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Prevention of Infectious Mastitis by Oral Administration of *Lactobacillus salivarius* PS2 During Late Pregnancy

Leónides Fernández, Nivia Cárdenas, Rebeca Arroyo, Susana Manzano, Esther Jiménez, Virginia Martín, and Juan Miguel Rodríguez

Department of Nutrition, Food Science, and Food Technology, Universidad Complutense de Madrid, Spain

Background. Previous studies have shown that oral administration of lactobacilli can be an efficient approach to treat lactational infectious mastitis. In this trial, we have evaluated the potential of *Lactobacillus salivarius* PS2 to prevent this condition when orally administered during late pregnancy to women who had experienced infectious mastitis after previous pregnancies.

Methods. In this study, 108 pregnant women were randomly assigned to one of 2 groups. Those in the probiotic group (n = 55) ingested daily 9 $\log_{10}$ colony-forming units of *L. salivarius* PS2 from approximately week 30 of pregnancy until delivery, whereas those in the placebo group (n = 53) received a placebo. The occurrence of mastitis was evaluated during the first 3 months after delivery.

Results. Globally, 44 of 108 women (41%) developed mastitis; however, the percentage of women with mastitis in the probiotic group (25% [n = 14]) was significantly lower than in the control group (57% [n = 30]). When mastitis occurred, the milk bacterial counts in the probiotic group were significantly lower than those obtained in the placebo group.

Conclusions. Oral administration of *L. salivarius* PS2 during late pregnancy appears to be an efficient method to prevent infectious mastitis in a susceptible population.

Clinical Trials Registration. NCT01505361.

Keywords. mastitis; prevention; probiotics; *Lactobacillus salivarius*; pregnancy.

The health effects of breastfeeding are well recognized and apply to mothers and infants in developed and developing countries [1]; therefore, this infant feeding option has been recommended by several prominent health organizations, including the World Health Organization, the American Academy of Pediatrics, the American College of Obstetricians and Gynecologists, and the American Public Health Association. However, numerous barriers to breastfeeding remain; among them, mastitis represents the first medical cause of undesired weaning [2]. Mastitis is generally used to define an infectious process of the mammary gland characterized by a variety of local and, in acute cases, systemic symptoms [3].

The lactating mammary gland harbors many microorganisms in dynamic equilibrium, including potential mastitis-causing agents [4]. Upon disturbance of this balanced state, infection can occur and recent studies suggest that mastitis is related to a mammary bacterial dysbiosis [5, 6]. Because resistance to antibiotics and formation of biofilms are common properties among mastitis-causing bacteria, many cases are refractory to antibiotic therapy. Therefore, alternative strategies are required to decrease mastitis incidence rates.

In previous studies, we reported that oral administration of selected lactobacilli strains to lactating women was an effective alternative to antibiotics for treating infectious mastitis [7–9]. In this context, the objective of this work was to evaluate the efficacy of oral administration of one of these strains, *Lactobacillus salivarius* PS2, to prevent the development of lactational mastitis in a population of pregnant women with a history of mastitis after a previous pregnancy.

MATERIALS AND METHODS

Design of the Study and Definition of Mastitis

A total of 108 pregnant women, aged 24–35 and at 27–32 weeks of gestation, participated in this study. All met the following criteria: normal pregnancy, healthy status, and a history of lactational mastitis after at least one previous pregnancy. Women ingesting probiotic supplements or receiving antibiotic treatment in the previous 30 days were excluded. All volunteers gave written informed consent to the protocol, which had been approved by the Ethical Committee of Clinical Research of Hospital Clínico San Carlos Madrid (Spain). The study was registered in the ClinicalTrials.gov database (NCT01505361). The study was designed as a double-blind randomized placebo-controlled trial. Volunteers were randomly assigned, in a 1:1 ratio, into 2 groups ("probiotic" and "placebo") of equal size, using a computer-generated allocation sequence. Volunteers in the probiotic group (n = 55) consumed a daily capsule with approximately 50 mg of freeze-dried probiotic (approximately 9 $\log_{10}$ colony-forming units [CFU] of *L. salivarius*

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Correspondence: J. M. Rodríguez, Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Universidad Complutense de Madrid, Madrid 28040, Spain (jmrodrig@ucm.es).

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PS2) from approximately 30 weeks of pregnancy until birth. The probiotic was manufactured by Biopolis (Valencia, Spain), and kept at 4°C throughout the study. The women in the placebo group (n = 55) only received the probiotic excipient (powdered milk). Two dropouts were registered in this group for reasons that were not related to the study. All volunteers were provided with diaries to record compliance with study product intake. Minimum compliance rate (percentage of the total treatment doses) was set at 86%.

Diagnosis of Mastitis and Breast Pain Score

Mastitis symptoms were assessed by midwives during the first 3 months after birth. "Acute mastitis" was defined as an acute inflammation of the breast with local (ingurgitation, pain, redness, reduced milk secretion) and systemic (flu-like) symptoms, whereas "subacute mastitis" was defined as a breast inflammation accompanied by other local symptoms (ingurgitation, needlelike and/or burning pain, reduced milk secretion) without systemic symptoms [5]. Both definitions include cases associated with high milk bacterial and leukocyte counts (>3 $\log_{10}$ CFU/mL and >4 $\log_{10}$ cells/mL, respectively). Leukocyte quantification was performed by optical microscopy after May-Grünwald-Giemsa differential staining. When mastitis occurred, volunteers were asked to score breast pain from 0 (extremely painful) to 10 (no pain), following a standard procedure [10].

Collection and Microbiological Analysis of the Milk Samples

Milk samples (10 mL) were collected and stored as described previously [8]. Samples were obtained between 91 and 100 days after birth from women who did not experience mastitis during the 3-month follow-up period. A sample was collected from women developing mastitis as soon as possible (<12 hours) after diagnosis of such condition.

Samples were spread onto Baird Parker, Columbia nalidixic acid, MacConkey, and Sabouraud dextrose chloramphenicol agar plates (bioMérieux, Marcy l'Etoile, France) and incubated for 48 hours at 37°C in aerobic conditions for selective isolation and quantification of the main agents involved in infectious mastitis. After bacterial counting, representatives of each colony morphology type were identified by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF; VITEK MS, bioMérieux) in Probisearch (Tres Cantos, Spain).

Isolation and Identification of *L. salivarius* PS2 in Milk Samples

Women were asked to provide a sample of milk at day 7 (±2 days) after birth. These samples were inoculated onto MRS (Oxoid, Basingstoke, United Kingdom) plates supplemented with L-cysteine (0.5 g/L) (MRS-Cys). Plates were incubated as described previously [7, 8]. Isolates identified as *L. salivarius* by MALDI-TOF were submitted to pulsed-field gel electrophoresis (PFGE) genotyping [8], and their profiles were compared with that of *L. salivarius* PS2.

Statistical Analysis

Per protocol analyses were performed in the assessment of the effectiveness of the probiotic treatment. Microbiological data were recorded as CFU/mL, and transformed to logarithmic values before statistical analysis. Continuous variables were expressed as mean and 95% confidence interval (CI) after checking for normality. One-way analysis of variance (ANOVA) followed by Bonferroni post test was used for comparison of age and starting time (week of pregnancy) of the study between probiotic and placebo groups, and to check the effect of the probiotic intervention and the type of mastitis (subacute and acute) on the breast pain score (BPS). Two-way ANOVA was used to investigate the effect of treatment and mammary gland health status on staphylococcal/streptococcal counts as well as the effect of treatment and mastitis type on BPS and to study the possible interaction of the 2 investigated variables. Proportions were compared using χ^2 or Fisher exact probability tests. A correlation analysis was performed to test the relationship between staphylococcal/streptococcal counts in milk and BPS. Statistical significance was set at $P < .05$. Statgraphics Centurion XVI version 16.2.04 (Statpoint Technologies Inc, Warrenton, Virginia) was used to carry out statistical analyses.

RESULTS

Homogeneity of the Groups

In this study, there were no significant differences between the probiotic and the placebo group regarding age, number of previous pregnancies, or week of pregnancy at day 0 (Table 1). Compliance to the probiotic or the placebo was >90% (Table 1). No adverse or side effects/events related to the ingestion of the probiotic were reported throughout the study.

Occurrence of Mastitis Among Participants in the Study

The occurrence of mastitis was evaluated during the first 3 months after delivery. The mean for the lactation day on

Table 1. General Description of Women Included in the Study (N = 108)

Characteristic	Probiotic	Placebo	*P* Value[a]
No. of subjects	55	53	
Age, y			
Mean (95% CI)	31.18 (30.70–31.66)	30.51 (30.02–31.00)	.17
Minimum/maximum	26/35	26/35	
Previous pregnancies, No.			
Mean (95% CI)	1.9 (1.2–2.5)	1.8 (1.3–2.4)	.20
Minimum/maximum	1/3	1/3	
Time of pregnancy, wk			
Mean (95% CI)	29.82 (29.49–30.15)	30.06 (29.74–30.37)	.29
Minimum/maximum	27/32	28/32	
Compliance, %			
Mean (95% CI)	93 (92–94)	95 (94–96)	
Minimum/maximum	90/100	92/100	

Abbreviation: CI, confidence interval.

[a] One-way analysis of variance.

Table 2. Mastitis Occurrence During the First 3 Months After Delivery Among the Women Included in the Study (N = 108)

Condition	Probiotic (n = 55) No.	Probiotic (n = 55) Frequency (%)	Placebo (n = 53) No.	Placebo (n = 53) Frequency (%)	*P* Value
Healthy	41	75	23	43	.001[a]
Mastitis	14	25	30	57	
Subacute	11	79	23	77	1.000[b]
Acute	3	21	7	23	

[a] χ^2 test to compare the proportion of mastitis cases between the probiotic and placebo groups.

[b] Fisher exact test to compare the proportion of subacute and acute mastitis cases between the probiotic and placebo groups.

which the first signs of mastitis appeared was 29 (range, 2–86 days). Globally, 44 women of 108 (41%) experienced mastitis; 77% of mastitis cases were classified as subacute, while acute cases accounted for the remaining 23% (Table 2). Statistically significant differences were found between the probiotic and the placebo groups in relation to mastitis frequency. The percentage of women with mastitis in the probiotic group (25% [n = 14]) was significantly lower than in the control group (57% [n = 30]) ($P = .001$, χ^2 test; Table 2).

Bacterial Counts in the Milk Samples

Globally, both the treatment (probiotic or placebo) and the breast health status (healthy, mastitis) had a significant influence ($P = .000$ for both main factors, 2-way ANOVA) on the bacterial counts detected in milk. When the mean bacterial counts recorded in milk samples from healthy women who had received the probiotic (n = 41; 2.65 [95% CI, 2.58–2.72] $\log_{10}$ CFU/mL) were compared with those of healthy women included in the placebo group (n = 23; 2.84 [95% CI, 2.77–2.91] $\log_{10}$ CFU/mL), a small (0.19 [95% CI, .09–.30] $\log_{10}$ CFU/mL) but significant difference was observed ($P < .001$, *t* test; Figure 1). The differences observed between both groups in the bacterial milk counts obtained from women with subacute mastitis were higher: The mean bacterial count in the probiotic group (n = 11) was 3.83 (95% CI, 3.70–3.97) $\log_{10}$ CFU/mL, whereas in the placebo group (n = 23) it was 4.61 (95% CI, 4.38–4.85) $\log_{10}$ CFU/mL ($P < .001$, *t* test; Figure 1). Similarly, higher mean bacterial counts were found in milk obtained from women with acute mastitis in the placebo (n = 7; 5.02 [95% CI, 4.51–5.53] $\log_{10}$ CFU/mL) than in the probiotic (n = 3; 3.79 [95% CI, 3.29–4.29] $\log_{10}$ CFU/mL) group ($P = .006$, *t* test; Figure 1).

Breast Pain Score

Most of the participants who experienced mastitis (43/44) scored their breast pain between 0 and 4 in a scale ranging from 0 (extremely painful) to 10 (no pain). There was a strong correlation between the reported BPS values and the milk bacterial counts ($r = -0.814$, $P = .000$, Pearson product-moment correlation; Figure 2). Consumption of *L. salivarius* PS2 had a

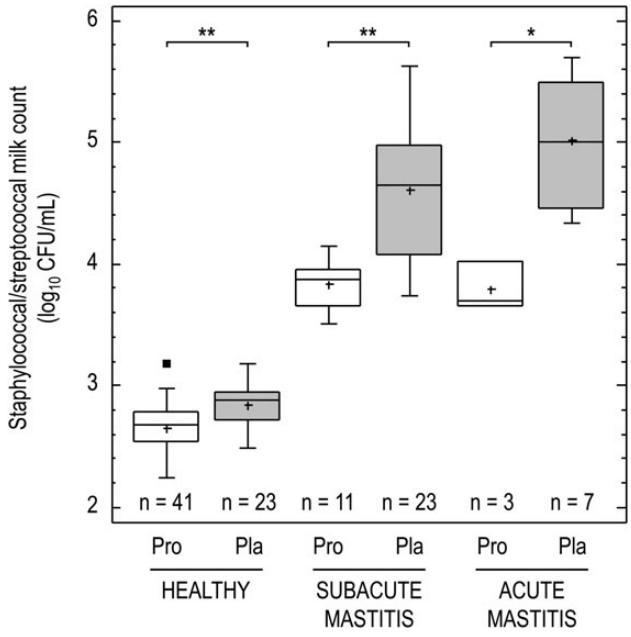

Figure 1. Staphylococcal/streptococcal counts in milk samples provided by the recruited women (N = 108). Differences in mean staphylococcal/streptococcal counts between probiotic (Pro) and placebo (Pla) groups for each status (healthy, subacute mastitis, and acute mastitis) were evaluated with *t* tests and are shown with horizontal lines inside the graph (*$P < .01$; **$P < .001$). Abbreviation: CFU, colony-forming units.

significant effect on BPS values ($P = .002$, 1-way ANOVA; Supplementary Table 1). Higher BPS values, which indicate less intense pain, were reported by women in the probiotic group (BPS = 2.8 [95% CI, 2.2–3.4]; n = 13) compared with those in

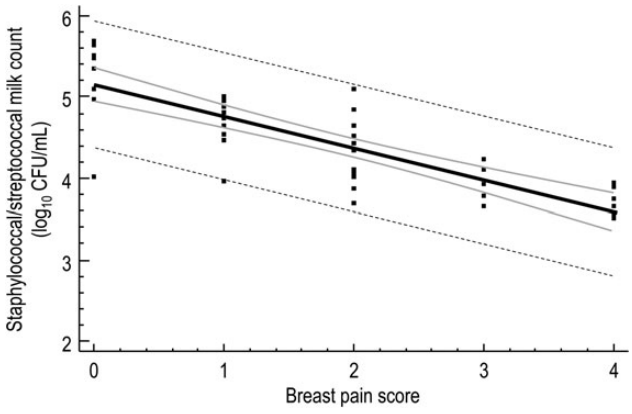

Figure 2. Correlation between staphylococcal/streptococcal milk counts and breast pain score (BPS) reported by women who developed mastitis during the study (n = 43). The correlation between both parameters (solid black line) follows the model: staphylococcal/streptococcal counts ($\log_{10}$ colony-forming units [CFU]/mL) = 5.138–0.389 × BPS; $r = -0.814$, $P = .000$. The 95% confidence interval for the mean staphylococcal/streptococcal counts as a function of the BPS is shown as a solid gray line, and 95% prediction intervals for new observations are shown as the outer dotted lines. Black squares represent milk samples.

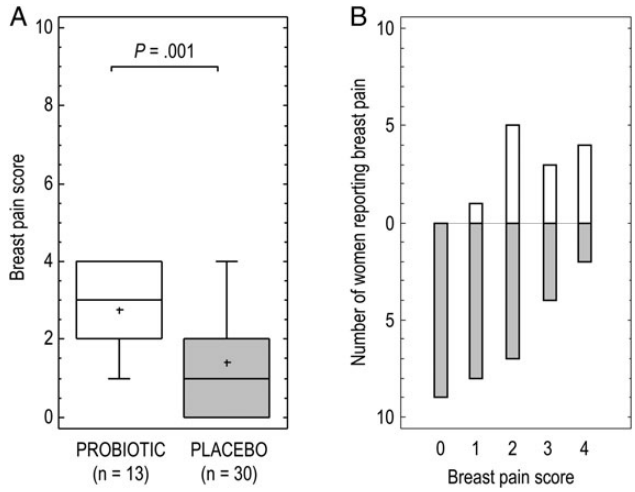

Figure 3. Distribution of the breast pain score (BPS) values reported by women who developed mastitis during the study. Breast pain categories were from 0 (extremely painful) to 10 (no pain). *A*, Differences between the participants of the probiotic and placebo groups (P = .001; 1-way analysis of variance). *B*, Distribution of BPS in the probiotic (n = 13; white bars) and the placebo (n = 30; grey bars) groups.

the placebo group (BPS = 1.4 [95% CI, .9–1.9]; n = 30) (P = .0012, 1-way ANOVA; Figure 3*A*). In contrast, the type of mastitis did not determine significant differences in the BPS values (P = .27, 2-way ANOVA; Supplementary Table 1).

Among all women with mastitis, those reporting lower BPS (more intense breast pain) were more frequently found in the placebo than in the probiotic group (P = .033, χ^2 test; Figure 3*B*). In fact, all women (n = 9) reporting extremely painful breasts (BPS = 0) belonged to the placebo group (Figure 3*B*).

Isolation and Identification of *L. salivarius* PS2 in Milk Samples

A subset of 29 and 24 women from the probiotic and the placebo groups, respectively, provided a sample of milk at day 7 after birth. *Lactobacillus salivarius* was isolated from 17 milk samples from the probiotic group (59%) and 3 milk samples from the placebo group (12.5%). In 15 of the 17 samples from the probiotic group, the *L. salivarius* isolates showed a PFGE profile identical to that of *L. salivarius* PS2. In contrast, none of the *L. salivarius* detected in milk samples from the placebo group showed a PFGE profile compatible with that of the assayed strain. None of the 15 women harboring *L. salivarius* PS2 experienced mastitis.

DISCUSSION

In this study, oral administration of *L. salivarius* PS2 to pregnant women with a history of mastitis after at least one previous pregnancy led to a mastitis rate (25%) that was significantly lower than that observed among women in the placebo group (57%). The decrease in mastitis incidence was similar for acute and subacute mastitis. To our knowledge, this is the first human clinical trial aimed at preventing lactational mastitis. The efficacy of selected *Lactobacillus* strains isolated from human milk for the treatment of mastitis has been previously reported [7, 8]. The probiotic treatments reduced the mean milk bacterial counts by approximately 2 $\log_{10}$ cycles, leading to a rapid improvement of the condition in most participating women. The final bacterial counts were approximately 2.5 $\log_{10}$ CFU/mL, a normal bacterial load in milk of healthy women [11].

Decreasing mastitis incidence would reduce the use of wide-spectrum antibiotics during lactation. Antibiotics are some of the main agents responsible for dysbiosis processes in the human microbiota, which may eventually lead to antibiotic-associated gastroenteritis and to infections of the oral cavity and genitourinary tract. More specifically, antibiotherapy during late pregnancy, intrapartum, or lactation negatively affects the intestinal, vaginal, and mammary microbiota of the mother, a fact that may have negative consequences to infant health by altering vertical bacterial transfer [12]. In a recent study, detection of lactobacilli or bifidobacterial DNA in the milk samples was significantly lower among women who had received antibiotherapy during pregnancy or lactation [13].

The existence of a site-specific microbiota in the mammary ecosystem during late pregnancy and throughout lactation [5] suggests that breast health may depend on the balance between the host state and its mammary-associated microbiota, although the exact events leading to the transition from colonization to infection are still ill-defined [14]. Host, microbial, and medical factors may play important roles in the protection against or predisposition to mastitis [5]. The composition of the milk microbiota is host-dependent [15–18], and strain-specific traits of some of its members, such as mechanisms for immune evasion [19, 20], virulence factors, or resistance to antibiotics [21, 22], may be essential in determining whether a woman will develop mastitis.

The host's genetic background may also play a key role in the lactation outcome as differences in selectin, Lewis antigens, and human milk oligosaccharide gene determinants may also predispose or protect against mastitis by altering neutrophil activation and production of reactive oxygen species [23]. It is known that human milk contains a wide spectrum of other biologically active substances, including eukaryotic antimicrobial peptides, such as cathelicidin LL-37. This peptide is expressed in the mammary gland and secreted in milk, and displays a relevant antimicrobial activity against potential mastitis-causing agents [24]. Polymorphisms or variations in the copy number or in the expression of genes encoding the biosynthesis of such antimicrobial peptides may be linked to mastitis susceptibility [25]. Finally, the existence of a genetic basis for host responses to bacterial intramammary infections has been widely documented in ruminants [26], while human granulomatous mastitis due to corynebacterial infection has been associated with a single-nucleotide polymorphism within the *NOD2* gene, impairing the neutrophil responses to Nod2 agonists [27].

The potential mechanisms by which some *Lactobacillus* strains are able to control mastitis-causing agents in the breast after oral administration have been reviewed recently [5]. Ingestion of probiotic strains during late pregnancy and/or breastfeeding increases immunoglobulin A and transforming growth factor–β2 levels in milk [28–30], which may control the local growth of mastitis-causing bacteria while limiting their ability to access or to damage the mammary epithelium. The characterization of the urine metabolic profile of lactating women with mastitis showed increased energy metabolism (lactate, citrate, formate, acetate, malonate) and decreased branched-chain amino acid catabolism (isocaproate and isovalerate) [9]. However, treatment of the same women with *L. salivarius* PS2 led to a sharp decrease in the excretion of lactose and, therefore, to a normalization of breast permeability. Changes in the levels of acetate and 2-phenylpropionate after probiotic intake suggested a immunomodulatory effect, whereas increased levels of malonate indicated an important antagonistic strategy of *L. salivarius* PS2 as this catabolite is a well-known repressor of the tricarboxylic acid cycle, which alters staphylococcal and streptococcal metabolism and negatively affects their survival, virulence, and ability for biofilm formation [31–33].

Local competitive exclusion and production of antimicrobials may also explain the control of mastitis-causing bacteria by certain lactobacilli [34–36]. This would imply that a *Lactobacillus* strain must be able to reach the mammary gland upon ingestion. In fact, it has been suggested that the presence of live bacteria in human milk is partially explained by bacterial transfer from the maternal gut to the mammary gland through an enteromammary pathway, involving complex interactions with immune cells [37]. An increased bacterial translocation from the gut to mesenteric lymph nodes and mammary glands in pregnant and lactating mice has been described previously [38]. In the same study, acridine orange staining of human milk and blood cytopreparations allowed the detection of bacterial cells in association with maternal mononuclear cells. Other studies have reported that oral administration of *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Lactobacillus fermentum*, and *L. salivarius* strains isolated from human milk to lactating women led to their presence in human milk [7, 8, 39]. Oral administration of a *Lactobacillus* strain to women during pregnancy resulted in colonization of their intestine and, subsequently, of their breastfed infant's gut [40]. Unfortunately, the role of milk bacteria as the potential source of the strain was not investigated.

Obviously, this clinical assay has strengths and weaknesses. The cohort was composed of women who developed mastitis after a previous pregnancy. As mastitis is a particularly painful and stressful experience for lactating women, the commitment of the volunteers with the trial was very high and was reflected in a very high compliance with the intake of the study product. However, the relatively small sample size, together with the fact that such women do not represent the pregnant population as a whole, limits the applicability of the findings to other cohorts. However, given the often recurrent nature of lactational mastitis and the significant differences in mastitis incidence observed between the probiotic and the placebo groups in this cohort, the administration of *L. salivarius* PS2 to pregnant women has the potential to improve overall quality of life, decrease antibiotic courses, and decrease healthcare costs. Preventive strategies aiming to reduce mastitis occurrence, such as the use of a target-specific probiotic strain, may contribute to extend the benefits of breastfeeding and are, therefore, relevant to public health.

Supplementary Data

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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