Effects of Probiotics on Enteric Flora and Feeding Tolerance in Preterm Infants

Soo Jeong Lee    Su Jin Cho    Eun Ae Park

Department of Pediatrics, College of Medicine, Ewha Womans University, Seoul, Korea

Key Words
Probiotics · Premature infants · Feeding tolerance · Lactobacillus acidophilus

Abstract
Background: Probiotics are live microbes that colonize the gastrointestinal tract and benefit the host. Preterm infants develop abnormal patterns of bowel colonization, and only a few clinical trials have reported the outcomes of preterm infants treated with probiotics. Purpose: We investigated the rate of colonization of Lactobacillus and the clinical variables affecting the colonization in preterm infants. Methods: Infants with gestational age less than 37 weeks treated at Ewha Womans University Hospital between March 2003 and July 2004 were eligible. Lactobacillus acidophilus (containing 10^8 CFU) was supplemented orally, mixed with breast milk or formula divided into three doses a day. Stool samples were collected before and 14 days after supplementation of the probiotic. Stool samples were anaerobically cultured on Rogosa agar and identified by Gram stain, catalase test and glucose fermentation test. Clinical characteristics were analyzed. Results: Seventy-three patients with an average gestational age of 33.0 ± 2.5 weeks were studied. Meconium was cultured in 46 patients and Lactobacillus was not detected. Probiotic supplementation began on 3.4 ± 6.8 days, and after 14 days of supplementation, Lactobacillus was cultured in an average of 3.01 × 10^8 CFU in the stool of 37.0% (27/73) of the patients. There was a tendency towards an increased incidence of sepsis in the Lactobacillus− group (p = 0.082). In the Lactobacillus+ group, a striking increase in feeding tolerance was detected. Conclusion: In preterm infants, with the administration of probiotics, 37% of the preterm infants had Lactobacillus colonized in the gastrointestinal tract and improved feeding tolerance. A double-blind study is in progress for further investigation into the effect on other systemic diseases in premature infants.

Introduction
A major difference between breast-fed and formula-fed newborn infants is the development of the intestinal flora, considered to be important for protection against harmful microorganisms and the maturation of the intestinal immune system. For healthy newborns, beneficial strains such as bifidobacteria form the majority of intestinal flora [1]. Early formation of such enteric colonies aids the normal development of the intestine by modulating intestinal mucosal immunity [2], regulation of systemic immune reactions [3], competitive inhibition of pathogenic bacteria [4], digestion of proteins and carbohydrates, formation of vitamins, and maturation and...
Feeding Tolerance in Preterm Infants

Probiotics Improve Enteric Flora and Feeding Tolerance in Preterm Infants

Probiotics are ‘live microbial feed supplements that beneficially affect the host by improving its microbial balance’ and have been described by Fuller [8] in 1989. Probiotics have various effects on the intestine such as changes in intestinal permeability [9], enhancing the mucosal immunoglobulin A responses [10], production of anti-inflammatory cytokines [11], and normalization of the intestinal microenvironment [12].

The lack of microbial diversity predisposes them to the acquisition of antibiotic resistant strains. Thus, one method of promoting bowel colonization with desirable flora is through the administration of probiotic bacteria. Clinical studies on probiotic treatment for premature infants remain preliminary. We hypothesized that probiotic treatment would enhance the colonization of the normal flora, increase feeding tolerance and decrease the incidence of NEC and neonatal sepsis. Other factors influencing the colonization of the normal flora were analyzed.

**Material and Methods**

This trial was performed in the NICU at Ewha Womans University Mok Dong Hospital in Seoul, Korea, between March 2003 and July 2004. All live-born infants admitted to the NICU with a gestational age less than 37 weeks were eligible. To observe a difference of 0.1 with a confidence of 0.90 and a variance of 0.5 in a population of 100 patients, the average number of premature infants at our institution per year, a total of 80 patients are needed. Due to dropouts, we analyzed 73 patients. The Lactobacillus acidophilus ATCC® 4356 (containing $10^8$ CFU) was administered orally mixed with breast milk or formula milk divided into three doses a day with informed consent of the parents. Feeding was initiated when the infant demonstrated a stable respiratory and cardiovascular status, usually on day 2–3 of life. When breast milk was unavailable, preterm formula was used. Bolus feeding was given every 3 h for infants less than 2,000 g and every 4 h for those greater than 2,000 g. Daily increment in feeding was 10 ml/kg/day if less than 1,500 g and 20 ml/kg/day if greater than 1,500 g.

Stool samples were collected once before administration of the probiotic and 14 days after starting administration. Stool samples were cultured on Rogosa SL agar [13] (BD, Sparks, Md., USA) in an anaerobic condition. After an incubation period of 48 h, colony counts were performed. Gram stain, glucose fermentation test and catalase test were performed to identify Lactobacillus. For the catalase test, 3% H$_2$O$_2$ was added to confirm the absence of air bubbles, and the glucose fermentation test was performed by checking the change of phenol red from red to yellow.

Gestational age, birth weight, clinical diagnosis, method of feeding, use of antibiotics, duration of total parenteral nutrition, mechanical ventilation, and the usage of umbilical catheters were noted. Gastrointestinal symptoms and signs such as feeding tolerance, abdominal distension, vomiting, mucoid stool, loose stool and otherwise abnormal stool patterns along with the incidence of sepsis or necrotizing enterocolitis (NEC) were noted. NEC was graded according to the modified Bell’s criteria. Feeding tolerance was defined as daily increment of feeding amount without abdominal distension, residual more than 50% of previous feeding at the time of next feeding, vomiting, or loose mucoid stool. In cases of abdominal distension, abdominal plain films were taken when clinically indicated to identify bowel distension (ileus) demonstrated as bowel distension presented as a radiologic finding by a pediatric radiologist at our institution. The patients were further divided into two groups: 27 infants (37.0%) who had Lactobacillus cultured from their stool after 14 days, and the other group with 46 infants (63.0%) without Lactobacillus. The clinical characteristics and gastrointestinal symptoms and signs were compared between the two groups.

Statistical analysis was done using SPSS 11.0, and the Student’s $t$ test, $\chi^2$ test, Wilcoxon rank sum test, and Mann Whitney $U$ test were performed accordingly. All numerical values are expressed as mean $\pm$ standard deviation. A p value $<$0.05 was considered to be statistically significant.

**Results**

**Colonization of Lactobacillus**

The average gestational age of the 73 patients was 33.0 $\pm$ 2.5 weeks. Stool cultures were performed to assess the colonization of Lactobacillus. We were able to culture the meconium of 42 patients before initial feeding and none of them grew Lactobacillus. All patients were given probiotics on day 3.4 $\pm$ 6.8 of life. Initial stool cultures done on day 2.8 $\pm$ 3.2 of life also demonstrated lack of Lactobacillus in the stool. After 14 days of supplementation of probiotics, on day 18.3 $\pm$ 9.5 of life, 27 patients (37.0%) demonstrated Lactobacillus in their stool and 46 (63.0%) did not. The average colony count of those who had Lactobacillus was $3.01 \times 10^8$ CFU. Lactobacillus colonies are characterized by the large size, creamy white color and buttery smell (fig. 1). Light microscopic examination after Gram stain demonstrated Gram-positive bacilli (fig. 2).
Clinical Characteristics of the Two Groups

The group with Lactobacillus cultured in the stool (Lactobacillus+ group, n = 27 patients) after 14 days of supplementation had an average gestational age of 33.3 ± 2.5 weeks and a mean birth weight of 1,829.2 ± 417.3 g. The group with no Lactobacillus cultured (Lactobacillus– group, n = 46 patients) had an average gestational age of 32.8 ± 2.5 weeks and a mean birth weight of 1,944.0 ± 499.1 g, which was not different between the two groups. The male:female sex ratio was 1:0.8 in the Lactobacillus+ group and 1:1.0 in the Lactobacillus– group. The percentage of vaginal delivery was 59.2% in the Lactobacillus+ group and 43.5% in the Lactobacillus– group. The 1-min Apgar score was not different between the two groups, i.e. 6.7 ± 1.5 for the Lactobacillus+ group and 6.0 ± 2.3 for the Lactobacillus– group. However, the 5-min Apgar score was higher in the Lactobacillus+ group, i.e. 8.4 ± 1.3 and 7.8 ± 1.6, respectively (p = 0.077). Breastfeeding was done in 55.6% in the Lactobacillus+ group and in 56.5% in the Lactobacillus– group, which was not statistically different.

No difference was found in the incidence of hyaline membrane diseases, intraventricular hemorrhage, cholestatic jaundice, persistent ductus arteriosus, and the usage of indomethacin. The incidence of NEC was 18.5% in the Lactobacillus+ group and 26.1% in the Lactobacillus– group, but this was not statistically significant. The incidence of culture-proven sepsis was significantly higher in the Lactobacillus– group including 9 cases (19.6%) versus 1 case (3.7%) in the Lactobacillus+ group (p < 0.05). The causative organisms were Staphylococcus epidermidis in the Lactobacillus+ group and 4 cases of Staphylococcus aureus and 1 case each of S. epidermidis, coagulase-negative staphylococcus, Streptococcus mitis, Klebsiella pneumoniae and Candida albicans in the Lactobacillus– group (table 1).

We then analyzed the duration of antibiotic therapy and colonization of Lactobacillus. Antibiotics were administered for 18.6 ± 12.5 days to 21 patients (80.8%) in the Lactobacillus+ group and for 20.3 ± 17.7 days to 38 patients (82.6%) in the Lactobacillus– group. No significant difference was found in the duration of antibiotic treatment between the two groups. However, when we analyzed the same data with different kinds of antibiotics, an interesting significant difference was found. Third-generation cephalosporins were used as clinically indicated in 8 patients (29.6%) in the Lactobacillus+ group and in 17 patients (37.0%) in the Lactobacillus– group. Vancomycin and imipenem were used as clinically indicated only in the Lactobacillus– group (p < 0.05). No difference was found in the duration of mechanical ventilation, use of umbilical catheters, and duration of total parenteral nutrition (table 2).

Enteral Feeding Tolerance and Gastrointestinal Symptoms

After supplementation of Lactobacillus for 14 days, feeding tolerance was improved in the Lactobacillus
Probiotics Improve Enteric Flora and Feeding Tolerance in Preterm Infants

Neonatology 2007;91:174–179 177

Comparisons of gastrointestinal symptoms were calculated by logistic regression analysis adjusting for the prior symptom status (table 3). The Lactobacillus– group showed an improvement in 17.4%, but the difference was not statistically significant. Vomiting and diarrhea improved in both groups after the administration of probiotics but not to a statistically significant degree. Abdominal distension manifesting as bowel distension on simple abdominal films improved in 40.8% in the Lactobacillus group after supplementation but in none out of the 10 patients (21.7%) in the Lactobacillus– group. Such difference was statistically significant (p < 0.01).

The increase in milk intake was compared every 3 days after Lactobacillus supplementation. No difference was found prior to supplementation, but after 3 days, the Lactobacillus+ group demonstrated a significant increase in tolerable milk intake between days 9, 12 and 15 (p < 0.05) (fig. 3).

Table 1. Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Disease</th>
<th>Lactobacillus+ (n = 27)</th>
<th>Lactobacillus– (n = 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDS</td>
<td>7 (25.9)</td>
<td>12 (26.1)</td>
</tr>
<tr>
<td>IVH &gt;grade 2</td>
<td>1 (3.7)</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>PDA</td>
<td>4 (14.8)</td>
<td>8 (17.4)</td>
</tr>
<tr>
<td>Indomethacin tx.</td>
<td>2 (7.4)</td>
<td>6 (13.0)</td>
</tr>
<tr>
<td>IHC</td>
<td>1 (3.7)</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>NEC</td>
<td>5 (18.5)</td>
<td>12 (26.1)</td>
</tr>
<tr>
<td>Proven sepsis</td>
<td>1 (3.7)</td>
<td>9 (19.6)*</td>
</tr>
</tbody>
</table>

Organisms

- S. aureus: 4
- S. epidermidis: 1
- CNS: 1
- S. mitis: 1
- K. pneumoniae: 1
- C. albicans: 1

Figures in parentheses are percentages. RDS = Respiratory distress syndrome; IVH = intraventricular hemorrhage; PDA = patent ductus arteriosus; IHC = intrahepatic cholestasis; CNS = coagulase-negative staphylococcus.

* p < 0.05.

Table 2. Duration and use of different treatments

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Lactobacillus+ (n = 27)</th>
<th>Lactobacillus– (n = 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of admission, days</td>
<td>38.8 ± 24.3</td>
<td>40.8 ± 35.5</td>
</tr>
<tr>
<td>Use of antibiotics</td>
<td>21/27 (77.8)</td>
<td>38/46 (82.6)</td>
</tr>
<tr>
<td>Use of third-generation cefalosporin</td>
<td>8/27 (29.6)</td>
<td>17/46 (37.0)</td>
</tr>
<tr>
<td>Use of vancomycin or imipenem</td>
<td>0/27 (0.0)</td>
<td>9/46 (19.6)*</td>
</tr>
<tr>
<td>Duration of antibiotics, days</td>
<td>18.6 ± 12.5</td>
<td>20.3 ± 17.7</td>
</tr>
<tr>
<td>Use of ventilator</td>
<td>9/27 (33.3)</td>
<td>18/46 (39.1)</td>
</tr>
<tr>
<td>Duration of ventilator, days</td>
<td>17.3 ± 15.4</td>
<td>27.4 ± 25.2</td>
</tr>
<tr>
<td>Use of UAC</td>
<td>7/27 (25.9)</td>
<td>10/46 (21.7)</td>
</tr>
<tr>
<td>Duration of UAC, days</td>
<td>6.0 ± 2.2</td>
<td>8.4 ± 4.9</td>
</tr>
<tr>
<td>Use of UVC</td>
<td>6/27 (22.2)</td>
<td>7/46 (15.2)</td>
</tr>
<tr>
<td>Duration of UVC, days</td>
<td>9.3 ± 7.3</td>
<td>17.1 ± 14.0</td>
</tr>
<tr>
<td>Use of TPN</td>
<td>25/27 (92.6)</td>
<td>43/46 (93.5)</td>
</tr>
<tr>
<td>Duration of TPN, days</td>
<td>27.1 ± 22.6</td>
<td>28.5 ± 26.9</td>
</tr>
</tbody>
</table>

Figures in parentheses are percentages. UAC = Umbilical artery catheter; UVC = umbilical vein catheter; TPN = total parenteral nutrition.

* p < 0.05.

Table 3. Abdominal symptoms and signs

<table>
<thead>
<tr>
<th></th>
<th>Lactobacillus+ (n = 27)</th>
<th>Lactobacillus– (n = 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor sucking power</td>
<td>15 (55.6)</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>7 (25.9)</td>
<td>7 (15.2)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1 (3.7)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td>Ileus on X-ray</td>
<td>14 (51.9)</td>
<td>10 (21.7)</td>
</tr>
</tbody>
</table>

Figures in parentheses are percentages. * p < 0.05.
Discussion

The intestinal mucosa appears to function as a defensive barrier, limiting the translocation of pathogenic bacteria present in the intestinal lumen. Trauma, immunosuppression and prematurity further increase intestinal permeability when mucosal barrier is impaired [14]. The normal flora in the intestine forms the intestinal mucosal barrier and equilibrates the intestinal microflora, inhibits growth of pathogenic bacteria, and modulates immune functions. The normal flora consists of approximately 500 bacteria, and the diversity is also important in the maintenance of the integrity of the microenvironment. Anaerobic bacteria are 10–1,000 times more abundant and rarely permeate to the extraintestinal environment, and thus, do not show pathogenic properties [15]. Specific anaerobes classified as lactic acid bacteria (L. acidophilus, Bifidobacterium) may play a protective role via immunologic mechanisms promoted by fermentation processes that metabolize varying quantities of lactic, acetic and formic acids, vitamin synthesis and production of antimicrobial bacteriocins and fatty acids [16].

Bifidobacterium is the main strain found in healthy breast-fed infants [1]. Breastfeeding plays an important role in the acquisition of a normal flora in newborn infants. Close contact with the mother also plays an important role in the formation of an intestinal microflora. Not only maternal immunoglobulin, various immune cells and antibacterial substances exist in the breast milk, but prebiotics such as bifidogenic factors are also found [17]. In spite of technological advances to add such natural protective factors to infant formulas, the microbiologic and immunologic superiority of breast milk is still incomparable [18]. Premature infants cared for in the NICU have many obstacles to overcome in establishing a normal flora. The colonization is delayed [2] and characterized by less diversity [6]. Prolonged and early antibiotic treatment, strict hand washing and a sterilized environment are necessary for the care of premature infants but hinder the colonization of bacteria in the gut. Less contact with the mother and less breastfeeding is also responsible for such differences. Antibiotic resistant bacteria colonize these premature infants and may cause systemic infections and spread to other patients. Such pathogenic bacteria play a major role in the pathogenesis of NEC [19]. The most common bacteria in the stool of infants in the NICU were coagulase-negative staphylococcus, S. aureus, Klebsiella species and enterococci. Anaerobes such as Clostridia were more common compared with bifidobacteria in breast-fed babies. Yeasts were also frequently found [20]. The number of different strains was decreased to less than 20 compared with 100 in healthy newborns, and such paucity in diversity results from antibiotic treatment which rids beneficial bacteria and the replacement with antibiotic resistant bacteria in the gut [6].

This paper presents some preliminary data of our study comparing the benefits of probiotics in the NICU. In order to investigate the rate of colonization of Lactobacillus, we started with the dosage of 10⁸ CFU, the usual pediatric dosage in infants in the NICU. Lactobacillus is an anaerobic organism residing in the small bowel and may be difficult to culture in stool, but we had stools positive for Lactobacillus and evaluated how other clinical factors such as enteral feeding tolerance, incidence of sepsis and NEC related to the difference in colonization. Molecular diagnosis with polymerase chain reaction was done in a different arm of the study and proved that our culture method was indeed demonstrating Lactobacillus. Seventy-three newborn infants with a gestational age less than 37 weeks were supplemented with L. acidophilus, and stool culture was performed to evaluate for the colonization in the intestines. No significant difference was found in the gestational age, birth weight, diagnosis, method of feeding, duration of antibiotic treatment, duration of total parenteral nutrition, and the duration of mechanical ventilation and use of umbilical catheters. The absence of an association of Lactobacillus colonization with breastfeeding needs to be further addressed in a larger trial. The 5-min Apgar score was lower in the Lactobacillus– group, and this may reflect the poor peri-
natal condition, which may have hindered the acquisition of *Lactobacillus* colonization.

The incidence of bacterial sepsis was significantly decreased in the *Lactobacillus*+ group, and this is in concordance with other studies suggesting that *Lactobacillus* may inhibit bacterial translocation of pathogenic bacteria into the systemic circulation. Organisms responsible for causing sepsis in the *Lactobacillus*– group were *S. epidermidis*, *S. aureus*, coagulase-negative *staphylococcus*, *S. mitis*, *K. pneumoniae*, and *C. albicans*, and these are the common organisms found in the NICU. The clinical diagnosis of sepsis was given to infants with poor feeding, lethargy or fever, and not all cases were confirmed microbiologically, which explains the high rate of sepsis in our data. Ampicillin and cefotaxime treatment is started intravenously in infants suspected of clinical sepsis, and the antibiotics are changed according to the culture and sensitivity results. Vancomycin and imipenem use was significantly related with no growth of *S. aureus*, *K. pneumoniae*, and *C. albicans*, and these are the common organisms found in the NICU. The clinical diagnosis of sepsis was given to infants with poor feeding, lethargy or fever, and not all cases were confirmed microbiologically, which explains the high rate of sepsis in our data. Ampicillin and cefotaxime treatment is started intravenously in infants suspected of clinical sepsis, and the antibiotics are changed according to the culture and sensitivity results. Vancomycin and imipenem use was significantly related with no growth of *S. aureus*, *K. pneumoniae*, and *C. albicans*, and these are the common organisms found in the NICU.

Although the incidence of NEC did not differ between the two groups, the *Lactobacillus*+ group demonstrated less abdominal distension and a more tolerable increase in enteral nutrition. Such feeding tolerance is important in premature infants since better enteral feeding correlates with less parenteral nutrition, less cholestatic jaundice and shorter duration of indwelling foreign bodies such as catheters. Further studies will reveal more beneficial effects of probiotic treatment to premature infants. The limitation of our study includes the absence of a placebo group, and this study was done as a preliminary study for a placebo-controlled double-blind study addressing the benefits of probiotics in premature and full-term infants.

### Conclusion

*L. acidophilus* supplementation to preterm infants colonizes the gastrointestinal tract and improves feeding tolerance. Invasive disease caused by *Lactobacillus* is possible but rare and should not interfere with the wide clinical use [21]. A double-blind randomized control multicenter trial should be conducted to test the effect in a larger number of preterm infants.

### References


