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PROBIOTIC BACTERIOTHERAPY IN CHRONIC INFANTILE DIARRHEA

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SUMMARY

A probiotic therapy based on the administration of a dairy product containing Lactobacillus casei *and* Lactobacillus acidophilus *was used against chronic diarrhea of 170 infants aging from 4 months - 4 years. The mean period during which the children suffered from diarrhea before the start of the probiotic treatment was of about 50 days. After 4 days of therapy the clinical symptoms had been eliminated. Until the end of the study none of the patients suffered a relapse. Bacterial counts in the feces suggested that the probiotic lactobacilli had restored the normal intestinal lactobacillus flora. The fermented product tested in this work can be considered a beneficial food.*

Introduction

Good health implies an equilibrium between bacterial populations in the gastrointestinal tract. Disturbance of this equilibrium will lead to intestinal disorders (Gebbers and Laissue, 1982) e.g. diarrhea. For three decades, members of the German Medical Association for Microbiological Therapy have used vaccines prepared with bacteria from common human flora as a therapeutic measure in chronic and recurrent infections. The main indications for this kind of therapy were catarrhal infections of the respiratory system, intestinal disorders, allergies and skin diseases as well as several less well-defined syndromes (Rusch *et al*., 1980). However, for a long time, starting at the end of the nineteenth century (Metchnickoff, 1907) lactobacilli, e.g. *Lactobacillus casei* and *Lactobacillus acidophilus*, are known to inhibit growth of pathogenic intestinal bacteria and therefore they are considered to be potential biotherapeutic agents against pathogens (González *et al*., 1993; Apella *et al*., 1992, Romero *et al*., 1990). Protective immunity is suggested to arise from intestinal mucosa (Nader de Macías *et al*., 1992, 1993). Only after the pathogen overcomes the non-specific host defense mechanisms, specific host defense mechanisms are activated and will produce antibodies, mainly $_{S}$IgA's (Perdigón *et al*., 1992). *L. casei* is able to stimulate phagocytosis both by the cell wall and the peptidoglycan, whereas it does not

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RESUMEN

La administración de un producto lácteo conteniendo Lactobacillus casei *y* Lactobacillus acidophilus *fue usada como terapia probiótica para diarrea crónica en 170 niños cuyas edades estaban comprendidas entre 4 meses y 4 años. El tiempo promedio de duración de diarrea en estos niños, antes del tratamiento probiótico, era de 50 días. Los síntomas clínicos se eliminaron al cabo de 4 días de terapia probiótica y ninguno de los pacientes incurrió nuevamente en diarrea hasta el final del estudio. Los recuentos bacterianos en heces sugirieron que los lactobacilos probióticos habían normalizado la flora láctica intestinal. El producto lácteo fermentado ensayado en este trabajo puede ser considerado un alimento beneficioso.*

RESUMO

A administração de um produto lácteo contendo Lactobacillus casei *and* Lactobacillus acidophilus *foi usada como terapia probiótica para diarréia crônica em 170 crianças cujas idades estavam compreendidas entre 4 meses e 4 anos. O tempo médio de duração de diarréia nestas crianças, antes do tratamento probiótico, era de 50 dias. Os sintomas clínicos foram eliminados em 4 dias de terapia probiótica e nenhum dos pacientes incorreu novamente em diarréia até o final do estudo. As contagens bacterianas em fezes constataram que os lactobacilos probióticos haviam normalizado a flora láctica intestinal. O produto lácteo fermentado testado neste trabalho pode ser considerado um alimento com muitos benefícios.*

produce changes in IgA. *L. acidophilus*, on the other hand, produces an increase in the levels of IgA without modifying phagocytosis (Morata de Ambrosini *et al*., 1998). An extracellular lectin-like substance is characterized in *L. casei*, when it is extracted, it does not show any stimulation of the immune system (Morata de Ambrosini *et al*., 1997). In order to eliminate diarrhea in children a simple probiotic dairy product prepared by addition of both *L. casei* and *L. acidophilus* to reconstituted milk, followed by a short fermentation process (metabolic activation of lactobacilli). In this study 170 infants suffering from chronic diarrhea were treated with this product and the microbiological and clinical results are presented.

Materials and Methods

Children

Criteria for inclusion in this study were: (i) age from 4 months to 4 years, (ii) diarrhea for more than 30 days, (iii) failure of previous conventional therapies (substitution of milk by soy milk or lactose-free milk, and sometimes antibiotic therapy), (iv) absence of antibiotic therapy during the last 7 days before probiotic therapy, (v) absence of malnutrition, (vi) bacterial origin of the diarrhea and (vii) no evidence of systemic infection. As controls, the clinical histories of the same patients selected for this study were used, as they all showed resistance to conventional therapy. Due to the results the infants selected were their own controls. Initially, 123 infants aging between 4 and 24 months (mean age: 10 months) constituted group A and 47 children aging between 24 and 48 months (mean age: 30 months) constituted group B. Since the collected results of both groups were not essentially different, all results were combined. All infants were ambulatory patients and received normal food at home. They belonged to a low socioeconomic stratum and all parents authorized the probiotic therapy. The CERELA (Centro de Referencia para Lactobacilos) Committee of Ethics approved the protocol used for these studies.

Diarrhea was defined as 3 or more unformed stools with a total volume of ≥200ml within 48 hr or a single voluminous (≥300ml) liquid stool.

Probiotic product

Lactobacillus casei subsp casei CRL 431 and *Lactobacillus acidophilus* CRL 730 were from the Cerela Culture Collection (CRL) stock, isolated initially from feces of healthy children, were used. Just before preparation of the probiotic product the bacilli had been cultured in MRS-medium at least twice in succession (De Man *et al*., 1960), concentrated and washed by centrifugation (4000g for 10 min in 0.1M phosphate buffer, pH 7), resuspended separately in 10-fold diluted reconstituted milk, and incubated anaerobically at 37°C during 4-6 hr. A mixture (the final probiotic dairy product) of equal amounts of each suspension (final concentration of each lactobacillus strain: 10^7-10^8 living cells/ml) was stored at 4ºC until used. The final probiotic dairy product was used before a maximum storage of 7 days.

Probiotic treatment

The total bacteriotherapeutic period consisted of 5 successive periods of 7 days: 3 periods of probiotic supplementation were alternated with periods without this supplementation. During the periods of supplementation the infants got daily 240ml skim milk supplemented with 15ml of the probiotic product.

Microbiological assays

Fecal samples were collected by rectal swabs before treatment, at the 7th day of the first period of probiotic supplementation, and at the 8th day after the end of the probiotic therapy. Each swab was placed into a Lapt-soft agar medium (Raibaud *et al*., 1961) containing agar (0.8% w/v) to maintain viability and each duplicate was carried to the laboratory under anaerobic conditions. Previously, the tare of each sterile swab was determined. On the other hand, each tube containing Lapt-soft agar medium was weighed. Finally the wet weight of each fecal sample was calculated and the results were expressed as Colony Forming Units per gram of feces (CFU/g). All samples were analyzed the day of collection. Dilutions of the samples were plated out (in duplicate) onto a selective *lactobacillus* medium (LSB; Rogosa *et al*., 1951). After incubation for 48 hr at 37ºC in CO_2 atmosphere colonies were counted and results expressed as colony forming units per gram of wet feces (CFU/g). To identify the *Lactobacilli* in feces, colonies from LBS agar plates were subcultured in MRS broth for 24-48 hr at 37ºC. Microorganisms were observed under the microscope to determine morphological characteristics and Gram reaction. Tests for catalase activity, carbohydrate utilization and growth at 37 and 45°C were carried out.

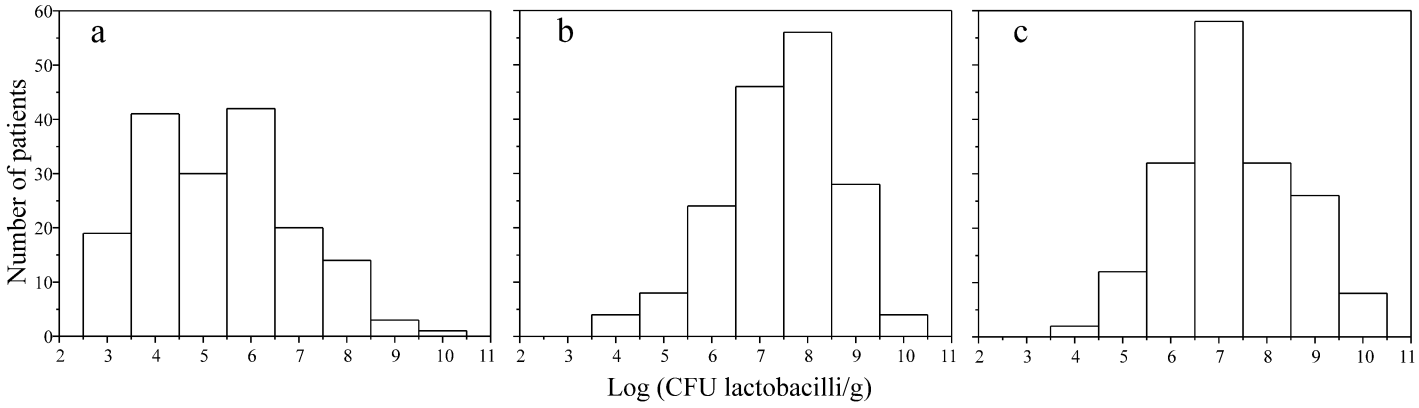

Figure 1. Frequencies of lactobacillus concentrations in fecal samples from infants (N= 170) before (1a), during (1b) and after (1c) probiotic bacteriotherapy. Counts of lactobacilli are given as the log of CFU/g wet feces.

Statistical analysis

Significant differences were tested using the Tukey HSD-test (Minitab Student R12; Rossman and Chance, 1998).

Results and Discussion

The data obtained from sequential ingestion of fermented milk by children (0-4 years old) suffering chronic diarrhea are presented in Figure 1. *Lactobacillus* counts in feces began before the first administration of fermented milk and are shown in Figure 1a. *Lactobacillus* concentrations obtained during and after probiotic treatment are shown in Figures 1b and 1c respectively. Since long-term probiotic therapy with rather high concentrations of *lactobacilli* might change the intestinal microflora, *lactobacilli* in feces were counted. Before therapy more intents showed counts of 10^4 and 10^6 *lactobacilli*/g (Figure 1a). By day 7 after the first supplementation period, the frequency peak was highest at fecal counts of 10^8 *lactobacilli*/g (Figure 1b). These data confirm that at least a part of the ingested *lactobacilli* pass the stomach. At the 8th day after the completed therapy the frequency peak was highest at fecal counts of 10^7 *lactobacilli*/g (Figure 1c). These results strongly suggest that in general the probiotic *lactobacillus* flora has restored the normal levels of *lactobacillus* flora. The presence of lectin-like substances in bacteria of the genus *Lactobacillus* has been rarely reported (Morata de Ambrosini *et al*., 1997; Mukai *et al*., 1992). These substances would play a critical role in the bacterial attachment to the gastrointestinal epithelium, and under these conditions the immune stimulation would be enhanced.

The mean period during which the infants suffered diarrhea just before the beginning of the probiotic treatment was of 51 ±33 days (N= 170; range 30-200 days). In all infants the probiotic therapy was very effective, since clinical symptoms were eliminated within the first period with probiotic supplementation. The therapeutic effect was seen after 4.1 ±1.6 days (N= 170; range 2-7 days). These results agree largely with those of Rusch *et al*. (1982), who proved the safety and efficacy of probiotic therapy in numerous case-reports. After the probiotic therapy till the end of this study none of the patients suffered again from diarrhea. This post-therapy period ranged from 34 days (last patient) to 630 days (first patient) with a median of 250 days. This observation could be explained by the remaining immune stimulating effect found, for both whole cells and cell walls of *L. casei*, after oral administration (Perdigón *et al*., 1992; Morata de Ambrosini *et al.*, 1998).

Univariate repeated measure (F-test) was 90.43 and its related significance's probability was $p=0.9\cdot10^{-15}$. Since the F-test was strongly significant, multiple comparisons (Tukey HSD-test) were used to determine the statistical significant differences between measures performed before, during and after treatment. Statistical significant differences were found between a) during and before treatment ($p=0.2\cdot10^{-4}$) and b) after and before treatment ($p=0.2\cdot10^{-4}$). No significant differences were found between after and during treatment (p=0.47). So, mean initial *lactobacillus* flora differs significantly from the mean flora during or after probiotic bacteriotherapy.

Some patients with high initial *lactobacillus* counts (up to 10^9-10^{10} UFC/g) also showed chronic diarrhea. Probably in these patients physiological deficiencies of the small bowel were involved; nevertheless, the positive probiotic effect in these patients is not well understood. The effectiveness of the therapy may be explained on the basis of adhesion and immunologic properties of our *lactobacilli*. The binding of the lectin-like structures to specific carbohydrates would explain the adhesion phenomenon of *lactobacilli* to epithelial cells (Morata de Ambrosini *et al.*, 1997). The hypothesis that these bacteria stimulate immune activities is justified by the results of well-controlled animal studies (Romero *et al.*, 1990; Perdigón *et al.*, 1992). Perdigón *et al*. (1998) demonstrated that feeding mice with *L. casei*, *L. acidophilus* or a mixture of both strains, produce a remarkable immunostimulatory effect on the host, both on the lymphocytic and reticuloendothelial systems. Production of secretory IgA is important because it constitutes the first line of defense against microbial pathogens (Tomasi and Bienenstock, 1968; Tomasi *et al.*, 1980).

The results presented, together with those obtained in previous studies using the same probiotic product in order to prevent diarrhea (González *et al.*, 1990) or to treat diarrhea due to post-gastroenteritis syndrome (González *et al.*, 1995), show that levels of 10^6 *lactobacilli*/g feces strongly contribute to paraimmunization (Mayr *et al.*, 1979). Our probiotic therapy confirms that daily consumption of high amounts of viable microorganisms alters the intestinal environment (Zoppi *et al.*, 1982). A successful probiotic ought not only to enhance the immune response, but also to be free from adverse side effects. Although further research is needed to elucidate the mechanism by which *L. casei* and *L. acidophilus* eliminate infantile diarrhea, clinical applications of our oral probiotic dairy product are possible.

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REFERENCES

Apella MC, González SN, Nader de Macías ME, Romero NC, Oliver G (1992) In vitro studies on the inhibition of growth of *Shigella sonnei* by *Lactobacillus casei* and *Lact. acidophilus*. *J. Appl. Bacteriol. 73*: 480-483.

De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol. 23*: 130-135.

Gebbers JO, Laissue JA (1982) Barrier function of the colonic mucosa: morphological aspects. *Microecol. Ther. 12*: 31-45.

González SN, Albarracín G, Locascio de Ruiz Pesce M, Malé M, Apella MC, Pesce de Ruiz Holgado A, Oliver G (1990) Prevention of infantile diarrhoea by fermented milk. *Microbiologie-Aliments-Nutrition 8*: 349-354.

González SN, Apella MC, Romero NC, Nader de Macías ME, Oliver G (1993) Inhibition of enteropathogens by lactobacilli strains used in fermented milk. *J. Food Prot. 56*: 773-776.

González SN, Apella MC, Cardozo R, Oliver G (1995) Biotherapeutic role of fermented milk. *Biotherapy 8*: 129-134.

Mayr A, Raettig H, Stickl H, Alexander M (1979) Paramunitat, Paramunisierung, Paramunitatsinducer. *Fortschr. Med. 97*: 1159-1165.

Metchnickoff, E (1907) The prolongation of life. Heinemann. London.

Morata de Ambrosini V, Salado C, González S, Oliver G (1997) Lectin-like substance in *Lactobacillus casei* CRL 431. *Microbiologie-Aliments-Nutrition 15*: 285-289.

Morata de Ambrosini V, González S, Perdigón G, P. de Ruiz Holgado A, Oliver G (1998) Immunostimulating activity of cell walls from lactic acid bacteria and related species. *Food Agric. Immun. 10*: 183-191.

Mukai T, Arihara K, Itoh H (1992) Lectin-like activity of *Lactobacillus acidophilus* strain JCM 1026. *FEMS Microb. Lett. 98*: 71-74.

Nader de Macías ME, Apella MC, Romero NC, González SN, Oliver G (1992) Inhibition of *Shigella sonnei* by *Lactobacillus casei* and *Lactobacillus acidophilus*. *J Appl. Bacteriol. 73*: 407-411.

Nader de Macías ME, Romero NC, Apella MC, González SN, Oliver G (1993) Prevention of infections produced by *E. coli* and *Listeria monocytogenes* by feeding milk fermented with lactobacilli. *J Food Prot. 56*: 401-405.

Perdigón G., Alvarez S, Médici M (1992) Systemic and local augmentation of the immune response in milk by feeding with milk fermented with *Lactobacillus acidophilus* and/or *Lactobacillus casei*. In Paubert-Braquet M, Dupont Ch, Paoletti R (Eds). *Foods, Nutrition and Immunity. Vol. 1. Dynamic Nutrition Research*. Karger. Paris. pp. 66-76.

Perdigón G, Nader de Macías ME, Alvarez S, Pesce de Ruiz Holgado A (1998) Systemic augmentation on the immune response in mice by feeding fermented milks with *L. casei* and *L. acidophilus*. *Immunology 63*: 17-23.

Raibaud P, Caulet M, Galpin JV, Mocquot G (1961) Studies on the bacterial flora of the alimentary tract of pigs II. Streptococci: selective enumeration and differentiation of the dominant group. *J. Appl. Bacteriol. 24*: 285-291.

Rogosa M, Mitchel DA, Weiseman RF (1951) A selective medium for the isolation and enumeration of oral and faecal lactobacilli. *J. Bacteriol. 62*: 132-133.

Romero NC, Nader de Macías ME, Apella MC, Oliver G (1990) Inhibition of *Listeria monocytogenes* by strains of lactic acid bacteria used as ferments. *Microbiologie-Aliments-Nutrition 5*: 175-179.

Rossman AJ, Chance BL (1998) *Workshop Statistics: discovery with data and Minitab*. Springer-Verlag, New York. pp. 279-325.

Rusch V, Hyde RM, Luckey TD (1980) Documentation on the identity, manufacture, safety, efficacy and stability of SYMBIOFLOR-products. *Microecol. Ther. 10*: 173-203.

Rusch V, Hyde RM, Luckey TD (1982) Microbiological therapy: oral and parenteral application of normal intestinal bacterial vaccines in humans and animals. *Microecol. Ther. 12*: 71-80.

Tomasi TB, Bienenstock J (1968) Secretory immunoglobulins. *Adv. Immunol. 9*: 1-5.

Tomasi T, Lawson B, Challacombe S, Mc Nabb P (1980) Mucosal immunity: the origin and migration patterns of cells in the secretory system. *J. Allergy Clin. Immunol. 65*: 12-19.

Zoppi G, Deganello A, Benoni G, Saccomani F (1982) Oral bacteriotherapy in clinical practice. I. The use of different preparations in infants treated with antibiotics. *Eur. J. Pediat. 139*: 18-21.