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Effects of probiotic intake on intestinal bifidobacteria of celiac patients

Flávia **MARTINELLO**, Camila Fontana **ROMAN** and Paula Alves de **SOUZA**

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ABSTRACT – Background – Healthy individuals exhibit a significantly higher concentration of faecal bifidobacteria in comparison to celiac patients. Even though there are potential benefits in probiotic usage, they have been little explored as an adjunctive therapy in celiac disease. **Objective –** This study aimed at the comparison of faecal bifidobacteria concentration and pH among celiac patients and healthy subjects before and after the daily intake of 100 g of yogurt containing probiotic for a thirty-day period. **Methods –** Feces from 17 healthy subjects and 14 celiac patients were analyzed, in which stool culture was performed for the isolation and quantification of faecal bifidobacteria. Furthermore, Gram's method was employed for the microscopic analysis of the colonies, while the identification of the *Bifidobacterium* genus was made through determination of the fructose-6-phosphate phosphoketolase enzyme. Faecal pH was measured using a calibrated pHmeter. **Results –** Faecal bifidobacteria concentration before probiotic consumption was significantly higher in healthy individuals ($2.3x10^8 \pm 6.3x10^7$ CFU/g) when compared to celiac patients ($1.0x10^7 \pm 1.7x10^7$ CFU/g). Faecal pH values did not show a significant difference. After the daily consumption of probiotic-containing yogurt both groups showed a significant increase in the concentration of faecal bifidobacteria, but healthy subjects presented significantly higher bifidobacteria concentrations ($14.7x10^8 \pm 0.2x10^8$ CFU/g) than the celiac group ($0.76x10^8 \pm 0.1x10^8$ CFU/g). The obtained pH values from both groups were not significantly different, being 7.28±0.518 for the celiac patients and 7.07±0.570 for healthy individuals after the probiotic intake. **Conclusion –** The probiotic supplementation significantly increased the number of bifidobacteria in the feces of celiac patients, although it was not sufficient to reach the concentration found in healthy individuals prior to its consumption.

HEADINGS – Probiotics. Celiac disease. Bifidobacterium. Hydrogen-ion concentration. Feces, microbiology. Microbial colony count.

INTRODUCTION

Patients with celiac disease (CD) have an intolerance to the polipeptide fragments of gluten, mediated by T lymphocytes. Gluten is a water-insoluble substance found in wheat flour, rye, barley and oats[30]. CD depends on genetic, immunological and environmental factors and it is characterized by total or partial atrophy of the intestinal villi and consequent poor absorption of nutrients[6,9,27]. Its prevalence in Brazil is shown to be 1/214[3].

CD diagnosis must be based on clinical, histopathological (gold standard) and serological examinations[3,27]. There are few studies on the intestinal microbiota role in CD, even though gliadin (a gluten peptide) and microorganisms similarly activate pro-inflammatory routes[12]. The information about the intestinal microbiota of celiac patients is mainly obtained from a stool sample examination[15].

Healthy subjects present a significantly higher concentration of bifidobacteria when compared to celiac patients, while faecal pH seems to remain the same in both situations[8].

The only effective and possible treatment for CD is dietary, throughout the exclusion of gluten from the diet, which allows the remission of symptoms and the restoration of the regular mucosa[1]. Without treatment, CD has a high morbi-mortality rate, with risks of developing complications such as anemia, infertility, osteoporosis and cancer, being the most prevalent the intestinal lymphoma[27]. Alternative treatments are also available and can be used simultaneously as palliatives, for instance, the use of probiotics, mainly *Lactobacillus* and *Bifidobacterium*[7,15,24].

The presence of bifidobacteria in the gastrointestinal tract seems to suffer variations throughout life and it is associated with beneficial effects to health, including the re-composition of the intestinal microbiota, the growth inhibition of pathogenic bacteria, regeneration of the epithelial barrier and anti-inflammatory effects[11,21,25,26,32]. Some species have the capacity of inhibiting the increased permeability induced by gliadin, weakening its cytotoxic effect and the host autoimmune response[10]. Smecuol et al. showed that celiac patients on a gluten diet had experienced beneficial effects related to gastrointestinal tract symptoms (such as constipation and gastroesophageal reflux) when consuming bifidobacteria in capsules before meals[28]. Other beneficial effects of bifidobacteria consumption with a gluten diet have been described, such as the reduction of human α-defensin 5 (HD-5) and paneth cells[20].

Bifidobacterium and *Lactobacillus* are widely used in several food products, such as yogurt, milk, cheese and dietetic supplements, upon which research has been increasing. Even though there are potential benefits in their usage, probiotics have been

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Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Florianópolis, SC, Brasil.
Correspondence: Camila Fontana Roman. Departamento de Análises Clínicas. Centro de Ciências da Saúde. Bloco JK, sala 109. Universidade Federal de Santa Catarina. CEP: 88040-900 – Florianópolis, SC, Brasil. Email: camila.fontana.roman@gmail.com

poorly explored as an adjunctive therapy in CD[17,32]. In this context, the hypothesis is that the intestinal microbiota of patients with controlled CD can be restored by the daily intake of probiotic-containing yogurt. The results of this study will allow the analysis of the necessity and efficacy of supplementation with probiotics to restore the intestinal microbiota equilibrium, with consequent reduction of gastrointestinal complications and infections, improving the quality of life of celiac patients.

METHODS

Study design

The Ethics Committee for Studies with Humans of the Federal University of Santa Catarina, Brazil, approved the experimental protocol for this study (number 772, 2010). The participants with CD were recruited in the local Association of Celiac People in Brazil (Associação dos Celíacos do Brasil – ACELBRA) during its monthly meetings. All celiac patients were on a controlled stage of the disease during the study, *i.e*., they were on a gluten free diet, without signals and symptoms of CD. The non-celiac participants were randomly recruited from the population.

Volunteers were submitted to a clinical and sociodemographic questionnaire and the research started by collecting the first stool sample to quantify bifidobacteria and measure faecal pH. Afterwards, each volunteer consumed one unit of probiotic-containing yogurt (100g) from Piá Essence, PIÁ®, Nova Petrópolis-RS) per day, having eaten in the fasting state at morning, during one month. The yogurt delivery was made weekly. After 30 days of consumption, feces were collected again in order to quantify bifidobacteria and measure faecal pH.

Exclusion criteria

The following exclusion criteria for the participation in the study were adopted: individuals with suspicion or diagnosis of autoimmune diseases; suspicion or diagnosis of diabetes; lactose intolerance; allergy to any excipient present in the yogurt; individuals who consumed products containing prebiotics and/or probiotics three months prior to the beginning of research, and individuals who presented fever, diarrhea and/or vomit three months prior to the beginning or during study.

Determination of faecal bifidobacteria content and pH

For the isolation and quantification of bifidobacteria and measurement of faecal pH, participants collected stool samples, which were sent to the laboratory and analyzed within 8 h after collection[13,29].

Feces aliquot (1 g) from each volunteer was diluted in 9 mL of distilled and deionized sterile water for the measurement of faecal pH in pHmeter PHTEK®. Another feces aliquot (1 g) from each stool sample was diluted in 9 mL of phosphate buffer. The mixture was homogenized five times using the anaerobic technique. From this dilution (10^{-1}), serial fold dilutions up to 10^{-7} were prepared. The stock phosphate buffer was previously prepared with 34 g of KH_2PO_4 in 500 mL of distilled and deionized water, having the pH adjusted to 7.2 with NaOH 1 N and the volume completed to 1 L with distilled water, being subsequently sterilized in an autoclave at 121°C during 18 minutes. For the dilution of the stool sample, the phosphate buffer was diluted a thousand times from the stock solution.

The culture media used for isolation of bifidobacteria was the RCA (Reinforced Clostridial Agar, DifcoTM BD) supplemented with antibiotics (nalidixic acid 2%, polymyxin B sulfate 0.85%, kanamycin sulfate 0.5%, iodoacetic acid 0.5%, 2,3,5-triphenyltetrazolium chloride 0.5% and amphotericin B 0.001%)[14].

The spread-plating of 100 µL from each dilution was prepared and the plates were incubated at 37°C for 72 hours under anaerobic conditions[14] using a commercial anaerobic atmosphere generation system (Anaerobac from Probac®), followed by counting of bifidobacteria colonies in the plates containing between 30 and 300 colony-forming units (CFU). For the confirmation of the *Bifidobacterium* genus, Gram staining was made, as well as catalase proof and fructose-6-phosphate phosphoketolase (F6PPK) reaction, as stated by Orban & Patterson (2000), for all isolated colony types[18].

The results from the bifidobacteria quantification were presented as CFU per gram of feces (CFU/g). To obtain the results, the number of CFU counted in each plate was multiplied by its respective dilution factor and corrected for the sample volume spread. They are expressed as mean ± standard deviation (n=17 for the control group and n=14 for the celiac group).

Determination of yogurt bifidobacteria content and pH

All lots of the yogurt Piá Essence donated were analyzed for the isolation and quantification of bifidobacteria and measurement of pH. One pot containing 100 g of yogurt was randomly selected from each lot and 1 g was diluted in 9 mL of distilled and deionized sterile water for measurement of pH in pHmeter PHTEK® previously calibrated.

Another yogurt aliquot (1 g) was also diluted in 9 mL of phosphate buffer. Serial dilutions were made from this solution as for the feces analysis. The spread-plating from each dilution, the counting of colonies, the confirmation of the genus and the expression of the results were made as previously described for the stool samples.

Statistical analysis

The statistical analysis was performed using the program GraphPad Prism® version 5.0 from 2007. For data distribution analysis, the D'Agostino normality test and Pearson Omnibus Normality Test were employed. Spearman's correlation coefficient was employed to verify the correlation among the bifidobacteria concentration, faecal pH and volunteers' ages. Wilcoxon test was used for the comparison of the results between groups. A significance level of 5% (P<0.05) was adopted for all tests.

RESULTS

The yogurt package informs that each 100 g of yogurt contains 10^8 CFU of *Lactobacillus acidophilus* and *Bifidobacterium lactis*. Amongst the yogurt lots available to the volunteers, the average concentration of bifidobacteria was $6.67x10^8 \pm 10.3x10^8$ CFU/g of yogurt. The average yogurt pH was 4.28±0.15 and there was a significant correlation between the bifidobacteria concentration and yogurt pH (P=0.0121). There was growth of Gram-positive bacillus colonies in every lot of yogurt, being all catalase negative and showing F6PPK activity.

Amongst the 17 healthy control individuals, 10 were female and seven were male, aged between 18 and 58 years (average of 26 years old). This group of individuals did not have any relatives with CD.

From the group of 14 celiac patients, 10 were female and four were male, with ages ranging from 18 to 60 years, being the average being 38 years old. The prevalence of CD in their families was

higher in first (father, mother and siblings) and second (grandparents, aunts, uncles and cousins) degree relatives. Two (14.4%) volunteers had first-degree celiac relatives, three (21.4%) had first and second-degree celiac relatives, one (7.1%) could not answer and eight (57.1%) did not have celiac relatives. The average age in which the diagnosis was made was 36 years old, where 100% of the patients had the small intestine biopsy done for confirmation of CD. A relation between faecal bifidobacteria concentration and age was not observed in any of the groups, either celiac or healthy subjects.

Seven (50%) of the 14 celiac patients received drug therapy after CD diagnosis, being calcium therapy the most prevalent in 57% of them, mainly related to women 30 years old or older.

During the stool sample examination from the volunteers, bifidobacteria colonies were observed, presenting round shape, smooth surface, pink to wine color and small to medium size. The colony morphology was similar between both groups, celiac and control (Figure 1). Bifidobacteria appeared in the form of short and long Gram-positive bacilli, with or without bifurcated ends V or Y-shaped, and as Gram-positive coccobacilli (Figure 2).

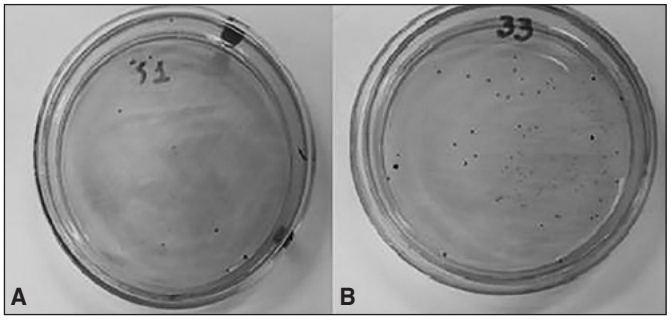

FIGURE 1. Bifidobacteria colonies in Reinforced Clostridial agar media supplemented with antibiotics. (A) Celiac patient plate. (B) Control subject plate.

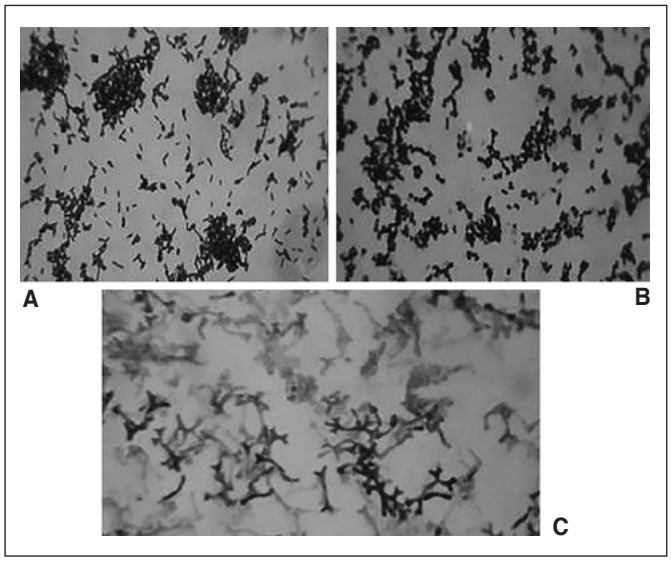

FIGURE 2. Micromorphology of bifidobacteria colonies stained by Gram method in optical microscopy in 1000 times increase. (A) Short Gram-positive bacilli, isolated, in pairs or grouped. (B) Gram-positive cocobacilli, isolated, in pairs or grouped. (C) Long Gram-positive bacilli with bifurcated ends V or Y-shaped.

The results of bifidobacteria quantification in the stool samples are shown in Figure 3. Healthy individuals presented a significantly higher concentration of bifidobacteria ($2.3x10^8 \pm 6.3x10^7$ CFU/g) before the probiotic-containing yogurt intake when compared to the celiac group ($1.0x10^7 \pm 1.7x10^7$ CFU/g) (Figure 3). Celiac patients presented, in average, 83% less bifidobacteria than healthy individuals. Still, celiac faecal pH (7.19±0.521) was not significantly different from the faecal pH of the control group (7.18±0.522).

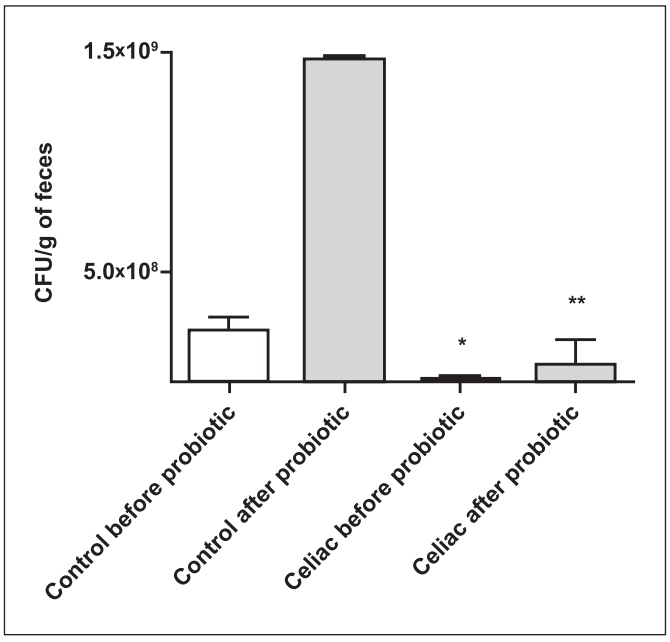

FIGURE 3. Number of colony forming units (CFU) of bifidobacteria per gram of feces from control and celiac groups, before and after probiotic intake. Results are expressed as mean ± standard deviation (control group n=17 and celiac group n=14). * $P<0.05$ nonparametric *t* test, when compared to the respective control group before probiotic intake or ** when compared to the respective control group after probiotic intake.

After the daily intake of 100 g of probiotic-containing yogurt for 30 days, healthy individuals presented a significantly higher bifidobacteria concentration ($14.7x10^8 \pm 0.2x10^8$ CFU/g) than celiac patients ($0.76x10^8 \pm 0.1x10^8$ CFU/g) (Figure 3). However, faecal pH of celiac patients (7.28±0.518) did not show significant difference from the faecal pH of healthy individuals (7.07±0.570) after the yogurt intake.

DISCUSSION

Several probiotic supplements can be found on the market; meanwhile it is still hard to find gluten free products for celiac patients. In this context, the product options for this research were limited. Amongst the companies for which support was requested, only PIÁ®, Nova Petrópolis-RS, provided the products. The average bifidobacteria concentration provided for the research participants ($6.67x10^8 \pm 10.3x10^8$ CFU/g of yogurt) is enough to bring benefits to their health, according to Vinderola & Reinheimer[31].

A number of factors can affect probiotic bacteria viability in yogurts. High carbohydrate concentrations added to the product before its fermentation can inhibit the bacteria, leading to long periods of fermentation and an underdevelopment of acidity[16].

Oliveira & Damin[16] found that the number of probiotic bacteria remained stable for at least seven days of storage. However, in this study volunteers consumed the probiotics up to their expiration date, which simulates the acquisition of products commercialized for the general population. A yogurt of a lot provided for the volunteers was randomly tested six days after its expiration date, in which a bifidobacteria concentration of $1.74x10^6$ CFU/g of yogurt was found. Coupled with the likely concentration of *Lactobacillus*, this would still be a probiotic food and bring benefits to people's health[19], including celiac patients.

The largest number of female celiac patients in this study is consistent with literature, which shows a higher prevalence of CD in women[3]. About 30% of celiac patients evaluated in this study have a relative with CD, which is similar to a study made with patients from Association of Celiac People in Brazil, section from Santa Catarina, (ACELBRA-SC) in 2004, revealing that 27% of associates had relatives with CD[3]. This data reinforces the idea that genetic determinants of CD are associated with environmental factors[15]. It is important to note that 100% of the celiac patients who participated in the research had the intestinal biopsy done for their diagnosis, which is recommended by the literature[30].

The poor intestinal absorption of most nutrients resulting from the inflammatory response on CD can explain why most celiac patients reported having osteoporosis and osteopenia[3,30]. It also explains why most participants of this research have replenished calcium and vitamin D after CD diagnosis. The supplementation with probiotic-containing yogurt could bring not only the benefits from the probiotics for celiac patients but also a greater amount of calcium absorbed from their diet.

The mechanisms of action of probiotics have not been completely elucidated, even though many have been suggested and possibly operate individually or associated[32]. There is evidence that probiotics have antimicrobial action, compete for limited nutritional resources from the intestinal microbiota, block adhesion of pathogens in the intestinal mucosa and have antitoxin effects of pathogens[22]. Bifidobacteria can also benefit people's health by lowering intestinal pH through the production of short chain fatty acids (acetate and lactate), thus inhibiting pathogenic bacteria growth. This is a digestive system self-mechanism for population control and selectivity of bacterial colonization[13]. Indeed, a significant correlation between faecal pH and bifidobacteria concentration was not seen in this study.

Macro and micromorphology of bifidobacteria colonies found in the stool samples were similar in both groups and were as described in the literature. However, the results show a significant lower quantity of bifidobacteria CFU per gram of feces of celiac patients than in the control group. Some studies show that allergic children and patients with atopic diseases are frequently colonized by a reduced number of bifidobacteria when compared to healthy children, showing a close relationship between bifidobacteria concentration and host immune disorders[5].

Nadal et al.[15] reported an imbalance in the intestinal biota of celiac children, especially the reduction of faecal *Bifidobacterium* spp. concentration. Similarly, Collado et al.[4] have reported that celiac children with active or inactive disease had inferior bifidobacteria counting than control groups for both analyzed samples, either feces or intestinal biopsy specimens. Therefore, this imbalance seems to be independent on the activity of the disease. This explains the lower bifidobacteria concentration found in feces of adult celiac patients in this study, all in a controlled phase of CD.

The results found in this study for bifidobacteria concentration without probiotic consumption show a significantly higher bifidobacteria count in healthy subjects when compared to celiac patients, which is consistent with literature[8]. Even after probiotic consumption, the faecal bifidobacteria count in celiac patients from this study has not reached the counting in healthy individuals without probiotic consumption (Figure 3).

The values of faecal pH for both groups before probiotic intake had no significant difference, having them remained very similar even after probiotic intake. These results suggest that the higher faecal bifidobacteria concentration after probiotic consumption did not increase intestinal fermentation, which would lower the pH and ease bifidobacteria growth[13]. However, it is worth noting that the pH from the control group was slightly more acidic than the pH from the celiac patients. The increase in bifidobacteria count favors the lowering of faecal pH due to the fermentation done by these bacteria[13]. The results of pH values from both groups, celiac and control, suggest that the smaller amount of bifidobacteria in the intestine of celiac patients is probably not related to faecal pH, but to the pathogenesis of CD. Thus, the relationship between bifidobacteria counting and CD has yet to be elucidated.

The maintenance of pH values before and after probiotic ingestion may be related to time or quantity/concentration of the daily-consumed probiotic, being suggested that probiotic effects are dose-dependent[19]. However, the recommended dose by the literature was consumed in this study, which is between 10^6 e 10^{11} CFU/day, depending on the desired effect[22].

In order to have the metabolism and intestinal content reflected in feces, variables must be taken into account, including intestinal motility, total fiber ingestion, intestinal secretion, and duration of dietetic intervention. Because of that, faecal pH may not exactly reflect colon pH. In fact, Bouhnik et. al.[2] have not considered the faecal pH as a good indicator of intestinal acidification, since it has not changed after ingestion of nondigestible carbohydrates by 200 healthy volunteers, despite the increase in the number of faecal bifidobacteria.

Although the healthy intestinal microbiota remains to be defined, there are many diseases related to its imbalance. In most cases, there is no information yet if microbiota imbalance has a triggering role or if it is a disease consequence. Anyway, both relationships lead to the hypothesis that an intervention to restore the microbiota to the healthiest state could mitigate the disease. The consumption of properly selected probiotics could be used with such role[23].

There is indication, amongst research to elucidate activity of bifidobacteria, that intestinal microbiota change can influence the typical inflammatory reactions in CD in a specie-specific way[4]. Therefore, it is thought that bifidobacteria has a great therapeutic potential, and manipulation of intestinal biota, as with probiotic supplementation, might improve quality of life of celiac patients.

However, it should be noted that the inclusion of a small number of participants, the evaluation of pH and bifidobacteria contents during a short period of time and the availability of molecular methods, more accurate to evaluate the intestinal microbiota, may be considered limitations of the present study. Therefore, we suggest that additional studies should be performed in order to evaluate all the aspects regarding intestinal microbiota and probiotic supplementation in CD.

It is still not clear why celiac patients who are in a controlled phase of the disease – i.e., on a gluten free diet, with restored intestinal villi and with no symptoms –, present less bifidobacteria.

CONCLUSION

The results obtained in the present study allow the conclusion that there is a lower bifidobacteria count in the intestinal microbiota of celiac patients, even when they are on a gluten free diet and consuming probiotic-containing food, when compared to the control group. This disturbance is independent on the faecal pH.

Supplementation with probiotics increased the number of faecal bifidobacteria, which reflects its intestinal concentration. Further research must be performed in order to evaluate the equilibrium of other bacteria (for instance, the pathogenics); to verify how long bifidobacteria count remains elevated after probiotic consumption; to correlate small intestine biopsy results with bifidobacteria concentration, since celiacs were on a gluten free diet; and evaluate if the microbiota imbalance was due to gluten contamination in food.

In summary, this information will help develop specific dietetic recommendations to celiac patients based on their microbiota composition.

Authors' contributions

Martinello F: survey execution. Roman CF: writing and translation of text. Souza PA: statistical analysis and writing of text.

Martinello F, Roman CF, Souza PA. Efeitos do consumo de probióticos sobre as bifidobactérias intestinais de pacientes celíacos. Arq Gastroenterol. 2017;54(2):85-90.

RESUMO – Contexto – Indivíduos saudáveis apresentam uma concentração de bifidobactérias fecais significativamente maior em comparação a pacientes celíacos. Apesar de haver benefícios potenciais no uso de probióticos na doença celíaca, estes têm sido pouco explorados como uma terapia adjuvante. **Objetivo –** Este estudo objetivou a comparação do pH e concentração fecal de bifidobactérias entre pacientes celíacos e indivíduos saudáveis antes e após o consumo diário de 100 g de iogurte contendo probiótico por um período de 30 dias. **Métodos –** Foram analisadas fezes de 17 pessoas saudáveis e 14 pacientes celíacos, tendo sido realizada a coprocultura para o isolamento e quantificação de bifidobactérias fecais. Além disso, o método de Gram foi empregado na análise microscópica das colônias, enquanto a identificação do gênero *Bifidobacterium* foi feita através da determinação da enzima frutose-6-fosfato fosfocetolase. O pH fecal foi medido usando um pHmetro calibrado. **Resultados –** A concentração de bifidobactérias fecais antes do consumo do iogurte probiótico foi significativamente maior em indivíduos saudáveis ($2.3x10^8 \pm 6.3x10^7$ UFC/g) quando comparada aos celíacos ($1.0x10^7 \pm 1.7x10^7$ CFU/g). Por outro lado, o pH fecal de ambos os grupos não apresentou diferença significativa. Após o consumo diário de iogurte contendo probiótico, ambos os grupos tiveram um aumento significativo na concentração de bifidobactérias fecais, entretanto indivíduos saudáveis apresentaram concentrações de bifidobactérias significativamente maiores ($14.7x10^8 \pm 0.2x10^8$ UFC/g) do que o grupo celíaco ($0.76x10^8 \pm 0.1x10^8$ UFC/g). Os valores de pH obtidos de ambos os grupos não foram significativamente diferentes, sendo de 7.28±0.518 para os pacientes celíacos e de 7.07±0.570 para os indivíduos saudáveis após o consumo do probiótico. **Conclusão –** A suplementação com probiótico aumentou significativamente o número de bifidobactérias nas fezes dos pacientes celíacos apesar de não ter sido suficiente para alcançar a concentração encontrada em indivíduos saudáveis antes do consumo de probióticos.

DESCRITORES – Probióticos. Doença celíaca. Bifidobacterium. Concentração de íons de Hidrogênio. Fezes, microbiologia. Contagem de colônia microbiana.

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