9	40.00	56.00	10.00	2.00	5.38 (0.05)	3.71 (0.02)	0.87 (0.02)	2.89 (0.12)
10	40.00	56.00	10.00	2.00	5.39 (0.10)	3.70 (0.03)	0.90 (0.04)	2.91 (0.05)
11	40.00	56.00	10.00	2.00	5.37 (0.07)	3.70 (0.01)	0.90 (0.03)	3.18 (0.03)
12	40.00	56.00	10.00	2.00	5.46 (0.06)	3.72 (0.01)	0.90 (0.02)	3.02 (0.01)

Table 2.

Experimental design matrix with the variables temperature (X1), moisture (X2) and mean values (standard deviation) of the responses (n = 3) pH (Y1), lactic acid produced (Y2) and lactic bacteria (Y3) after fermentation of the protein supplement

Variables			Responses		
Trial	Temperature (°C)	Moisture (%)	pH	Lactic acid (%)	Lactic bacteria (CFU g ⁻¹)
1	40.00	56.00	3.83 (0.01)	1.47 (0.01)	2.4 (0.1) x10 ⁹
2	45.00	56.00	3.92 (0.01)	1.33 (0.01)	3.3 (0.3) x10 ⁸
3	40.00	76.00	3.49 (0.01)	1.56 (0.02)	3.5 (0.2) x10 ⁹
4	45.00	76.00	3.69 (0.01)	1.08 (0.01)	2.8 (0.1) x10 ⁸
5	38.96	66.00	3.64 (0.01)	1.66 (0.02)	3.8 (0.1) x10 ⁹
6	46.04	66.00	3.82 (0.01)	1.36 (0.01)	3.5 (0.1) x10 ⁸
7	42.50	51.86	3.96 (0.01)	1.38 (0.03)	2.1 (0.2) x10 ⁹
8	42.50	80.14	3.66 (0.01)	1.06 (0.01)	2.8 (0.1) x10 ⁸
9	42.50	66.00	3.76 (0.01)	1.34 (0.02)	3.2 (0.1) x10 ⁸
10	42.50	66.00	3.76 (0.01)	1.31 (0.03)	3.1 (0.2) x10 ⁸
11	42.50	66.00	3.75 (0.01)	1.35 (0.06)	3.6 (0.1) x10 ⁸
12	42.50	66.00	3.75 (0.01)	1.36 (0.13)	3.7 (0.1) x10 ⁸

Fig. 5. Response surface for the pH response (Y1) as a function of temperature (X1) and moisture (X2).

Fig. 8. Behavior of the lactic acid production with experimental values x predicted by the model.

Fig. 6. Contour curve for the pH response (Y1) as a function of temperature (X1) and moisture (X2).

Fig. 9. Response surface (A) for the response of lactic acid production (Y2) as a function of temperature (X1) and moisture (X2).

Fig. 7. Standardized effects (tcalc) of the variables: mean, temperature, moisture on lactic acid production.

Fig. 10. Contour curve for the response of lactic acid production (Y2) as a function of temperature (X1) and moisture (X2).

at the 5% level. The existence of the behavior presented a variation explained by adjusted coefficient of determination $R^2 = 91.36\%$. The effects of temperature and humidity on the pH response can be seen in Fig. 5 (response surface) and Fig. 6 (contour curve). There was a decrease in the pH value with low temperature and high moisture.

Lactic acid production ranging from $1.06 \pm 0.01\%$ to $1.66 \pm 0.02\%$ was higher for the conditions of Trial 5 (Tab. 2). In equation 2, lactic acid production predicted by the model is presented as a function of the coded variables. The temperature, moisture and interaction between the two presented potentially important effects for lactic acid production (Fig. 7). The behavior of lactic acid produced can be observed in Fig. 8.

(Eq. 2): $Y_2 = 1.34 - 0.13 X_1 + 0.08 X_1^2 - 0.08 X_2 - 0.06 X_2^2 - 0.09 X_1 X_2$

The adjusted model of the line was significant at the 5% level and presented a variation explained by adjusted coefficient of determination $R^2 = 94.61\%$. The response surfaces for the lactic acid production response are presented in Figs. 9 and 10. There was an increase in lactic acid production at low temperatures and central moisture within the studied ranges.

The amount of probiotics per gram of the product ranged from $2.8 \pm 0.1 \times 10^8$ to $3.8 \pm 0.1 \times 10^9$ (CFU/g). Variables had no effect on this response. The adjusted model of the line was insignificant at the 5% level determining the non-existence of behavior.

With all the analyses' result, the software provided new formulations optimized and originated through models constructed using the coded values. The formulated model accepted in this research presented the value of 38.96°C and 66.00% moisture to obtain an approximate pH of 3.66 and lactic acid production of 1.69 in the final product.

DISCUSSION

Fermentation was observed in all trials. During fermentation lactic acid increases and consequently pH reduces (Hu et al., 2008). Pollen was one of the supplement ingredients and,

with fermentation, the exine, the microstructure which protects the pollen, can be partially destroyed and the rich nutrient content assimilated and used (Ariizumi & Toriyama, 2011). All ingredients become bioavailable, making the fermented product more digestible (Tonheim et al., 2007) and stable, and restricting the growth of pathogenic microorganisms (Pattabhiramiah, Reddy, & Brueckner, 2012). The formulated supplement was fermented, providing the final product with a nutritional, protected and probiotic.

The final pH value of the fermented product ranged from 3.49 ± 0.01 to 3.96 ± 0.01 . The pH in fermented diets after two weeks is 4.51 to 4.82 (Ellis & Hayes Jr., 2009). In bee bread pH ranged from 3.8 to 4.3 (Herbert & Shimanuki, 1978) and 3.93 to 4.23 (Barene, Daberte, & Siksna, 2015) and bee bread fermented for fifteen days produced in the laboratory from 3.89 to 4.24 (Ríos et al., 2014). While in laboratory-produced fermented bee bread for twenty days pH ranged from 3.83 to 4.25 (Araneda et al., 2014). In pollen analyzes the pH ranged from 4.1 to 5.9 (Herbert & Shimanuki, 1978) and in the dehydrated pollen 4.12 to 5.35 (Barreto, Funari, & Orsi, 2005). pH rapidly decreased in the developed supplement during the five days of fermentation because of the active fermentation performed by the microorganisms.

Lactic acid production by fermented supplement ranged from 1.06 ± 0.01 to $1.66 \pm 0.02\%$, a value close to that found in the literature. Laboratory-fermented pollen according to findings by Ríos et al. (2012) varied between $0.28 \pm 0.08\%$ and $2.26 \pm 0.08\%$ and findings by Ríos et al. (2014) between $0.34 \pm 0.05\%$ and $2.00 \pm 0.07\%$. It is difficult to compare these results because of the differences in the method of execution, substrate and microorganisms used. The microorganisms produce substances, in particular lactic acid, that acidify the substrate (Leroy & Vuyst, 2004; Hu et al., 2008). It is appropriate to verify the adaptability and compatibility of the selected cultures to the substrate used (Saad, Cruz, & Faria, 2011). The formulated supplement was suitable for the activity of bacteria and yeasts and, consequently, the medium was

acidified with high lactic acid values.

An anaerobic environment was used for the fermentation of the supplement, a medium similar to the fermentation of the pollen when stored in the comb. The anaerobic condition was an essential parameter for both the rapid fermentation of the product and the temperature, and moisture significantly affected the permanence and activity of the microorganisms in the medium.

The temperature 38.96°C had a representative effect on the pH reduction and lactic acid production. Microorganisms are found over a wide temperature range; those below the minimum are inhibited and those above the maximum value do not survive. The ideal range for the activation and survival of acid lactic bacteria is comprised between 30 - 40°C (Siqueira, 1995; Gandra & Gandra, 2007; Silva et al., 2010). The temperature set for the fermentation of the supplement is in accordance with the ideal temperature of growth and activity of the acid lactic bacteria.

Moisture at 66% used affected the fermentative responses. Hu et al. (2008) and Ríos et al. (2012) verified the existence of the relationship between high moisture, pH reduction and increased lactic acid, which corroborated with the results found. According Gandra & Gandra (2007), water must be available for microorganisms to perform metabolic activities. In the fermented supplement developed, the high moisture allowed efficient activity of the microorganisms during the fermentation process.

There was no effect on the amount of inulin (1%, 2% and 3%) used on fermentative responses. Higher bacteria activity was observed when 1% of inulin was used in a product with characteristics close to bee bread (Vamanu et al., 2010). Honey, a natural prebiotic that stimulates growth and activity of bacteria (Vamanu et al., 2010), was one of the ingredients of the fermented supplement developed. The fermentative activity of the bacteria occurred effectively, possibly with the collaboration of 2% of inulin plus honey.

The amount of probiotic (7.50%, 10.00% and 12.50%) added to the supplement had no sig-

nificant effect on the fermentative responses. Mixed cultures and available substrate are important variables for fermentation, so the presence of one strain favors the development of the other (Gandra & Gandra, 2007; Martín & Cuenca, 2009). Ellis & Hayes Jr (2009) used *Lactobacillus bulgaricus*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *Streptococcus thermophilus* and *Bifidobacterium bifidum* to promote fermentation of the diet and to suggest that nutritional value and product palatability improved. Therefore, the type of crop and the combination of crops to be used, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* spp. *bulgaricus*, *Bifidobacterium animalis* spp. *lactis*, *Lactobacillus casei shirota* and *Saccharomyces cerevisiae*, interfered more in the fermentation than the used amount of 10%.

The concentration higher than 10⁸ CFU/g present in the fermented supplement developed corroborates the concentration indicated for probiotic products by the National Health Surveillance Agency (2008). Commercial probiotic preparations use mean concentrations of 10⁹ CFU/g (Kazimierczak-Baryczko & Szymaś, 2006; Szymaś, Łangowska, & Kazimierczak-Baryczko, 2012). Regardless of the value added to the developed fermented supplement, the final product of all the trials presented ideal CFU/g to be considered as probiotic products. Bacteria reproduced and colonized the existing substrate in ideal amounts for the medium in which they were located.

It was possible to develop a fermented protein supplement optimized under laboratory conditions in five days. The method developed to ferment the supplement provided ideal conditions for efficient activity of the microorganisms. The developed supplement possessed fermentative properties similar to bee bread, natural food of the honeybees.

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