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NRC Research Press

ARTICLE

Decrease in lactobacilli in the intestinal microbiota of celiac children with a gluten-free diet, and selection of potentially probiotic strains

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Abstract: The intestinal microbiota would be implicated in pathology associated with celiac disease caused by an abnormal immune system reaction against gluten present in cereal grains. The objectives of this work were to detect through basic methods the changes in the composition of the most common genera of bacteria from the intestinal microbiota of symptom-free celiac disease children with a gluten-free diet compared with healthy children from Tucumán and to select lactobacilli (Lb) strains with probiotic potential from the feces of healthy children. Results demonstrated that the feces of celiac children with a gluten-free diet showed significantly lower counts of Lb ($P < 0.05$) compared with healthy children, while enterobacteria tended to increase in celiac children. On the basis of these results, isolation of some Lb from the feces of healthy children was carried out. Thus, 5 Lb strains were selected because of their high resistance percentages to gastrointestinal tract conditions. In addition, their autoaggregation and hydrophobicity properties were evaluated: *Lactobacillus rhamnosus* (LC4) showed the highest percentage of autoaggregation while *Lactobacillus paracasei* (LC9) showed high hydrophobicity. Based on these results, LC4 and LC9 were selected, and their use as potential probiotic strains to improve signs and symptoms associated with celiac disease is discussed. This is the first study performed in Argentina concerning the relationship between intestinal microbiota and celiac disease in celiac children with a gluten-free diet. In addition, the development of a probiotic food addressed towards celiac patients and designed with Lb isolated from the feces of healthy children from our province represents a promising alternative to improve the quality of life of celiac patients.

Key words: intestinal microbiota, celiac disease, *Lactobacillus*, probiotics.

Résumé : Il appert que la microflore intestinale serait impliquée dans la pathologie associée à la maladie cœliaque, qui est causée par une réaction immunitaire anormale envers le gluten présent dans les grains céréaliers. Les objectifs du présent ouvrage étaient de détecter au moyen de méthodes de base des variations dans la composition des genres de bactéries les plus fréquents de la microflore intestinale d'enfants cœliaques asymptomatiques sous alimentation sans gluten (ECASG) comparativement à des enfants sains (ES) de Tucumán et de sélectionner à partir de selles d'ES des souches de lactobacilles (Lb) présentant un potentiel probiotique. Les résultats ont démontré que les selles d'enfants cœliaques à l'alimentation dépourvue de gluten renfermaient des comptes de Lb significativement inférieurs ($P < 0,05$) à celles d'enfants sains, tandis que les entérobactéries avaient tendance à être plus nombreuses chez les enfants cœliaques. Ainsi, on a sélectionné 5 souches de Lb en vertu de leur taux élevé de résistance aux conditions sévissant dans le tube digestif. Par ailleurs, on a évalué leurs attributs d'autoagrégation et d'hydrophobicité. À cet égard, *Lactobacillus rhamnosus* (LC4) présentait le plus haut pourcentage d'autoagrégation tandis que *Lactoacillus paracasei* (LC9) était le plus hydrophobe. En s'appuyant sur ces résultats, LC4 et LC9 ont été retenus et l'on a traité de leur usage possible en tant que souches probiotiques destinées à améliorer les signes et les symptômes associés à la maladie cœliaque. Il s'agit de la première étude réalisée en Argentine portant sur la relation entre la microflore intestinale et la maladie cœliaque chez des patients cœliaques suivant un régime sans gluten. En outre, la préparation d'aliments probiotiques destinés aux patients cœliaques et conçus avec des Lb isolés de selles d'enfants sains de notre province représente une solution prometteuse pour améliorer la qualité de vie des patients cœliaques. [Traduit par la Rédaction]

Mots-clés : microflore intestinale, maladie cœliaque, *Lactobacillus*, probiotiques.

Introduction

The human gastrointestinal tract is a complex ecosystem comprising hundreds of bacterial species. Intestinal microbiota plays a key role in health and disease (Gibson and Roberfroid 1995; Moreau and Gaboriau-Routhiau 2001) by metabolizing nutrients and activating innate and adaptive immunity (Sanz 2010). The composition of this ecosystem is highly influenced by the diet, an important environmental factor leading to bacterial diversity (Ley et al. 2008; Liszt et al. 2009).

Celiac disease (CD) is an immune-mediated small intestinal enteropathy triggered by gluten ingestion in genetically sus-

Received 21 July 2014. Revision received 11 October 2014. Accepted 14 October 2014.

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ceptible individuals, with a prevalence of 1% (Rubio-Tapia et al. 2012) in the United States. In Argentina, a prevalence of 1.26% of celiac (1/79 children) diagnosed by histopathology has been estimated (Mora et al. 2012). CD classically includes gastrointestinal symptoms such as diarrhea, malabsorption, and weight loss. A gluten-free diet (GFD), currently the only treatment for CD, is effective in the vast majority of patients (Nachman et al. 2009; Murray et al. 2004). CD has a multifactorial pathogenesis involving genetic, immunological, and environmental factors, and the role of intestinal bacteria in its development and treatment is not fully understood yet (Collado et al. 2007; Medina et al. 2008). In contrast to healthy individuals, the gut microbiota of celiac patients is characterized by higher numbers of Gram-negative bacteria and lower numbers of Gram-positive bacteria (De Palma et al. 2009). The former activate pro-inflammatory pathways while the latter, including lactobacilli (Lb) and bifidobacteria, inhibit toxic effects induced by other species or gluten antigens (Laparra et al. 2013) on the gastrointestinal tract. In this sense, probiotics, defined as "live microorganisms ingested in adequate amounts which are capable of exerting a beneficial effect on the health of the host" (FAO/WHO 2002), would be a promising alternative to improve gut microbiota dysbiosis and chronic intestinal inflammation in celiac patients. The dietary habits of a population, which are closely associated with the socio-economic environment, are one of the main factors determining the diversity of the human gut microbiota (Bäckhed et al. 2005; De Filippo et al. 2010). Long-term dietary treatments aimed at food-related diseases can affect the composition of resident microbiota and thus its functional relationships with various organs and tissues of the host. The objectives of this study were (*i*) to detect through basic methods the changes in the composition of the most common genera of bacteria from the intestinal microbiota of symptom-free celiac disease children with a gluten-free diet (CCGFD) compared with healthy children (HC) from Tucumán, and (*ii*) to select from the feces of HC Lb strains with probiotic potential based on in vitro evidence. The survey of the intestinal microbiota of celiac patients represents the first work in this topic in our province.

Materials and methods

Subjects and fecal sampling

This experimental study was conducted between May 2011 and April 2012. A total of 30 children were included in the study: 15 healthy children (HC) (mean age, 6.5 years; range, 2–11 years) with no family history of food intolerance, and 15 symptom-free children (mean age = 7.5, range: 3–14 years) with a previously confirmed diagnosis of celiac disease and a gluten-free diet (CCGFD: celiac children with a gluten-free diet). CD was diagnosed on the basis of clinical symptoms, positive serology markers (antigliadin and antitransglutaminase antibodies), and signs of severe enteropathy by duodenal biopsy examination and positive response to a GFD. CCGFD patients had adhered to a GFD for a period longer than 6 months, and their clinical symptoms, serological tests, and histopathological evaluation of duodenal biopsy specimens were negative. The children included in the study had not been treated with antibiotics for at least 1 month before the sampling time. The study was approved by the Ethical Committee of the Facultad de Medicina of the Universidad Nacional de Tucumán, which regulates clinical research in humans. Children were enrolled in the study after the written informed consent of their parents.

Fecal samples from the 2 groups of children under study were collected in sterile recipients, diluted 10-fold in sterile physiological solution, and homogenized. Aliquots of this dilution were kept at −80 °C for further immunological studies.

Intestinal microbiota bacterial counts

Successive dilutions (1:100) in 0.1% peptone water were performed with the fecal samples collected. Dilutions were plated in duplicate in the following culture media: Brain Heart Agar (for total aerobic bacteria), Brain Heart Agar supplemented with vitamin K and hemin (for total anaerobic bacteria), Rogosa agar (for Lb), and Violet Red Bile Glucose agar (for enterobacteria). Plates for Lb, enterobacteria, and total aerobic bacteria were incubated aerobically at 37 °C. Total anaerobic bacteria were incubated anaerobically at 37 °C with Anaerocult® gas generating envelopes for anaerobic incubation (Merck, Argentina). The plates were counted 48–96 h after incubation and reported as colony-forming units per gram of feces (CFU/g feces).

Isolation of Lb

Lb isolation was performed from fecal suspensions in sterile physiological solution (1/10 *m*/*v*) which were seeded in duplicate in LBS medium (semidefined medium for the selective isolation and enumeration of Lb from foods and intestinal, vaginal, and dental flora) in plates that were incubated at 37 °C for 48 h. Bacteria were isolated by successive seeding with the cross-streak method and a collection of 5 strains was obtained. Microbiological purity and identification of bacteria were tested by Gram staining, catalase test, appearance of colony and cell morphology, and fermentation profiles of carbohydrates (Oliszewski et al 2006; Gusils et al 2004). Although several genera of lactic acid bacteria grew in the LBS medium, only those identified as *Lactobacillus* were selected for further studies.

Biochemical identification of Lb

Selected strains were transferred to MRS liquid culture medium and incubated at 30 °C for 48 h. After 3 successive passages, sugar fermentation profile was analyzed using the API 50 CHL system (BioMerieux) according to manufacturer's instructions. The gallery was incubated under aerobic conditions at 37 °C for 24–48 h. The biochemical profile of each strain, based on the observation of the color developed in each of the tubes, was analyzed by apiweb® software provided by the manufacturer.

Autoaggregation assays

The autoaggregation capacity of strains was assessed according to the technique described by Vandevoorde et al. 1992. Briefly, cultures of each Lb strain, grown for different time periods and under various growth conditions, were centrifuged (6000*g*, 15 min), washed with PBS (pH 7), and resuspended in the same buffer to an optical density (OD) of 0.6 ± 0.05 at 600 nm. Variation in OD at 600 nm of cell suspensions was monitored every 1 h for 4 h without agitation of the suspensions during spectrophotometric determinations.

Autoaggregation percentage was calculated by the following expression:

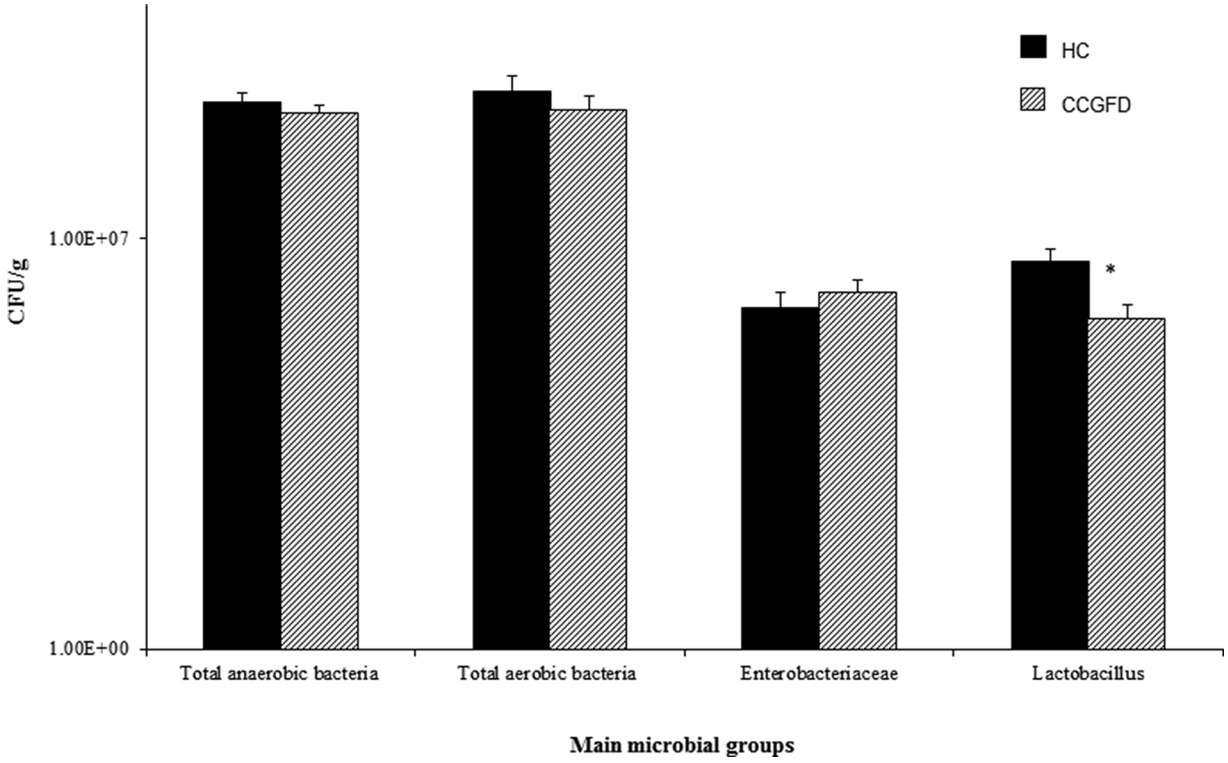
$$\text{Autoaggregation (\%)} = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100$$

where $OD_{initial}$ is OD at initial time (t = 0) of the autoaggregation assay, and OD_{final} is OD at each time point after the beginning of the assay (t = 1, 2, 3, or 4 h).

Hydrophobicity assays

The degree of hydrophobicity was assessed by the microbial adhesion to hydrocarbons (MATH) method according to a previous report (Juárez Tomás et al. 2005). The hydrophobic solvent xylene was employed. The strains to be studied were activated using 2 successive passages, and an overnight culture of each strain was centrifuged for 10 min at 10 000 rpm. Bacterial pellets were washed and resuspended in saline to obtain an optical density (OD_{600}) of 0.5–0.7. The solvent was added to each cell suspension and the mixture was vortexed vigorously for 1 min. Then suspensions were kept still to allow the immiscible solvent and

Fig. 1. Bacterial counts (CFU/g) of the most common microbial groups in fecal samples of celiac children with a gluten-free diet (CCGFD) and of healthy children (HC). Data are the means of 3 independent experiments from each sample. Each experimental group was composed of 15 individuals. Median values and ranges are given. *, indicates a significant difference at $P < 0.05$, by Tukey's test after ANOVA.



aqueous phase to separate. The lower aqueous layer was carefully removed using Pasteur pipettes and transferred to clean tubes. Absorbance was measured at 600 nm and each solution was vortexed for 90 s. Then the tubes were allowed to rest for 15 min to allow the separation of the 2 phases, and the OD_{600} of the aqueous phase (lower phase) was measured again.

Hydrophobicity percentage was obtained from the following calculation:

$$\%\ \text{hydrophobicity} = \frac{(OD_{before} - OD_{after})}{OD_{before}} \times 100$$

The strains were classified into 3 categories: high (71%–100%), medium (70%–36%), and low (35%–0%) hydrophobicity (24).

Simulated gastrointestinal digestion

The resistance to gastrointestinal digestion of 5 Lb strain isolates was tested by a sequential protocol. Overnight cultures were adjusted to 1×10^8 CFU/mL and washed twice with PBS. Then, 0.5 mL of simile saliva solution (2 mg/mL lysozyme in PBS, pH 7.00) was added to 1.750 mL of each cell suspension, and they were incubated for 5 min at 41 ± 0.5 °C. After that, 2.250 mL of simile gastric juice (125 mmol/L NaCl, 7 mmol/L KCl, 45 mmol/L $NaHCO_3$, 3 g/L pepsine, pH 2.00) was added, pH was adjusted to 3.00–3.50, and the suspensions were incubated for 1 h at 41 ± 0.5 °C. Prior to 2 h of incubation at 41 ± 0.5 °C, 3 mL of simile intestinal juice (0.75% (*m*/*v*) bile salts and 2 mg/mL pancreatin, pH 8.00) were added and pH was adjusted to 8.00. The tubes were centrifuged (10 000*g*, 10 min, 4 °C) and pellets were then resuspended in 1750 mL of PBS. At the beginning and end of the assay, bacterial suspensions were plated on MRS and incubated at 37 °C for 48 h. A reduction greater than 2 log in bacterial counts indicates no survival to simulated conditions in the gastrointestinal tract.

Statistical analysis

Significant differences between means were determined by Tukey's test after analysis of variance (ANOVA) with a Minitab Statistic Program, release 12 for Windows. A *P* value of <0.05 was considered statistically significant.

Results

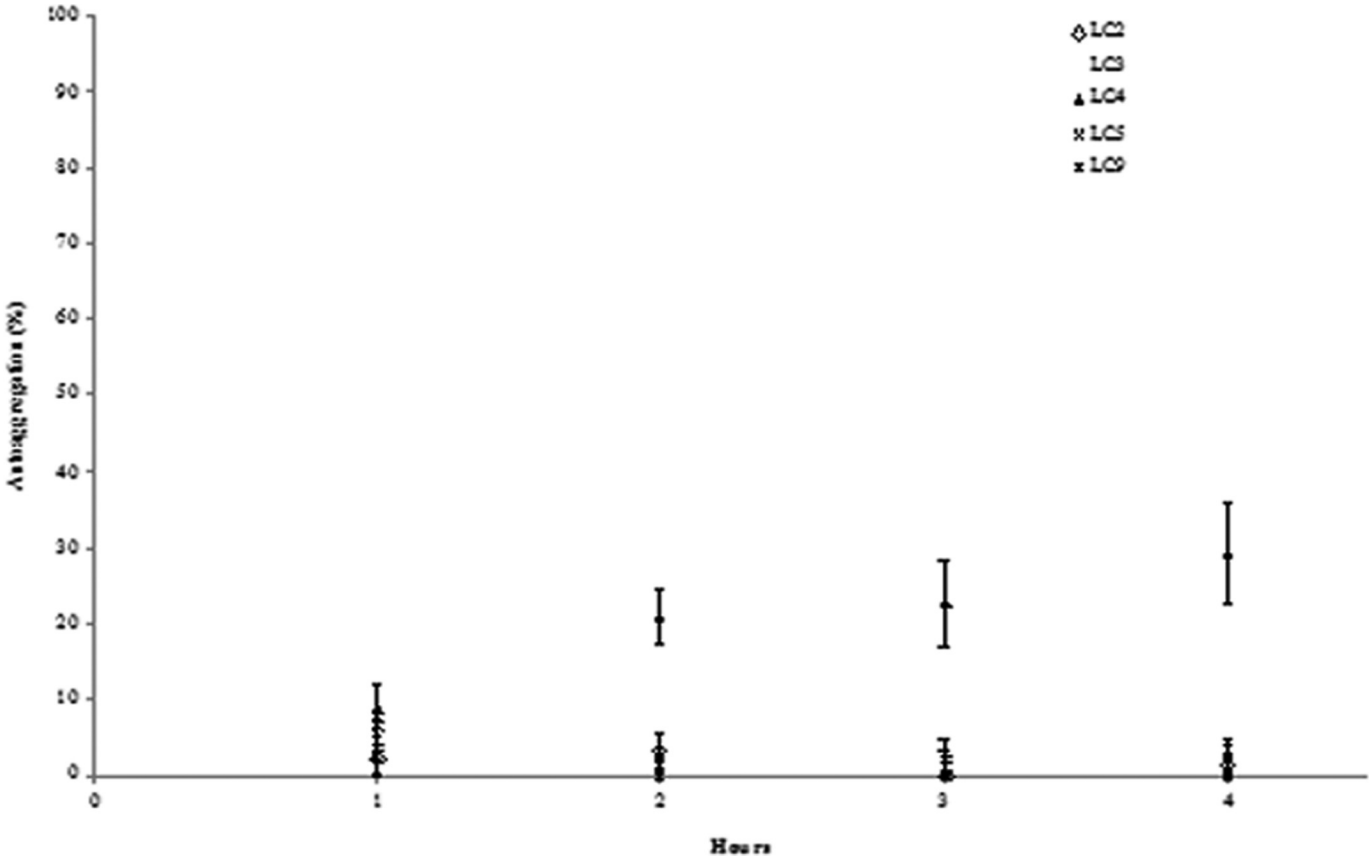
Intestinal microbiota bacterial counts

Selective media were used to enumerate the most common culturable bacterial genera present in fecal samples. A comparison of these bacterial genera between CCGFD and HC is shown in Fig. 1. The total count of aerobic and anaerobic bacteria showed no significant differences between the 2 groups analyzed. Thus, anaerobic bacteria count was $(2.09 \pm 9.08) \times 10^9$ CFU/g for HC and $(1.37 \pm 5.47) \times 10^9$ CFU/g for CCGFD, while aerobic bacteria were $(3.31 \pm 2.57) \times 10^9$ for HC and $(1.54 \pm 5.47) \times 10^9$ for CCGFD. The enterobacteria population tended to increase in CCGFD compared with that in HC; however, no significant differences were found between the 2 groups (HC = $(6.60 \pm 5.23) \times 10^5$ CFU/g; CCGFD = $(1.18 \pm 7.69) \times 10^6$ CFU/g). In contrast, Lb counts in the CCGFD group revealed significantly lower values ($P < 0.05$) than those in the HC group (HC = $(4.00 \pm 2.45) \times 10^6$; CCGFD = $(4.38 \pm 3.14) \times 10^5$). F1

Lb isolation from fecal microbiota and biochemical identification of *Lactobacillus* spp.

Lb isolation from fecal samples of HC was performed in LBS medium. The colonies developed were large, white, raised, convex, opaque, with well-defined edges, which correspond to the characteristics presented by Lb in this culture medium and agree with the description of the genus indicated by Kandler and Weiss 1992. Microscopic examination and Gram staining allowed identification of Gram-positive strains from 5 pure colonies grown in MRS. The catalase test was negative in all cases. Biochemical iden-

Fig. 2. Autoaggregation results of lactobacilli strain isolates from the intestinal microbiota of healthy children. The autoaggregation percentages obtained for each sample of bacterial culture were plotted as a function of the time of duration of the autoaggregation assay (0–4 h). Error bars represent standard deviations of the mean values of results from 3 replicate experiments.



tification of 5 Lb strains was performed using the API-50 CHL system, a study of the fermentation of 49 sugars, according to manufacturer's specifications. The results obtained, which constitute the biochemical profile of the strain, are used for its identification. This technique allowed the identification of LC2, LC3, LC4, and LC5 as *Lactobacillus paracasei*, while LC9 was identified as *Lactobacillus rhamnosus*.

Evaluation of autoaggregation of *Lactobacillus* spp.

The autoaggregation ability of probiotic strains appears to be a necessary requirement for their adherence to intestinal epithelial cells, which occupy specific locations to prevent potential colonization by pathogens (Kandler and Weiss 1992; Boris et al. 1997). Autoaggregation percentages obtained for each Lb strain were plotted as a function of the time of duration of the autoaggregation assay (0–4 h). Autoaggregation was assayed based on the settling characteristics of the microorganisms. LC2, LC3, LC5, and LC9 showed constant turbidity and an autoaggregation percentage below 5%, while LC4 showed precipitate formation and autoaggregation close to 30%. In view of these results, LC4 seems to be more appropriate than the other strains for its use as a probiotic. The results are expressed in Fig. 2. F2

Hydrophobicity test

The ability of probiotic strains to adhere to tissues is considered an important factor in the colonization of different environments of the human body (Del Re et al. 2000). Some characteristics of the bacterial surface can be studied to infer strain adhesion properties. Therefore, in this work we studied the hydrophobicity of Lb isolates. LC2 and LC3 showed low hydrophobicity with values of 5.80% ± 0.12% and 2.94% ± 4.16%, respectively, while LC4 had a moderate hydrophobicity percentage of 21.91% ± 9.7%. The LC9 strain showed the highest hydrophobicity rate: 61.18% ± 1.66%. Based on this property, LC9 would favor adhesion to epithelial intestinal cells, while LC4 would also have probiotic potential.

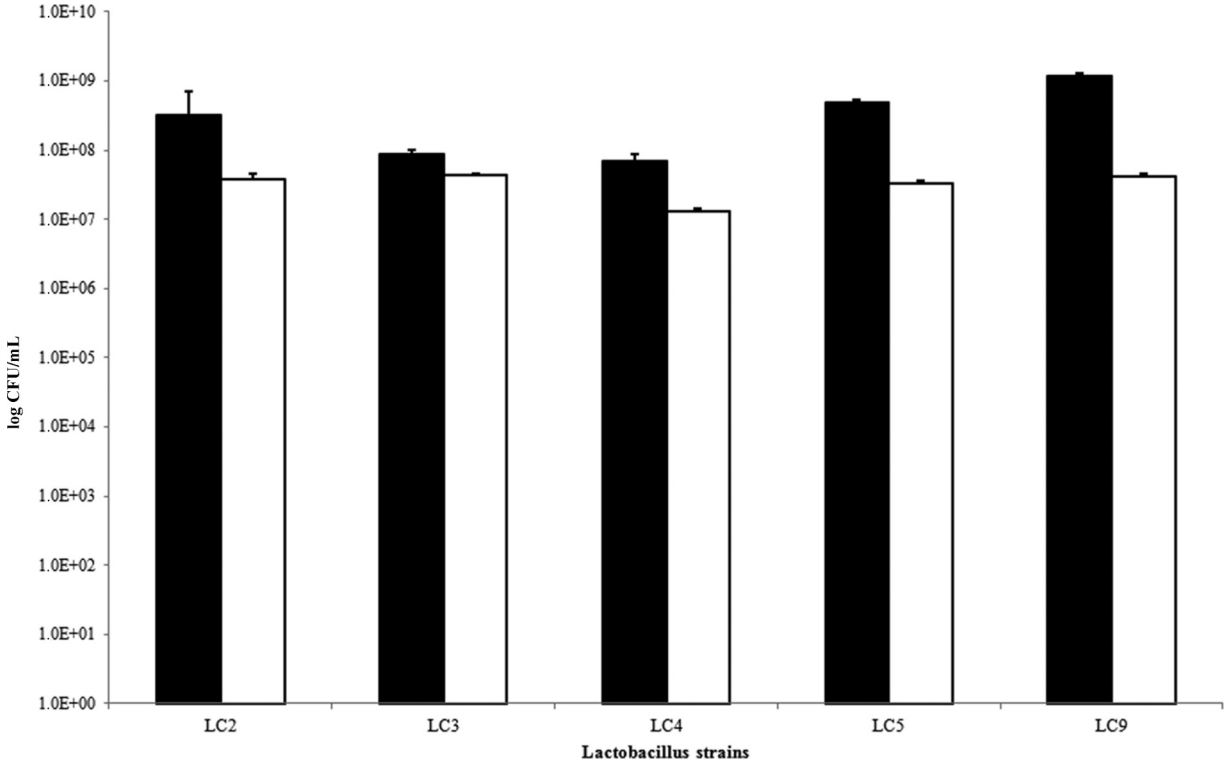
Resistance to gastrointestinal tract conditions

Bile salts and heartburn are some of the barriers to be overcome by microorganisms that act as probiotics. That is why in this study the isolates were subjected to a treatment that simulated stomach conditions (pH 3) and intestinal secretions (pancreatin, pepsin, pH 8). This is a modification of the technique of Madureira et al. 2005, which combines the effect of exposure to gastric juice followed by exposure to bile salts over a 120 min period. This assay was aimed at simulating 2 situations that occur during transit through the gastrointestinal tract: passage through the stomach followed by the effect of bile salts on the small intestine. All Lb studied showed high resistance to the conditions of the gastrointestinal tract; LC3 showing the highest viability value after exposure to low pH and intestinal secretions (98%), followed by LC4 (90%), LC2 (89.28%), and LC5 (86.52%). The LC9 strain was least resistant but still maintained 80% viability in the assay conditions. Figure 3 shows the counts for all strains at the beginning and end of the assay. F3

Discussion

The relationship between intestinal microbiota and diet and that between the microbiota and health or disease have been extensively studied in the last few years (Quigley 2013). Diet has an important impact on the bacterial diversity of the intestinal microbiota (Ley et al. 2008; Liszt et al. 2009) by influencing its com-

Fig. 3. Resistance to gastrointestinal digestion of 5 lactobacilli strain isolates. Results are expressed in log CFU/mL. Black bars indicate values at the beginning of the assay, white bars are the values at the end of the assay. Error bars represent standard deviations of the mean values of results from 3 replicate experiments.



position and function and consequently the health of the host, particularly in the case of patients suffering from food-related diseases. In this sense, some studies showed that the microbiota could be an important factor involved in the etiology of CD, and changes in bacterial genera were observed in the microbiota of celiac children compared with healthy ones. Genetic predisposition is also important in this enteropathy, and its relation to microbiota was evaluated in some reports (Sellitto et al. 2012; De Palma et al. 2012). In a previous work by De Palma et al. (2009), it was reported that Lb and bifidobacteria decreased in adults on a GFD compared with healthy individuals. In addition, a reduction in bifidobacteria and Lb compared with Gram-negative bacteria was detected in children with active CD and particularly in patients with a GFD treatment (Nadal et al. 2007). In this work we studied the most common bacterial genera present in the feces of HC and in those that, with a previous diagnosis of CD, were subjected to a GFD for a period no shorter than 6 months. Changes in the most common bacterial genera were found in the CCGFD group. Thus, bacterial counts performed in this study showed a significant decrease ($P < 0.05$) in Lb population in infants undergoing a GFD in relation to healthy individuals; bifidobacteria were not evaluated in this work. No significant differences in enterobacteria were found between HC and CCGFD, in contrast with other studies (Nadal et al. 2007). This fact may be due to the basic methodology employed in this work.

Previous investigations (Sellitto et al. 2012; De Palma et al. 2012; Nadal et al. 2007) as well as this work would indicate that dietary therapy in celiac patients could contribute to a reduction in the number of beneficial bacteria such as Lb and bifidobacteria and increase the count of enterobacteria (Fig. 1), which are characteristic of microbiota associated with the active phase of CD (De Palma et al. 2012; Nadal et al. 2007; Collado et al. 2009). On the basis of these considerations, a GFD would allow the recovery of intestinal histology but would not be fully effective in normalizing the intestinal dysbiosis associated with the intestinal microbiota of celiac patients. Furthermore, although the immunosuppressive effects of GDF can benefit celiac patients prone to Th1-type proinflammatory response, they may be detrimental to the defense against pathogens and to the regulation of the immune response in chronic processes. In this scenario, probiotics with immunomodulatory properties and capable of improving host resistance to infection may play an important role in enhancing or restoring the compromised microbial balance that characterizes the intestinal microbiota of celiac patients (de Sousa Moraes et al. 2014). *Lactobacillus* is considered the genus with the largest number of species with probiotic characteristics and, therefore, with beneficial health properties. Thus, 5 strains of Lb with probiotic potential from healthy eutrophic children were isolated. The requirements for a microorganism to be considered a probiotic are diverse and include resistance to the conditions of the gastrointestinal tract and the ability to adhere to intestinal epithelial cells. The physicochemical characteristics of the bacterial cell wall and the nature of the surface to which bacteria adhere influence Lb self-aggregation and adhesion. These properties are very important in the selection of probiotic strains. *Lactobacillus casei* (LC4) showed 30% autoaggregation with considerable hydrophobicity, although *Lactobacillus rhamnosus* (LC9) showed the highest hydrophobicity level. Taken together, the results of the in vitro assays allow us to consider that LC9 and LC4 present the best features for their potential use as probiotics. On the other hand, further studies should be conducted to evaluate the immunomodulatory properties of these lactic acid bacteria, employing both in vitro and in vivo assays, since their effect on the immune system is one of the main factors responsible for the beneficial influence of probiotics on human health.

A recent study showed that the percentage of celiac patients in the pediatric population in Argentina was higher than expected (Mora et al. 2012), but no studies on the intestinal microbiota in

celiac patients have been conducted. This is the first study in Argentina concerning the relationship between intestinal microbiota and CD in celiac children with a GFD. Treatment with probiotics does not aim at substituting the GFD but at attenuating the altered inflammatory parameters in celiac individuals and favorably modifying the composition of the intestinal microbiota, thus reducing the counts of Gram-negative bacteria with greater pathogenic and inflammatory impact.

Since the intestinal microbiota is affected by feeding and eating habits dependent on the social, cultural, religious, and economic characteristics of a population, the development of a probiotic food addressed towards celiac patients and designed with Lb isolated from the feces of healthy children of our province represents a promising alternative to improve the quality of life of celiac patients.

Acknowledgements

This work was supported by grants from Beca "Ramón Carrillo-Arturo Oñativia" del Ministerio de Salud de la Nación Argentina a través de la Comisión Nacional Salud Investiga. The authors declare that there are no conflicts of interest concerning the publication of this article.

References

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. 2005. Host-bacterial mutualism in the human intestine. Science, **307**: 1915–1920. doi:10.1126/science.1104816. PMID:15790844.

Boris, S., Suárez, J.E., and Barbés, C. 1997. Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. J. Appl. Microbiol. **83**: 413–420. doi:10.1046/j.1365-2672.1997.00250.x. PMID:9351223.

Collado, M.C., Calabuig, M., and Sanz, Y. 2007. Differences between the fecal microbiota of celiac infants and healthy controls. Curr. Issues Intest. Microbiol. **8**: 9–14. PMID:17489434.

Collado, M.C., Donat, E., Ribes-Koninckx, C., Calabuig, M., and Sanz, Y. 2009. Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease. J. Clin. Pathol. **62**: 264–269. doi:10.1136/jcp.2008.061366. PMID:18996905.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., and Massart, S. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc. Natl. Acad. Sci. U.S.A. **107**(33): 14691–14696. doi:10.1073/pnas.1005963107. PMID:20679230.

De Palma, G., Nadal, I., Collado, M.C., and Sanz, Y. 2009. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. Br. J. Nutr. **102**(8): 1154–1160. doi:10.1017/S0007114509371767. PMID:19445821.

De Palma, G., Capilla, A., Nova, E., Castillejo, G., Varea, V., and Pozo, T. 2012. Influence of milk-feeding type and genetic risk of developing coeliac disease on intestinal microbiota of infants: the PROFICEL study. PLoS One, **7**: e30791. doi:10.1371/journal.pone.0030791. PMID:22319588.

de Sousa Moraes, L.F., Grzeskowiak, L.M., de Sales Teixeira, T.F., and Gouveia Peluzio Mdo, C. 2014. Intestinal microbiota and probiotics in celiac disease. Clin. Microbiol. Rev. **27**: 482–489. doi:10.1128/CMR.00106-13. PMID:24982318.

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Lett. Appl. Microbiol. **31**: 438–442. doi:10.1046/j.1365-2672.2000.00845.x. PMID:11123552.

FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization). 2002. Guidelines for the evaluation of probiotics in food. London/Ontario/Canada: FAO/WHO. Available from ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.

Gibson, G.R., and Roberfroid, M.B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. **125**: 1401–1412. PMID:7782892.

Gusils, C., Perez Chaia, A.B., and Oliver, G., and González, S. 2004. Microtechnique for identification of lactic acid bacteria. *In* Public health microbiology: methods and protocols. Vol. 268. Humana Press, Totowa, N.J., USA. pp. 453–458.

Juárez Tomás, M.S., Wiese, B., and Nader-Macías, M.E. 2005. Effects of culture conditions on the growth and auto-aggregation ability of vaginal *Lactobacillus johnsonii* CRL 1294. J. Appl. Microbiol. **99**: 1383–1391. doi:10.1111/j.1365-2672.2005.02726.x. PMID:16313411.

Kandler, O., and Weiss, N. 1992. Regular nonsporing Gram-positive rods. *In* Bergey's manual of systematic bacteriology. 10th ed. Vol. 2. *Edited by* P.H.A. Sneath, M.S. Mair, M.E. Sharpe, and J.G. Holt. The Williams and Wilkins Co., Baltimore, Md., USA. pp. 1208–1260.

Laparra, J.M., Olivares, M., and Sanz, Y. 2013. Oral administration of *Bifidobacterium longum* CECT 7347 ameliorates gliadin-induced alterations in liver iron mobilisation. Br. J. Nutr. **110**(10): 1828–1836. doi:10.1017/S0007114513001098. PMID:23657133.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., and Gordon, J.I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. **6**: 776–788. doi:10.1038/nrmicro1978. PMID:18794915.

Liszt, K., Zwielehner, J., Handschur, M., Hippe, B., Thaler, R., and Haslberger, A.G. 2009. Characterization of bacteria, clostridia and *Bacteroides* in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting. Ann. Nutr. Metab. **54**: 253–257. doi:10.1159/000229505. PMID:19641302.

Madureira, A.R., Pereira, C.I., Truszkowska, K., Gomes, A.M., Pintado, M.E., and Malcata, F.X. 2005. Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. Int. Dairy J. **15**: 921–927. doi:10.1016/j.idairyj.2004.08.025.

Medina, M., De Palma, G., Ribes-Koninckx, C., Calabuig, M., and Sanz, Y. 2008. *Bifidobacterium* strains suppress in vitro the pro-inflammatory milieu triggered by the large intestinal microbiota of coeliac patients. J. Inflamm. **5**: 19. doi:10.1186/1476-9255-5-19. PMID:18980693.

Mora, M., Litwin, N., Toca, M., Azcona, M., Solís Neffa, R., Battiston, F., et al. 2012. Prevalence of celiac disease: multicentric trial among pediatric population from five urban districts in Argentina. Arch. Argent. Pediatr. **110**: 490–496. doi:10.5546/aap.2012.eng.490. PMID:23224306.

Moreau, M.C., and Gaboriau-Routhiau, V. 2001. Influence of resident intestinal microflora on the development and functions of the gut-associated lymphoid tissue. Microb. Ecol. Health Dis. **13**: 65–68. doi:10.3402/mehd.v13i2.8009.

Murray, J.A., Watson, T., Clearman, B., and Mitros, F. 2004. Effect of a gluten-free diet on gastrointestinal symptoms in celiac disease. Am. J. Clin. Nutr. **79**(4): 669–673. PMID:15051613.

Nachman, F., Maurino, E., Vazquez, H., Sfoggia, C., Gonzalez, A., Gonzalez, V., et al. 2009. Quality of life in celiac disease patients: prospective analysis on the importance of clinical severity at diagnosis and the impact of treatment. Dig. Liver Dis. **41**(1): 15–25. doi:10.1016/j.dld.2008.05.011. PMID:18602354.

Nadal, I., Donat, E., Ribes-Koninckx, C., Calabuig, M., and Sanz, Y. 2007. Imbalance in the composition of the duodenal microbiota of children with coeliac disease. J. Med. Microbiol. **56**: 1669–1674. doi:10.1099/jmm.0.47410-0. PMID:18033837.

Oliszewski, R., Van Nieuwenhove, C., Gonzalez, S., and Perez Chaia, A.B. 2006. Identificación y caracterización tecnológica de bacterias ácido lácticas ailadas de leche de cabra y quesos artesanales del Noroeste argentino. Revista Argentina de Lactología. Santa Fe: Ediciones UNL. **24**: 47–58.

Quigley, E.M. 2013. Gut bacteria in health and disease. Gastroenterol. Hepatol. **9**: 560–569. PMID:24729765.

Rubio-Tapia, A., Ludvigsson, J.F., Brantner, T.L., Murray, J.A., and Everhart, J.E. 2012. The prevalence of celiac disease in the United States. Am. J. Gastroenterol. **107**(10): 1538–1544. doi:10.1038/ajg.2012.219. PMID:22850429.

Sanz, Y. 2010. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult humans. Gut Microbes, **3**: 135–137. doi:10.4161/gmic.1.3.11868. PMID:22555465.

Sellitto, M., Bai, G., Serena, G., Fricke, W.F., Sturgeon, C., Gajer, P., et al. 2012. Proof of concept of microbiome-metabolome analysis and delayed gluten exposure on celiac disease autoimmunity in genetically at-risk infants. PLoS One, **7**: e33387. doi:10.1371/journal.pone.0033387. PMID:22432018.

Vandevoorde, L., Christiaens, H., and Verstraete, W. 1992. Prevalence of coaggregation among chicken lactobacilli. J. Appl. Bacteriol. **72**: 214–219. doi:10.1111/j.1365-2672.1992.tb01826.x. PMID:1568947.