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# Fermented or unfermented milk using *Bifidobacterium animalis* subsp. *lactis* HN019: Technological approach determines the probiotic modulation of mucosal cellular immunity

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ABSTRACT

Functional foods are important sources of probiotic delivery, mainly by fermented milk products. The physiological benefits attributed to bifido bacteria are their abilities to interfere with the adhesion of pathogenic species to surfaces of intestinal cells, and to enhance the host's immune function through their metabolic activities. However, the effects of a technological approach — fermentation or addition of probiotic in milk, and its efficacy in health are rarely taken into consideration. Hence, fermented or unfermented milk using *Bifidobacterium animalis* subsp. *lactis* HN019 were administered to BALB/c mice for 14 days. After that, the architecture of the gut was histologically investigated, and the related immune cells were examined by flow cytometry and immunofluorescence. Increase in mucus and cellularity production, changes in immune pattern and preservation of mucosal epithelia in health BALB/c mice were observed only in the fermented milk group. This suggested that changes in functionality of bifidobacteria and/or the metabolites produced by the fermentation process are the keys to improving beneficial effects in the host of the gut mucosa.
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## 1. Introduction

The intestinal homeostasis is maintained by immunomodulation of the intestinal mucosa. This modulation is promoted by the interaction of indigenous microbioma with medicines, food and a large variety of orally taken substances, including probiotics (Yan & Polk, 2011).

Since 2002, probiotics have been defined as "live microorganisms which, when administered in adequate amounts, confer unspecified health benefits on the host" (FAO/WHO, 2002) without attention to their interactions with the matrix where they were delivered, or the changes undergone by the technology applied to the development of a probiotic product, as fermentation. The probiotic health benefits are strain-specific, and should confer at least, one of the most significant health effects. Metabolic effects of fermentation of indigestible dietary fiber (Nilsson, Stman, Holst, & Björck, 2008), positive influence on transit of luminal contents by peristalsis (Matsumoto et al., 2012), competition with pathogenic microbes for nutrients and binding sites on mucosal epithelial cells (Candela, Biagi, Turroni, Vitali, & Brigidi, 2008) and modulation of the host's immune response (Mazmanian, Liu, Tzianabos, & Kasper, 2005) were described. The probiotics of human consumption are preferentially of human origin, they are non-pathogenic, and they survive gastrointestinal transit (Hardy, Harris, Lyon, Beal, & Foey, 2013; Ouwehand et al., 2008). On the other hand, the exact mechanism of action is not fully understood (Collado, Isolauri, Salminen, & Sanz, 2009).

The probiotics of genera *Bifidobacterium* are believed to be beneficial to human health (Collado et al., 2009) because of their abilities to interfere with the adhesion of pathogenic species to surfaces of intestinal cells, even though their abilities to enhance the host's immune function are attributed as the major physiological benefits to host (Gopal, Prasad, & Gil, 2003).

The hallmark of mucosal immunity is the B-lymphocyte $IgA^+$, where the clonal expansion of this B-lymphocyte is associated to the modulation of the mucosal immune response which may also be promoted by probiotic microorganisms, without enhancement of the inflammatory immune response. The probiotics may regulate the innate immune response upwardly or downwardly, in order to maintain the gut homeostasis (Galdeano, de Le Blanc, Vinderola, Bonet, & Perdigon, 2007; Macpherson, Geuking, & McCoy, 2011).

Functional foods are the main delivery forms of probiotics; although the differences between fermented (FBM) or unfermented (UFBM) milk benefits to health-related subjects are not often taken into consideration. The probiotic activity is changed not only by the strain specificity,

*Abbreviations:* CFU, colony forming units; CM, control milk; CW, control water; FBM, fermented bifido milk; FBMHT, fermented bifido milk heat treated; PP, Peyer's patches; UFBM, unfermented bifido milk.

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but also by the technological process used and the food matrix where the bacteria are delivered (Ranadheera, Baines, & Adams, 2010; Sánchez et al., 2009).

This study was aimed at investigating and comparing the immune effects in the gut mucosa induced by different food technological processes that were applied to the same food matrix (skimmed milk), using identical probiotic strains and bacterial counts (*Bifidobacterium animalis* subsp. *lactis* HN019) higher than 8.00 $\log_{10}\cdot\text{CFU}\cdot\text{mL}^{-1}$ in healthy BALB/c mice.

## 2. Materials and methods

### 2.1. Product design

Fermented and unfermented milk were produced with *B. animalis* subsp. *lactis* HN019 (DuPont-Danisco, Madison, USA), and skimmed milk and water were used as control. In summary, skimmed milk UHT (Molico®, Nestlé, Araçatuba, SP, Brazil) was purchased from a local market. Afterwards, the milk was inoculated with 1 mL of $2.4 \times 10^{-2}$ g/mL probiotic culture previously hydrated and activated for 15 min in a water bath at 37 °C to obtain the same 9.00 $\log_{10}\cdot\text{CFU}\cdot\text{mL}^{-1}$ counts of microorganism in each final product. Three technological processes were employed. They are as follows:

i. Fermentation of milk by *B. lactis* HN019: fermentation was conducted at 37 °C, until the milk reached pH 4.7 and was controlled by CINAC system (*Cynetique d'acidification*, Ysebaert, Frépillon, France) (Spinnler & Corrieu, 1989). When pH4.7 was reached, the fermented milk was cooled until 10 °C, and stored in a refrigerator at 4 °C. The product which resulted denoted fermented bifido milk (FBM).
ii. Addition of milk with *B. lactis* HN019: after inoculation of the milk the product was stored in a refrigerator at 4 °C — unfermented bifido milk (UFBM).
iii. Fermentation of milk by *B. lactis* HN019 followed by heat treatment: fermentation was conducted as described in (i) above, and subsequently, the product was heat-treated at 60 °C for 40 min in Thermomix (Vorwek& Co. KG, TM31, Wuppertal, Germany). After cooling, heat-treated fermented bifido (FBMHT) was stored in a refrigerator at 4 °C.

*B. lactis* HN019 enumeration was carried out in each product before the animal trial, in order to ensure a recommended dose (WGO, 2009). It counted higher than 8.00 $\log_{10}\cdot\text{CFU}\cdot\text{mL}^{-1}$ in UFBM and FBM, and the heat caused a destruction of live bacteria in FBMHT.

### 2.2. Animals and protocol design

Twenty-five healthy eight-week-old male BALB/c mice were in five different groups ($n = 5$). Mice were fed with commercial food plus: (i) FBM, (ii) UFBM, (iii) FBMHT, (iv) control milk (UHT Molico®, Nestlé, Araçatuba, SP, Brazil) or (v) control water. The refrigerated samples were replaced daily and administrated ad libitum for two weeks. Animal weights were measured daily. Protocol design was approved by the Institution of Animal Care and Use Committee from the Pharmaceutical Sciences Faculty, São Paulo University (CEEA/FCF/14/2009 — protocol n. 210), and was replicated twice.

### 2.3. Histological sections

The mice were sacrificed in a $CO_2$ chamber (Insight Equipamentos, RibeirãoPreto, Brazil), and the large intestine tissues were prepared for histological studies. They were fixed in formaldehyde, dehydrated using a graded series of ethanol and xylene, and embedded in paraffin. The colon was sectioned and stained by Hematoxylin–Eosin (HE) and Alcian Blue (AB). The histological analyses were performed through a blind approach. The colon was analyzed by a light microscopy (Olympus America Inc., Center Valley, PA, USA). The sections were digitally processed using a photometric methodology (Photometrics system coolSNAP cf and software metaVue v. 6, 3r7, Photometrics, Tucson, AZ, USA). Four different areas were considered for each lamina, and the percentages of threshold areas were calculated.

### 2.4. Evaluation of Peyer's patches

The small intestine was collected, and the Peyer's patches were counted twice by hand which was performed blindly.

### 2.5. Flow cytometry of the gastrointestinal tract (GIT)

The small and large intestines were minced and incubated for 90 min at 37 °C in a digestion buffer containing 0.7 $\text{mg}\cdot\text{mL}^{-1}$ collagenase IV (Sigma-Aldrich, St. Louis, USA). A large particulate matter was removed by passing cell suspension through a small loose nylon wool plug, and the cells were examined. Dendritic cells, macrophages, $CD3^+CD4^+$ cells, $CD3^+CD8^+$ cells, B-2 B cells and B-1 B cells were determined by flow cytometry, using FACSCanto II (Becton Dickinson, San Jose, USA). In order to determine the membrane markers, the cells were labeled with monoclonal antibodies (Mabs) against mouse CD11b–APC (eBioscience, San Diego, USA), IgM–PE (eBioscience, San Diego, USA), IgD–PE (BD Pharmingen, San Jose, USA), e CD5–PerCy5 (BD Pharmingen, San Jose, USA), CD3–PE (BD Pharmingen, San Jose, USA), CD4–APC (BD Pharmingen, San Jose, USA), CD8a–FITC (BD Pharmingen, San Jose, USA), and CD11c–FITC (eBioscience, San Diego, USA) and F4/80–PerCP (eBioscience, San Diego, USA). FlowJo (Tree Stars) was used for analysis of flow cytometry data. To distinguish auto-fluorescent cells from cells expressing low levels of individual surface markers, upper thresholds were established for auto-fluorescence by staining samples with fluorescence-minus-one (FMO) control stain sets (Herzenberg, Tung, Moore, Herzenberg, & Parks, 2006). In these sets, a reagent for a channel of interest was omitted.

### 2.6. Immune histology of colon

The number of IgA positive cells, macrophages and dendritic cells were determined on histological slices using a direct immunofluorescence assay. After deparaffinization, using xylene and rehydration in a decreasing gradient of ethanol, paraffin sections (4 μm) were incubated with a 1:100 dilution of α-chain monospecific antibody conjugated with FITC (Sigma, St. Louis, MO, USA), CD11b–FITC (eBioscience, San Diego, USA), F4/80–PE (eBioscience, San Diego, USA), CD11c–FITC (eBioscience, San Diego, USA), and TLR4–FITC (eBioscience, San Diego, USA) for 90 min at room temperature, and observed with a fluorescent light microscope. The number of fluorescent cells was counted in 30 fields at 1000× magnifications. The results were expressed as the number of positive fluorescent cells per ten fields of vision.

### 2.7. Statistical analyses

All the results are representative of at least, two independent experiments. Data were analyzed through STATISTICA version 8.0 (StatSoft Inc., Tulsa, USA). ANOVA was performed to compare the means of two groups, and Kruskal–Wallis test was used for comparison of three or more groups that were applied. Newman–Keuls test was used to compare the difference in weight, and $P < 0.05$ was considered significant.

## 3. Results and discussion

### 3.1. Weight profile and histology differences into the gut mucosa

The weight profiles during the two week ingestion of CW, CM presented no statistic variation throughout the experiment (Table 1). Despite that FBM and FBMHT had shown a similar final weight, in the

**Table 1**
BALB/c mouse weight's[a] (g) before fermented milk intake (D0), and after products wereconsumed at days 7 (D7) and 14 (D14).

| | CW | CM | UFBM | FBM | FBMHT |
|---|---|---|---|---|---|
| D0 | $28.22 \pm 1.34^{a}$ | $30.04 \pm 2.61^{a}$ | $30.00 \pm 0.94^{a}$ | $29.64 \pm 0.89^{a}$ | $30.39 \pm 1.73^{a}$ |
| D7 | $28.61 \pm 1.18^{a,b}$ | $30.44 \pm 1.75^{b,c}$ | $29.32 \pm 2.35^{a}$ | $30.47 \pm 1.08^{c,d}$ | $30.81 \pm 1.78^{d}$ |
| D14 | $28.45 \pm 1.06^{a}$ | $30.65 \pm 1.40^{a}$ | $28.48 \pm 2.24^{c}$ | $30.40 \pm 0.98^{b}$ | $31.64 \pm 1.26^{b}$ |

CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat-treated.

[a] Mean values (N = 5) ± standard deviation with different letters in the same line are significantly different; Newman–Keuls test was used to compare the differences in weight, $P \leq 0.05$.

middle of experiment, FBMHT exhibits an increase in body weight and represents the biggest weight variation during the protocol with the tendency to increase weight in the first week and lose it in the next week (Fig. 1B and Table 1), the FBMHT trend line has indicated an increase on the final weight about 0.9 g, similar to FBM. On the other hand, UFBM reveals a decreasing trend line that shows in the end to be less of 1.4 g. This effect could be promoted by a decrease in the capacity of nutrient absorption by the destruction of gut epithelia (Fig. 1) (Acheson & Luccioli, 2004).

Histology of the colon was performed to evaluate if the effect of technological differences employed in probiotic products – UFBM, FBM and FBMHT– interfered with the integrity of the intestinal mucosa, considering that matrix, probiotic and dose were the same (Grzeskowiak, Isolauri, Salminen, & Gueimonde, 2011). FBM promoted an increase of ~15% on cellular influx, and restored the gut epithelium when compared to the controls. It also promoted an increase of ~7% when compared to the UFBM (Fig. 2). The UFBM showed a little destruction on gut epithelium (Fig. 2A-d), probably by the mucus decrease when compared to FBM and CM (Fig. 2B-d and C). Moreover, digital images showed a similar

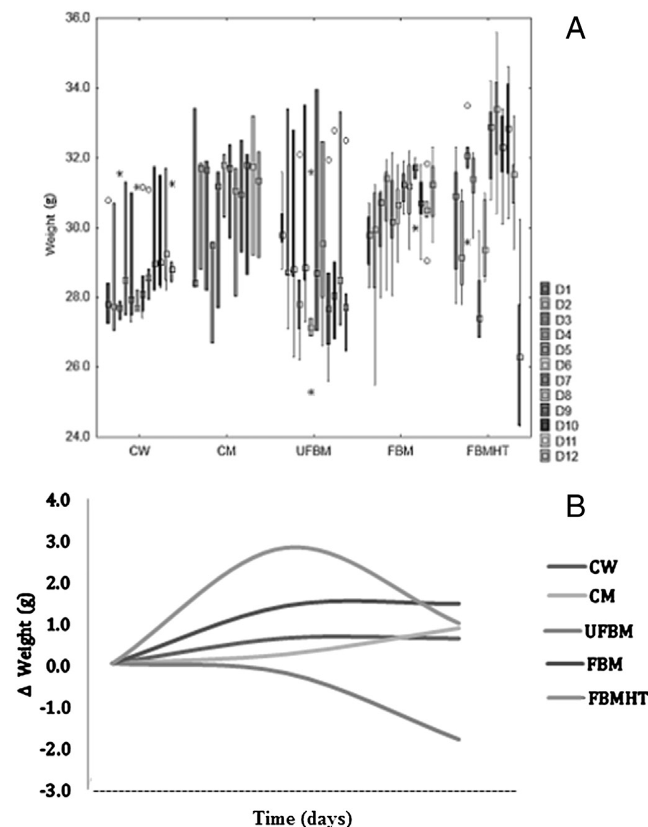



**Fig. 1.** A) Box plot of weight by time (days) shows the weight monitoring in BALB/c mouse after products intake for 14 days (N = 5). B) Trend line of gain/loss of weight in BALB/c mouse after product intake for 14 days (N = 5). CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated.

cellular infiltration in animals that consumed water, milk and unfermented milk ($P \leq 0.05$) in comparison to those that ingested fermented milk or heat-treated fermented milk (Fig. 2C).

In order to enhance mucus production (Grzeskowiak et al., 2011) and start low grade inflammation (Perdigon, Galdeano, Valdez, & Medici, 2002), these data suggested that metabolites produced during the *B. lactis* HN019 fermentation (Bogsan et al., 2013) or modification of the probiotic functionality are required, and play a key role in the interaction between host and functional foods.

### *3.2. Effects in immune activation in the gut mucosa*

Considering the differences induced in mucosal preservation and cellular infiltrate due to the product consumption described earlier, the amounts of PP were affected as evident in Fig. 3. The number of PP in the small intestine of BALB/c mice fed with FBM had a significant increase ($P \leq 0.05$) when compared with those fed with CW, CM and UFBM. These data corroborate with the hypothesis that it is not just the viable microorganism or metabolites produced during fermentation, but a combination of both that are able to perform the best interaction between the probiotic and the host. Non-viable cells plus fermentation metabolites could stimulate the PP formation despite that no statistical differences were noted (FBMHT), merely metabolites produced during the fermentation process (Bogsan et al., 2013) were not enough to properly stimulate the immune system (Fig. 4). The combination of both, viable cells and fermentation metabolites, could perform the best interaction taking into account that the technical process could change the probiotic functionality based on differences of the physiological state of the bacteria promoted by cold and acid stress. It should be noted that: CM had pH 6.68; UFBM had pH 6.03; FBM had pH 4.52; and FBMHT had pH 4.62 after 24 h of cold storage.

Bearing in mind that the infection of PP resulted in reduced lymphocyte egress independent of TNF or gamma-IFN ratio (Pabst, 2013), and caused the observed increase in colon cellularity and PP numbers in FBM mice group, the investigation of immune pattern was essential, and the results were presented in Fig. 4.

According to published literature (Galdeano & Perdigon, 2004; Vitiñi et al., 2000), the percentage of T cells showed the same ratio between all products (Fig. 4). Indeed, when these populations were compared between the different groups, it was observed that helper T cell ($CD3^{+}CD4^{+}$) had a significant increase in percentage when compared to the control and FBM. Moreover, cytotoxic T cell ($CD3^{+}CD8^{+}$) had no significant differences in the percentage of immune cells (*data not shown*). Conversely, when the DC, TLR4 and macrophages' expressions were scrutinized, the UFBM showed a significant decrease in the number of these cells.

B cells are divided among B2 cells (marginal zone and follicular), B-1 cells (B1-a and B1-b) and Regulatory B cells. They are distinguished also by tissue presence and cytokine profile (Baumgarth, 2011). B1 cells (B-1a and B-1b) are significant sources of antibodies specific for bacterial cell wall components that liberate IgA without T cell activation (Macpherson & Harris, 2004).

Subtypes of B cells were also analyzed, and the results could be seen in Fig. 5. Results suggested a change in the pattern of B cells' activation. Animals that ingested water or milk showed predominance in B1 cells (Galdeano & Perdigon, 2004; Vitiñi et al., 2000), while those that

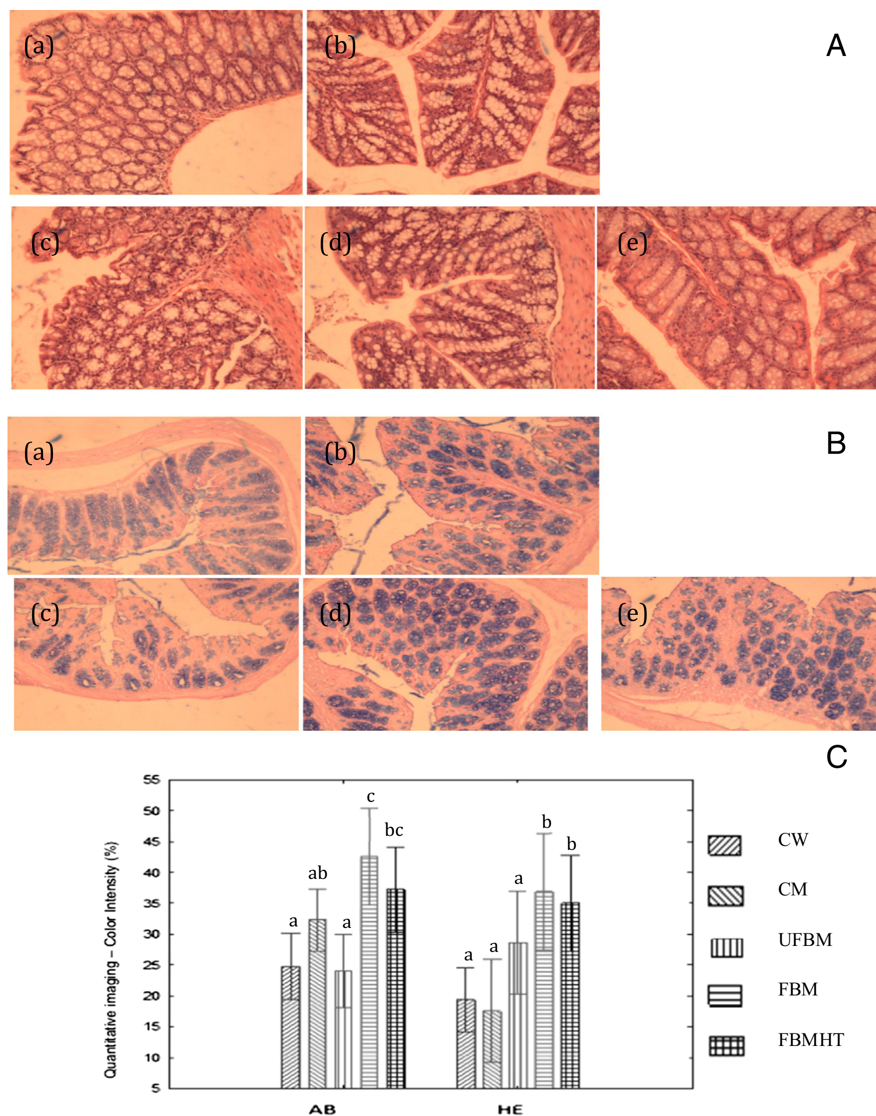



**Fig. 2.** A) Histologic sections of colon stained by Hematoxylin–Eosin (HE). B) Histologic sections of colon stained by Alcian Blue (AB). Analyzed by Olympus BX60 microscope with lens 10×/0.30 Ph1 UplanFl and condenser of 1.25× Photometrics coolSNAPcf through the system and software version 6.5r7 metaVue. (a) Control water; (b) control milk; (c) unfermented bifido milk; (d) fermented bifido milk; (e) fermented bifido milk heat-treated. C) Color intensity (in %) of cellular infiltrate and mucus thickness in BALB/c mice colon after 14 days of product intake (N = 5) in control water (▨; CW); control milk (▧; CM); unfermented bifido milk (▥; UFBM); fermented bifido milk (▤; FBM); fermented bifido milk heat treated (▦; FBMHT). Mean values ± standard deviation (n = 6) with different letters are significantly different; $P < 0.05$.

ingested UFBM, FBM and FBMHT showed a decrease in B2:B1 relationship. In contrast, B1 cells maintained prevalence in controls and FBM groups, and changed the pattern in UFBM and FBMHT, while the population of B2 cells was similar to those of B1 cells. This fact was emphasized when the FBM intake was led. Since T independent activation of B1 cells was maintained when compared with UFBM or FBMHT consumptions, it is possible to infer that adaptive immunity activation was triggered through an increase in T independent activation of B2 cells.

Peyer's patches (PP) are clusters of lymph nodes located in the small intestine. Their main function is to present antigens to initiate the mucosal immune function. This system has an excellent IgA response to the presence of bacteria symbionts within the mucosa; although, the systemic immune response is differently affected (Macpherson & Uhr, 2004). In the PP, probiotics increase B cells' differentiation of IgA plasmocytes to deliver sIgA to the intestinal lumen through the gut mucosa (Castillo, de Moreno de Le Blanc, Galdeano, & Perdigón, 2012; Gogineni, Morrow, & Malesker, 2013). The function of sIgA is to protect the mucosa from indigenous microbiota, and from food contaminated with pathogenic microbes. The PP number could be modified through extrinsic factors like nutrition, stress and age (Van Kruiningen, West, Freda, & Holmes, 2002).

Experiments in which cellular components (B and T lymphocytes and dendritic cells) were purified from different secondary lymphoid structures and reconstituted in vitro showed that the IgA switch was much more efficient when leukocytes (primarily dendritic cells) were derived from Peyer's patches than when they were derived from other cellular sources. This suggests that $IgA^{+}$ B cell induction takes place locally within the mucosa, although the system is primitive in terms of T independence and the superfluity of compartmentalized B, T, and follicular zones within the intestinal lymphoid follicles (Macpherson & Uhr, 2004).

The literature shows that macrophages and DC percentage are increased after the fermentation of probiotic milk intake in the peripheral blood (Gill & Rutherfurd, 2001). Moreover, in the intestinal mucosa, these values are not well described because the mucosal stimulation is strictly strain-dependent (Mileti, Matteoli, Iliev, & Rescigno, 2009). In the analysis of the innate immunity through DC and macrophages, and colon macrophages and DC, an increase in cell numbers was observed in

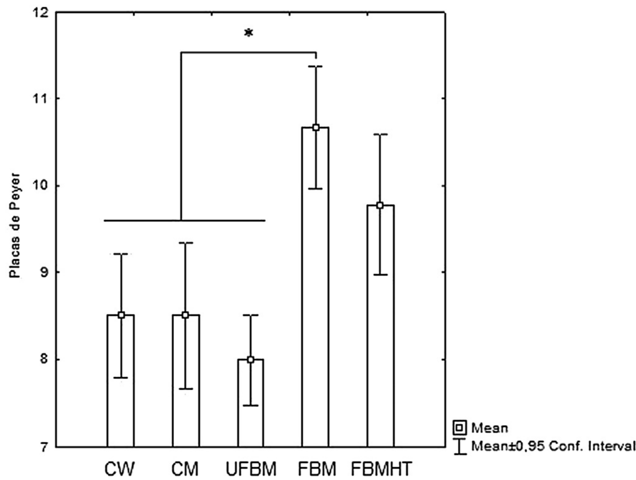
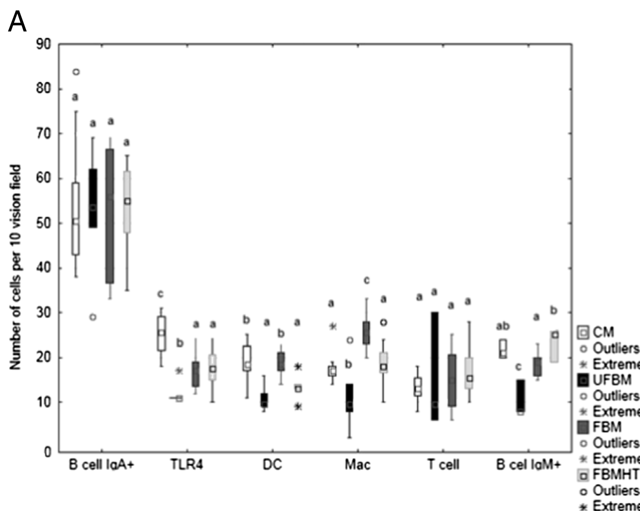



**Fig. 3.** Number of Peyer's patches in BALB/c mouse after product intake for 14 days (N = 5). CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated. Tukey test; $P < 0.05$.




B

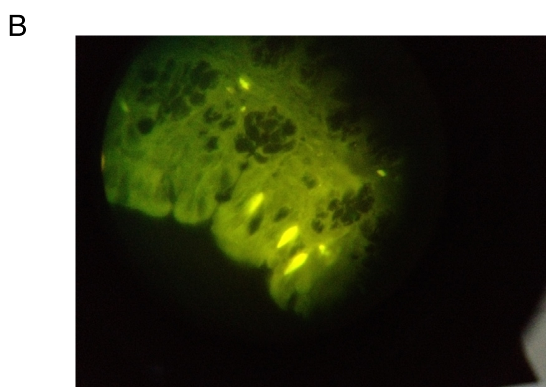


**Fig. 4.** A) Number of positive cells per ten fields of vision in the gut mucosa of BALB/c mice fed with control milk (CM, □), unfermented bifido milk (UFBM, ▣), fermented bifido milk (FBM, ■) and fermented bifido milk heat treated (FBMHT, ■) analyzed per colon immunohistochemistry (N = 6). Abbreviations: T CD4$^+$: T helper cells CD4 positive; T CD8$^+$: T cells cytotoxic CD8 positive; DC: dendritic cells CD11c positive, MÆ: macrophages CD11b positive and B: B cells B220 positive. Mean values ± standard deviation (N = 5) with different letters are significantly different; $P \leq 0.05$ intra-group. B) Green cells identify IgA$^+$ B cell through immunohistochemistry of histological sections of BALB/c mice's large intestine of animals fed with fermented milk in 40×magnification observed in the immunofluorescence microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

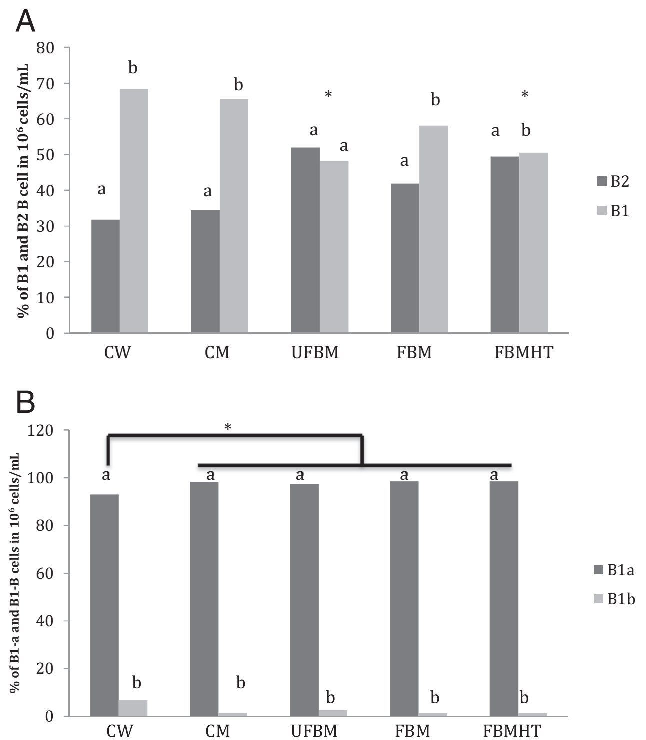



**Fig. 5.** A) Distribution of B cell subtypes — B2 (■) and B1 (■), in the gut mucosa of BALB/c mice fed with CW, CM, UFBM, FBM and FBMHT for 14 days. Abbreviations: CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk; FBMHT: fermented bifido milk heat treated; B1: cells B IgM$^+$ IgD$^+$ CD5$^+$; B2: cells B IgM$^+$ IgD$^+$ CD5$^+$. B) B-1 cell subtype distribution — B1-a (■) and B1-b (■), in the gut mucosa of BALB/c mice fed with CW, CM, UFBM, FBM and FBMHT for 14 days. Mean values ± standard deviation (N = 5) with different letters are significantly different; $P \leq 0.05$ intra-group; mean values ± standard deviation (N = 5) with * are significantly different; $P \leq 0.05$ inter-groups. CW: control water; CM: control milk; UFBM: unfermented bifido milk; FBM: fermented bifido milk. B1-a B cell IgM$^+$ IgD$^+$ CD5$^+$, B1-b B cell, IgM$^+$ IgD$^+$ CD5$-$.

the consumption of FBM when compared to the control milk; in contrast, they had a significant decrease when UFBM was consumed (Fig. 4).

De Moreno De Leblanc et al. (2008) demonstrated in animals that consumed *L. casei* fermented milk had an increase in bifidobacteria population, and a significant increase in IgA$^+$ plasmocytes. In spite of this, in the current study, neither IgA$^+$ plasmocytes nor T cells have shown significant differences between the groups, whereas IgM$^+$ plasmocytes and TLR4$^+$ cells have shown an increasing expression in FBM group (Fig. 4). These data could infer that FBM stimulates the innate immunity, while UFBM does not. Therefore, what was believed to be a lack of food antigen presentation could be reported to be a change in the activation pattern, and related to an increase in the PP number as observed in Fig. 3.

Finally, neither the probiotic nor variation in the products' manufactures exerts influence in B1 cell modulation. It is the selected matrix that exerts this influence, as milk matrix significantly decreased B1-b expression (Fig. 5). Moreover, it may be that mucus production and intraperitoneal–mucosal recirculation (Margry, Wieland, van Kooten, van Eden, & Broere, 2013) require further studies. Therefore, it could be postulated that changes in bacteria functionality as promoted by the fermentation process and food matrix employed denote a key factor in signaling B cell activation pattern.

## 4. Conclusions

The data from this study suggest that, even in the use of the same matrix, probiotic strain and dosage, variations will arise in the technological

approach in order to produce a probiotic dairy product that affects the immune activation pattern in BALB/c mice. Considering the fact that not all microorganisms have the same effects on the host, it is not possible to infer that the effects found with one probiotic strain will be equally found by another, neither is it possible to conclude that there will be similarities in their applications to products throughout the different technological processes – fermented or not. Thus, the administration of probiotic strains needs to be carefully evaluated, and the questions of if they should be fermented or not, and which matrix could be more suitable to promote the desired effect should be taken into consideration.

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