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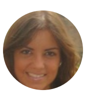

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The Probiotic Bacterial Strain *Lactobacillus fermentum* D3 Increases In Vitro the Bioavailability of Ca, P, and Zn in Fermented Goat Milk

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Abstract We determined calcium, magnesium, phosphorus and zinc levels in a total of 27 samples of commercial goat- and cow-milk fermented products and 9 samples of a goat-milk fermented product with addition of a probiotic bacterial strain, *Lactobacillus fermentum* D3, manufactured experimentally by our research group. Atomic absorption spectroscopy with flame atomization and UV/VIS spectrophotometry were used as analytic techniques. The results of an in vitro digestion process showed that the bioavailability of calcium, phosphorus, and zinc was significantly higher in our fermented milk containing the probiotic bacterial strain than it was in commercial goat-milk fermented products. Furthermore, our product showed a significantly higher bioavailability of calcium and zinc compared to goat- and cow-milk fermented products made with other microorganisms. We conclude that, in in vitro assays, strain D3 seems to increase the bioavailability of these minerals and that this new product may constitute a better source of bioavailable minerals compared to other products already on the market.

Keywords Fermented goat · Cow-milk products · Mineral composition · Dialyzable fraction · Probiotic starter culture

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Introduction

The fact that people are more concerned about their health and quality of life is leading to the search of new foods with health-giving properties which might delay or even prevent the onset of certain illnesses. Among these foodstuffs, goat milk is currently being subjected to numerous studies as it is easy to digest and tolerate and high in nutritional value. It contains high-quality, absorbable proteins, particularly greater quantities of seric proteins than are to be found in cow milk [1], which may make it more readily digestible [2]. Goat milk provides more short- and medium-chain fatty acids (caproic, caprylic, and capric), which contribute to an easier utilization of the fat at the digestive level, together with essential fatty acids such as linoleic, linolenic, and arachidonic [3]. As far as minerals are concerned, it affords highly available calcium (Ca) and phosphorus (P), partly due to their being found in association with casein in the milk. It also favors iron absorption and its deposition in target organs [4]. Apart from this, goat milk is a good source of vitamins, noteworthy among which are A, D, E, thiamine, riboflavin, and niacin. Other minor components stand out either for their functional activity (conjugated linoleic acid, antioxidants, and bioactive peptides, for example) and/or their influence on the flavor of dairy products. Goat milk is eminently tolerable and thus advisable both for children and for the elderly, due to the high digestibility of its proteins and fat. In addition, it has been demonstrated that goat milk reduces low-density lipoprotein (LDL) and total cholesterol levels [3, 5], maintaining high-density lipoprotein (HDL) cholesterol, triglyceride, and transaminase (GOT and GPT) levels within physiological limits.

India is at the forefront of the goat milk production of the world while Spain is in sixth place, together with other European countries such as France and Greece [6].

In general, estimates of the total element content in food are insufficient and its bioavailability needs to be considered. Mineral bioavailability especially has gained increasing interest in the field of nutrition. In vivo studies are both expensive and laborious, and the possibility of measuring certain parameters during the experiments is often limited [7–10]. On the other hand, methods of simulated digestion in vitro constitute a suitable alternative for calculating the percentage that is transformed into absorbable forms in the digestive tract (bioaccessibility) [8]. These procedures are rapid, usually inexpensive, and allow individual experimental variables to be easily controlled [11]. The results are usually expressed as dialyzable fraction under given experimental conditions such as pH, enzyme addition, and temperature [7, 9, 12].

The aim of this work was to undertake a comparative study of the in vitro bioavailability of Ca, magnesium (Mg), P, and zinc (Zn) in commercial goat- and cow-milk fermented products. It is well-known that probiotics have a number of beneficial health effects in humans and animals, including the stimulation of the immune system and the enhancement of the bioavailability of nutrients [13, 14]. Therefore, we compared these commercial fermented products with a fermented goat-milk product to which our research team added not only the traditional yoghurt starters, *Lactobacillus bulgaricus* spss. *delbruickii* and *Streptococcus thermophilus*, but also a potentially probiotic lactic bacterium, *Lactobacillus fermentum* D3. The results also provide information concerning the mineral contents of the various cow- and goat-milk products assayed. These data are useful for completing the composition tables of the foodstuffs in question, in which information about the elements of new functional fermented products is still scarce. Moreover, we consider that this additional information concerning the composition of goat milk and its fermented products is essential for the success of industries producing goat milk, as well as for the marketing of these products.

Materials and Methods

Probiotic Strain

Strain D3 was isolated from goat milk and identified as *L. fermentum* on the basis of biochemical tests, sugar fermentation reactions (API 50 CHL), and the sequencing of its 16S rRNA gene. *L. fermentum* D3 resists gastric pH and bile salts and has antimicrobial and immunomodulating properties.

Sample Collection

We analyzed a total of 8 samples of different fermented goat milks. Although the number of fermented goat-milk products on the market is limited, all the commercial brands available in the Granada supermarkets were included. We also analyzed a total of 19 samples of fermented cow-milk products. Several brand names of each product, representing the most widely accepted and most frequently consumed in Spain by different income groups, were selected for testing. For every one of the referred commercial brands, four samples were collected for carrying out the present study. We also analyzed 9 samples of the fermented goat-milk product experimentally developed by us with the addition of *L. fermentum* D3. Fermentation was conducted under standard conditions using commercial strains of *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* as starters. Strain D3 of *L. fermentum* was added after fermentation at 10^7 viable bacteria per milliliter. All samples were carefully handled to avoid contamination, and the appropriate quality-assurance procedures and precautions were followed to ensure the reliability of the results.

ASS Instrumentation and Conditions

We used a Perkin-Elmer 1100B double-beam atomic-absorption spectrometer equipped with deuterium-arc-background correction (Perkin-Elmer, Norwalk, CT, USA). Measurements were made at 213.9 nm for Zn, 285.2 nm for Mg, and 422.7 nm for Ca using various hollow cathode lamps (Perkin-Elmer). P was measured at 750 nm with a UV/VIS spectrophotometer. A Selecta multiplace digestion block (Selecta SA, Barcelona, Spain) and Pyrex tubes were used for sample mineralization. A Moulinex blender (Moulinex, Bagnolet, France) was used to homogenize the samples. A shaken, thermostatic water bath (Selecta SA, Barcelona, Spain) was used for in vitro assays. Bidistilled deionized water with a specific resistivity of 18 MΩcm from a Milli-Q system (Millipore, Milford, MA) was always used.

Materials

To decrease the risk of contamination, polypropylene vessels and plastic pipette tips were used, glassware being reduced to a minimum. All the materials were washed in nitric acid and rinsed several times with bidistilled deionized water.

Chemicals

Standard solutions of Ca, Mg, P, and Zn (1.000 ± 0.002 g/L) (Tritisol, Merck, Darmstadt, Germany) were used and diluted as necessary to obtain working standards. High-quality concentrated nitric acid (65 %), perchloric acid (70 %), and vanadium pentaoxide (Merck) were used for sample mineralization. All reagents used were of analytical grade. A chemical modifier ($LaCl_3$) was needed for Ca measurements. All solutions were prepared from analytical reagent-grade chemicals (Suprapur, Merck, Germany). To simulate gastric and intestinal digestion, we used pepsin (Sigma P7000), porcine pancreas (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), pancreatin (Sigma P1500) porcine pancreas (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), and bile salts (Sigma B8756). A pepsin solution was prepared by dissolving 16 g of pepsin in 100 mL of 0.1 mol/L HCl; pancreatin–bile extract mixture was prepared by dissolving 4 g of pancreatin and 25 g of bile extract in 1 L of 0.1 mol/L $NaHCO_3$. Sodium hydroxide (0.5 M) and hydrochloric acid (6 M) were used to adjust pH. Cellulose dialysis tubing (43×27 mm) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) was used. The dialysis tubing was freed of trace-metal impurities by boiling in 2 %*w*/*v* $NaHCO_3$, 0.1 %*w*/*v* sodium dodecyl sulphate, and 0.01 M ethylenediaminetetraacetic acid disodium salt (EDTA) for 10 min and then in bidistilled deionized water for 10 min before being washed thoroughly in bidistilled deionized water and preserved in 20 % ethanol solution.

Sample Preparation and Analysis

A 2-g portion of homogenized sample was treated with 8 mL of HNO_3–$HClO_4$ (4:1) and a few micrograms of V_2O_5 as a catalyst in Pyrex tubes placed in the digestion block and heated to 60 °C for 45 min, 90 °C for 30 min, and finally 120 °C for 60 min. The solutions were cooled to room temperature, transferred to a calibrated flask, and diluted with bidistilled deionized water to a final volume of 25 mL. All analyses were made in duplicate.

Method for Estimating the Ca, Mg, P, and Zn Dialyzable Fraction In Vitro

Bioaccessibility of Ca, Mg, P, and Zn from the fermented milk samples was determined by using an in vitro method, involving a simulated gastrointestinal digestion procedure with suitable modifications [15–18]. To simulate gastrointestinal digestion, a 10-g portion of homogenized sample was added to 80 mL of bidistilled deionized water, and pH was adjusted to 2.0 with 6 M HCl. Freshly prepared pepsin solution (3 mL) was added to the sample, and the mixture was made up to 100 g with bidistilled deionized water. This was then incubated in a shaken thermostatic water bath for 2 h at 37 °C. After this process of simulated gastric digestion, duplicate 20-g samples of pepsin digest were transferred to 250-mL Erlenmeyer flasks. The dialysis tubing, containing 25 mL of bidistilled deionized water and a quantity of $NaHCO_3$ equivalent to the titratable acidity measured previously, were placed in the flasks and incubated in the shaken bath at 37 °C for 30 min, after which 5 mL of the pancreatin–bile extract mixture was added to each flask and incubated for 2 h. The dialysis tubing was removed and the dialyzate was weighed. Zn, Ca, Mg, and P were determined in the dialyzate by FAAS and UV/VIS under the same conditions as those described above. Titratable acidity was defined as the number of equivalents of 0.5 M NaOH required to titrate the quantity of combined pepsin-digests pancreatin–bile extract mixture to pH7.5. This was determined in a 20-g aliquot of the pepsin digest to which 5.0 mL of pancreatin–bile extract mixture had been added with 0.5 M NaOH at 20 °C. All digestions were done in duplicate, and blanks were prepared and analyzed following the same procedure. Mineral (Zn, Ca, Mg, and P) availability was calculated as the percentage of element dialyzed of the total amount present in the aliquot (percent dialyzability).

The analytical characteristics of the methods applied to determine P, Zn, Ca, and Mg in fermented goat- and cow-milk products have been described elsewhere [19, 20].

Statistical Analysis

The statistical package *SPSS 15.0 for Windows* (Chicago, IL, USA) was used to establish the statistical significance of differences in data values. Results are expressed as the arithmetic mean±standard deviation. The normal distribution of variables and the homogeneity of variances were verified by the Kolmogorov–Smirnov and Levene tests, respectively. Comparisons were made using Student's *t* test for parametric data and the Kruskall–Wallis test for non-parametric data. Additionally, correlations were established by Pearson's and Spearman's tests, for parametric and non-parametric conditions, respectively, and by regression models. The results were considered to be statistically significant at $p<0.05$.

Results and Discussion

Ca, Mg, P, and Zn Levels in Fermented Goat- and Cow-Milk Products

The levels of Ca, Mg, P, and Zn determined in the fermented goat- and cow-milk products together with those found by other authors are set out in Table 1. The total concentrations of the minerals studied did not differ significantly between

Table 1 Total quantity of Ca, Mg, P, and Zn present in fermented milks: a comparison between the results of this study and those reported by other researchers [21–25]

Reference	Ca (μg/g)	Mg (μg/g)	P (μg/g)	Zn (μg/g)
Cow				
This study	2,098.5±389.9	91.21±28.94	755.4±171.3	5.82±1.140
Martín-Diana et al. [22]	1,010.0±60.00	96.00±2.000	–	4.600±0.100
Ceballos et al. [29]	1,135.8±158.0	94.00±14.60	870.4±76.80	4.630±0.680
Güler and Sanal [24]	1,145.0±96.0	406.0±20.00	1,009.0±48.00	7.280±0.690
Goat				
This study	1,831.4±504.2	116.1±39.54	852.5±119.3	6.23±0.61
Park [21]	1,405.00	149.0	1,253.0	3.370
	1,287.00	148.0	1,164.0	4.100
Martín-Diana et al. [22]	1,110.0±60.00	106.0±1.000	–	4.500±0.100
Güler [23]	1,455.0±3.600	587.0±11.72	1,052.0±24.00	6.850±0.250
Ceballos et al. [29]	1,585.7±158.0	129.2±14.60	1,189.7±76.80	5.280±0.680
Güler and Sanal [24]	1,356.0±94.00	399.0±48.00	1,277.0±53.00	5.160±0.470

the goat- and cow-milk products ($p>0.05$); only in the goat-milk samples did the levels of P tend to be significantly higher ($p=0.055$). The fermented goat milk contained less Ca than the cow milk, although the difference was not statistically significant. The mean quantity of Ca in the samples of fermented goat milk (1,831.4 μg/g) and cow milk (2,098.5±389.9 μg/g) were higher in our study than those found by other authors [21–25].

Our results with regard to Mg revealed that it was present in higher quantities in fermented goat than cow milk, although the differences were not statistically significant. The total content in cow milk found in our study (91.21±28.94 μg/g) was lower than that found by other authors [22, 24, 25], and the mean quantity found by us in fermented goat milk was 116.1 μg/g, which is also lower than the values given by some other authors mentioned in Table 1.

Total P content was higher in fermented goat milk to a significant degree ($p=0.055$). The mean quantities of P found by us in both goat (852.5±119.3 μg/g) and cow (755.4±171.3 μg/g) milk were lower than those determined by other authors (Table 1). Chen et al. [26], on the other hand, found values of 1,092.8 and 788.6 μg P/g in two different types of fermented milk from Tibet, with our values for fermented goat milk falling within this range and cow milk below it.

In our study, the mean quantity of Zn found in the fermented cow-milk products (5.82±1.14 μg/g) was higher than that found by Martín-Diana et al. [22] and Ceballos et al. [25] but lower than that found by Güler et al. [24] (Table 1).

Bioaccesibility: the In Vitro Ca, Mg, P, and Zn Dialyzable Fraction

Some authors have indicated, conversely to the results of our study, that one of the nutritional advantages to fermented goat milk compared to cow milk lies in its higher mineral content and better use made of them by the human body, both during the digestive and metabolic processes. Thus, by using a suitable animal model, different authors have obtained results demonstrating this better use of the minerals contained in goat milk, an effect which they attribute both to differences in the mineral content itself and to the protein and fat composition of the two types of milk [27–31].

The results for the Ca, Mg, P, and Zn dialyzable fraction obtained in this study after digestion in vitro are set out in Table 2. The bioavailability of Ca, Mg, and P in the fermented goat-milk products studied was not significantly different from that of the same minerals in the cow-milk products. Only the bioavailability of Zn was significantly higher in the fermented goat-milk products.

As far as Ca in vitro is concerned, its bioavailability proved to be slightly higher in goat (33.0±11.3 %) than in cow (31.2±11.0 %) milk, although the differences were never statistically significant. Other authors, however, have encountered a significantly better nutritional use of minerals deriving from goat than from cow milk [29, 32].

With regard to Mg in vitro, in our study, we found that despite the data showing that fermented goat milk

Table 2 Bioaccessibility of Ca, Mg, P, and Zn in fermented goat- and cow-milk products

Element	Type of milk	*N*	Mean±SD	Range
Ca %	Goat	17	32.98±11.34	17.78–51.22
	Cow	18	31.21±11.04	19.33–58.17
Mg%	Goat	16	32.76±8.07	21.40–46.68
	Cow	18	40.07±16.34	25.07–90.11
P %	Goat	17	68.51±13.54	39.54–88.33
	Cow	19	72.54±13.06	48.29–94.47
Zn %	Goat	17	36.11±9.81	15.26–52.58
	Cow	18	25.95±7.49*	13.68–38.53

* $p=0.001$

Table 3 Bioaccessibility of Ca, Mg, P, and Zn in commercial fermented goat-milk products and our own experimental one

Element	Type of milk	*N*	Mean±SD	Range
Ca %	Industrial culture	8	24.71±7.78	17.78–42.81
	Own culture	9	39.60±9.28*	23.67–51.22
Mg%	Industrial culture	8	29.31±8.21	21.40–44.82
	Own culture	9	36.22±6.70	22.47–46.68
P %	Industrial culture	8	61.37±15.10	39.54–78.9
	Own culture	9	74.23±9.36**	60.32–88.33
Zn %	Industrial culture	8	29.97±8.05	15.26–38.07
	Own culture	9	41.01±8.44***	24.95–52.58

* $p=0.002$

** $p=0.041$

*** $p=0.012$

contained more Mg than cow milk, its mean bioavailability was in fact less in the former (Table 2), although not significantly so. On the other hand, Moreno et al. [32] and López-Aliaga et al. [30] found greater bioavailability of Mg in fermented goat-milk products than in those made with cow milk.

Campos et al. [29] demonstrated that goat milk facilitated the nutritional use of Ca and P in both healthy rats and those with intestinal resection compared to cow milk and a milk-free diet. In our study, P was the mineral that showed most bioavailabilty in fermented goat milk, but although the data indicated that goat milk contained more P than cow milk, its bioavailability was less though not significantly so. Remeuf [33] reported that soluble P in goat milk accounted for 39 % of the total, which is a considerably lower quantity than the figure of 68.5±13.5 % found by us. Pérez-Llamas et al. [34] found P bioavailability values between 38 % and 44 % in cow-milk baby foods, which is also lower than the figure of 72.54±13.06 % encountered in our study.

Only the percentage of Zn was significantly higher in the fermented goat-milk products (36.1±9.8 %) compared to those made with cow milk (26.0±7.5 %), and it was also more bioaccessible ($p<0.05$). This coincides with the information provided by other studies that have shown the better nutritional use of minerals deriving from goat rather than cow milk [35, 36]. Velasco-Reynold et al. [12] obtained a mean value of Zn bioavailability of 25.2 % in duplicate diets, while Cámara et al. [37] reported that the bioavailability of this mineral in school meals, as determined by digestion in vitro, ranged between 5.78 % and 31.45 %. Thus, it can be seen that the results of our study with regard to the bioavailability of Zn in cow-milk products are similar to those mentioned above, while the mean bioavailability values obtained in goat milk is considerably higher, which strengthens its claim to be a more functional food. This may be related to the fact that it forms more soluble compounds with other components of the milk, which may act as chelating agents in the fermented goat-milk products and would then facilitate a wider diffusion of this mineral through the dialysis membranes used in our study.

The Influence of Probiotic Starters on Ca, Mg, P, and Zn Bioavailability in Fermented Goat- and Cow-Milk Products In Vitro

Among the most important bioactive components of fermented dairy products are probiotic microorganisms, which, according to recently conducted studies, are beneficial to the health. Further to this, numerous economic indicators reveal that products manufactured with probiotic microorganisms are at the forefront of innovation in the functional food sector [38]. Several authors have reported an enhancement in mineral bioavailabity, particularly of Ca, caused by different probiotic microorganisms used in the fermentation process [39, 40].

In this study, we have developed in our laboratory a fermented goat-milk product using the traditional bacteria for the

Table 4 Bioaccessibility of Ca, Mg, P, and Zn in products fermented with different probiotic cultures

Element	Probiotic starters	*n*	Mean±SD	Range
Ca %	*Lactobacillus bulgaricus*+*Streptococcus thermophilus*	15	30.58±12.50	17.78–58.17
	Microorganisms used in kéfir+*Bifidobacterium*	11	27.33±6.90[a]	20.94–41.37
	Own culture	9	39.60±9.28[b]	23.67–51.22
Mg%	*Lactobacillus bulgaricus*+*Streptococcus thermophilus*	15	39.52±18.00	21.40–90.11
	Microorganisms used in kéfir+*Bifidobacterium*	11	33.00±9.19	23.19–53.41
	Own culture	8	36.22±6.70	22.47–46.68
P %	*Lactobacillus bulgaricus*+*Streptococcus thermophilus*	16	68.99±13.70	39.54–88.65
	Microorganisms used in kéfir+*Bifidobacterium*	11	69.58±15.98	42.83–94.47
	Own culture	9	74.23±9.36	60.32–88.33
Zn %	*Lactobacillus bulgaricus*+*Streptococcus termophilus*	16	28.22±7.01[c]	14.77–38.53
	Microorganisms used in kéfir+*Bifidobacterium*	11	25.77±8.78[c]	13.68–38.07
	Own culture	9	41.01±8.44[d]	24.95–52.58

Non-coincidence in superscript letters following the data on one mineral denotes statistically significant differences ($p<0.05$)

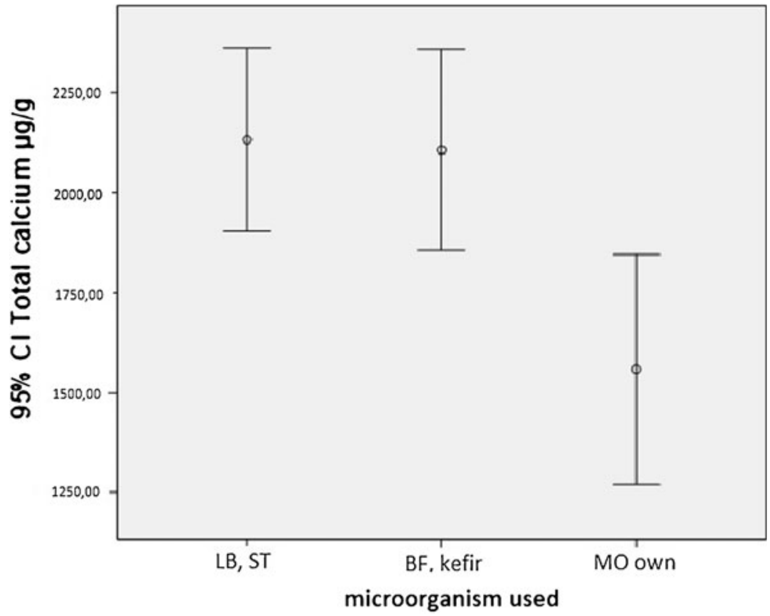

Fig. 1 Total Ca levels±95 % of their confidence intervals (CI) for the three types of sample chosen *LB*, *ST*=*Lactobacillus bulgaricus* and *Streptococcus thermophilus, BF*, *Kefir*= *Bifidobacterium* and the microorganisms used to make kefir, *MO own*=experimental culture with *Lactobacillus fermentum* D3

initial fermentation process (*L. bulgaricus* ssp. *delbruickii and S. salivarius* ssp *thermophilus*), followed by the addition of a probiotic bacterium isolated and catalogued by our research group, *L. fermentum* D3. We then compared this fermented goat-milk product with others already on the market and found that although on some occasions the total mineral content was higher in goat milk fermented with the industrial cultures, the percentage of dialyzed mineral was higher in fermented milks made with the addition of our bacterial isolate, these differences being statistically significant for Ca (p=0.002), P (p=0.041), and Zn (p=0.012) (Table 3). These results show that the bioavailability of Ca, Mg, and P, and possibly also Mg, probably due to the fermenting effect exerted by the *L. fermentun* D3 strain on the components of the fermented goat milk, would enhance the levels of chelating substances, possibly casein phosphopeptides and/ or short-chain fatty acids, which are able to maintain these elements in a soluble state. Perez-Conesa et al. [41] found that the probiotic bacteria *Bifidobacterium longum* and *Bifidobacterium bifidum* lowered pH compared with a control diet containing no probiotics and that Ca absorption was related to pH in the colon. Rehka et al. [40] found that the fermentation of soymilk with five strains of probiotic lactic-acid bacteria (*L. acidophilus* B4496, *L. bulgaricus* CFR 2028, *L. casei* B1922, *L. plantarum* B4495, *and L. fermentum* B4655) with the yeast *Saccharomyces boulardii* made Ca more soluble. Further work is needed to investigate the possible modes of action of our isolate, *L. fermentum* D3, on mineral absorption.

To observe the influence of the various bacteria on the process of bioavailability in vitro of minerals in fermented

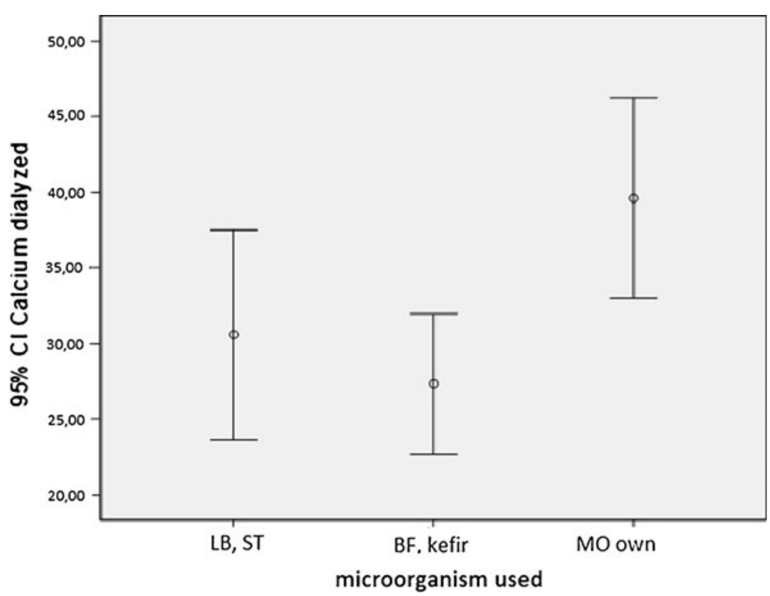

Fig. 2 Percentage levels of Ca dialyzed±95 % of their confidence intervals (CI) for the three types of sample chosen *LB*, *ST*=*Lactobacillus bulgaricus* and *Streptococcus thermophilus, BF*, *Kefir*= *Bifidobacterium* and the microorganisms used to make kefir, *MO own*=experimental culture with *Lactobacillus fermentum* D3

goat- and cow-milk products, we divided them into three groups: (a) products fermented with the traditional bacteria *L. bulgaricus* ssp. *delbruickii* and *S. salivarius* ssp *thermophilus*; (b) kefirs and products fermented with *Bifidobacterium*: (c) a product developed experimentally with the addition of *L. fermentum* strain D3. Table 4 shows the bioavailability of the elements studied according to the bacteria used as starter culture. The results of this study also revealed the benefits of our new probiotic bacterium with regard to bioavailability.

As far as Ca is concerned, it can be seen that although the total quantity present in milks fermented with the novel bacterium were lower than in the other two groups of fermented milks, it showed higher bioavailability (Figs. 1 and 2), and that the differences were significant when compared with the group fermented with *Bifidobacterium* and the microorganisms used in the kefir. Furthermore, the total quantity and percentage of Zn dialyzed were significantly higher in the milks fermented with our novel isolate compared to the other two groups.

Conclusion

This study affords new data concerning the content and in vitro bioavailability of Ca, Mg, P, and Zn in goat- and cow-milk fermented products. Although further studies are required, both in vitro and in vivo, these preliminary results are encouraging and seem to demonstrate an increased bioavailability of Ca, P, and Zn in our product compared to other fermented milks, due to the activity of our novel probiotic *L. fermentum* D3, even when the total quantity of the mineral in question is lower, as is the case of Ca.

The experimental design described constitutes a suitable strategy to assess the benefits that goat-milk fermented products might provide and thus, with a view to its marketing prospects, to achieve a better compliance of nutritional requirements.

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