Effect of Administration of Bifidobacteria and Lactic Acid Bacteria to Newborn Calves and Piglets

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ABSTRACT

The effect of oral administration of bifidobacteria and lactic acid bacteria on newborn livestock was investigated. Oral administration of Bifidobacterium pseudolongum or Lactobacillus acidophilus to calves improved BW gain and feed conversion over that of untreated controls. The frequency of diarrhea occurrence was decreased in the group fed probiotics. However, there was no significant difference between calves administered B. pseudolongum and those administered L. acidophilus. Under conditions of feeding without antibiotics, the frequency of diarrhea was decreased markedly by administration of probiotics. The BW gain of piglets was significantly greater during both the suckling and weaning periods for the group fed probiotics than for the control group. That effect was more evident during the suckling period than during the weaning period. Under conditions of feeding without antibiotics, the frequency of piglet mortality was decreased markedly by administration of probiotics. The probiotics tested here had useful effects, including improved BW gain, feed conversion, and fecal condition of both newborn calves and piglets.

(Key words: probiotic, Bifidobacterium, Lactobacillus acidophilus)

Abbreviation key: DCP = digestible CP, MIC = minimum inhibitory concentration.

INTRODUCTION

Antibiotics have been widely used to promote growth of livestock. For newborn calves and piglets, administration of antibiotics is useful to prevent infections caused by pathogenic bacteria. However, the use of antibiotics in livestock can have serious consequences, such as the development of resistant populations of bacteria. Subsequent use of the same antibiotics for therapy may be ineffective. Also, residual antibiotics in dairy foods, meat, eggs, and milk are unacceptable.

Recently, the use of probiotics has attracted the attention of researchers (1). Lactobacillus acidophilus, Streptococcus faecalis, and other lactic acid bacteria have been tested as probiotics for livestock (7, 10, 19). Pollmann et al. (20) observed that administration of L. acidophilus was an effective way to promote BW gain and feed conversion in piglets. Ozawa et al. (19) reported that the administration of S. faecalis to calves and piglets promoted colonization by beneficial bacteria and decreased the occurrence of detrimental bacteria, such as Salmonella, in the intestine. Clostridium butyricum and Bacillus subtilis have also been tested as probiotic agents (5, 6, 8). However, few reports exist concerning the use of bifidobacteria as a probiotic for animals (9, 17).

Since its discovery by Tissier (25) in feces of breast-fed infants, Bifidobacterium, a prominent genus of bacteria in the human intestines, has been studied by many researchers. Bifidobacterium is a beneficial and important genus of intestinal bacteria in humans (23). Subsequently, many species of Bifidobacterium have been found in various animals, suggesting that these bacteria are a common component of the intestinal flora of animals as well as humans (11, 12, 13, 18, 27). Use of Bifidobacterium strains as probiotic agents is an attractive possibility that is expected to provide beneficial effects for livestock.

This study examined whether administration of bifidobacteria or lactic acid bacteria was effective in promoting the growth of new-
MATERIALS AND METHODS

Bacterial Strains

In this study, *Bifidobacterium pseudolongum* M-602 isolated from chicken feces, *Lactobacillus acidophilus* LAC-300 and *Enterococcus faecium* FA-5 from human feces, *Bifidobacterium thermophilum* S-501 from piglet feces, and *Bifidobacterium animalis* M-95 from rat feces were used as probiotics. Each strain was cultured for 16 h at 37°C in liquid medium containing glucose, yeast extract, casein peptone, K2HPO4, KH2PO4, and L-cystine. The culture solutions were centrifuged, washed twice with distilled cool water, and freeze-dried. Dried cells were mixed with dried dextrin to adjust the cell counts.

Trial 1

Forty-five Holstein calves (15 per experiment; three replications) at 7 d after birth were used to determine the effects of administration of *B. pseudolongum* M-602 or *L. acidophilus* LAC-300. Each experiment in three replications was carried out in different seasons. In each experiment, 15 calves were assigned randomly to three treatments: 1) a group administered *B. pseudolongum* M-602, 2) a group administered *L. acidophilus* LAC-300, and 3) an untreated control group. Each calf was placed in an individual pen enclosed with an iron fence and kept independently at the same farm to prevent cross-contamination among calves. For the groups fed bifidobacteria or lactobacilli, *B. pseudolongum* M-602 or *L. acidophilus* LAC-300 was administered every morning with milk replacer at a dose of $3.0 \times 10^9$ cfu. All calves tested were treated with the probiotics during trial 1 from 7 to 56 d of age. For the initial 35 d of the test (from 7 to 42 d of age), a milk replacer (Morinaga Calf Milk; Morinaga Milk Industry Co., Ltd., Tokyo, Japan) was fed; from 14 to 56 d of age, a starter (New Starter; C. Itoh Feed Mills Co., Ltd., Tokyo, Japan) was fed. Water and dried grass were available for ad libitum consumption throughout the feeding period. Also, all calves tested here were fed colostrum generally after birth. The calculated analysis of the milk replacer was 25.5% CP, 17.0% crude fat, 1.0% crude fiber, 10.0% crude ash, 80.0% calcium, 60.0% phosphate, 24.0% digestible CP (DCP), and 100.0% TDN. The milk replacer also contained 20 g of colistin sulfate/tonne and $4.2 \times 10^6$ units of zinc bacitracin/tonne. The calculated analysis of the starter was: 17.0% CP, 2.5% crude fat, 10.0% crude fiber, 9.0% crude ash, 60.0% calcium, 40.0% phosphate, 15.0% DCP, and 74.0% TDN. The starter also contained 44.4 g of chlortetracline/tonne. The BW gain and feed conversion were observed until calves were 56 d of age. To determine the effect of probiotic administration on fecal condition, the feces of all calves were observed twice (morning and early night) every day for the initial 28 d of the test, and a numerical fecal score (0 = normal, 1 = soft, and 2 = scours) was assigned. The mean fecal score (per day per calf) of each group was used to calculate the mean fecal score of all calves and for group comparison.

Trial 2

The effect on calves of the administration of probiotics under feeding conditions without antibiotics was investigated. Nineteen newborn calves were divided into two groups, a group fed probiotics and a control group. Each calf in the group fed probiotics was administered a cell mixture containing $1 \times 10^{10}$ cfu of *B. thermophilum* S-501, $1 \times 10^{10}$ cfu of *E. faecium* FA-5, and $1 \times 10^9$ cfu of *L. acidophilus* LAC-300 with milk replacer every morning. All calves tested were placed in individual pens; milk replacer without antibiotics and water were available for ad libitum intake. The BW gain, feed conversion, and health condition were observed for 28 d. The condition of feces was observed every day, and the occurrence of diarrhea in calves throughout the trial period was recorded.

Trial 3

The effects of administration of probiotics on 328 piglets (103 to 122 per experiment; three replications) were investigated. Each experiment was carried out in a different season. In each experiment, newborn piglets were allotted at random to three treatments, as in trial
1. The control group consisted of 12 to 14 litters, and treatment groups fed *B. pseudolongum* or *L. acidophilus* consisted of 6 to 7 litters. Each litter consisted of 4 to 5 piglets. For the group fed bifidobacteria or lactobacilli, each piglet was administered *B. pseudolongum* M-602 or *L. acidophilus* LAC-300 at a dose of $3 \times 10^8$ cfu/d per piglet during the suckling period and $3 \times 10^9$ cfu/d per piglet during the weaning period. During the suckling period, all piglets were kept with the sow in the same litter and were fed on milk from the sow and an early milk replacer (Hello A; C. Itoh Feed Mills Co., Ltd.) for ad libitum intake. During the weaning period, test piglets were removed from the sow and were fed only a late milk replacer (Hello B; C. Itoh Feed Mills Co., Ltd.). Water was available for ad libitum intake throughout both periods. The calculated analysis of the early milk replacer was 22.0% CP, 3.5% crude fat, 2.0% crude fiber, 8.0% crude ash, .60% calcium, .50% phosphate, 20.5% DCP, and 87.0% TDN. The milk replacer also contained 40 g of colistin sulfate/tonne, 30 g of morantel citrate/tonne, and 44 g of tylosin phosphate/tonne. Calculated analysis of the late milk replacer was 18.0% CP, 3.0% crude fat, 4.0% crude fiber, 8.0% crude ash, .60% calcium, .50% phosphate, 16.0% DCP, and 80.0% TDN. The late milk replacer also contained 20 g of colistin sulfate/tonne and 30 of morantel citrate/tonne. The BW gain and feed conversion were observed until 56 d of age.

**Measurement of Minimum Inhibitory Concentration of Antibiotics**

The minimum inhibitory concentration (MIC) of the antibiotics that were present in the milk replacer or starter was tested against *B. pseudolongum* M-602 and *L. acidophilus* S-501 by the paper disk method. The bacteria cultured in GAM broth (Nissui Seiyaku Co. Ltd., Tokyo, Japan) supplemented with 2% glucose were plated on GAM agar medium, and those plates were dried for 1 h at 37°C. Paper disks supplemented with each antibiotic at several concentrations were placed on the surface of each plate. The plates were incubated for 2 or 3 d at 37°C. The MIC was determined based on the appearance of a clear zone around each paper disk. The minimum concentration of each antibiotic inhibiting growth in this assay was compared with the concentration of antibiotic in milk replacer or starter used in the trial. The tentative criterion for judgment of antibiotics resistance was whether the MIC was higher than the concentration of antibiotics in the feed.

**Statistical Analysis**

Data for BW, BW gain, feed intake, feed conversion, and fecal score for trials 1 and 3 were analyzed by standard procedures of analysis of variance to ascertain whether significant differences in those measurements in each of the trials were caused by the probiotic treatments. When the difference was significant ($P < .05$), an unpaired $t$ test procedure was used to compare each mean of treatments. Also, data for trials 2 and 4 were analyzed by unpaired $t$ test procedures to determine whether differences existed between probiotic and control groups. In each experiment, a probability of $< .05$ was considered to be statistically significant.

**RESULTS**

**Trial 1**

The results of trial 1 are shown in Table 1. All calves tested were in good health during the experiments. Also, the mean initial BW was 47.5 for calves treated with *B. pseudolongum*, 46.3 for calves treated with *L. acidophilus*, and 46.4 for untreated control calves. No significant difference existed among groups. At the end of the test period, BW was different between the control group and the groups fed probiotics. The BW gain of calves in the
BIFIDOBACTERIA FOR CALVES AND PIGLETS

TABLE 1. Performance of calves fed probiotics.

<table>
<thead>
<tr>
<th>Item</th>
<th>Bifidobacterium pseudolongum</th>
<th>Lactobacillus acidophilus</th>
<th>Control</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calves, no.</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Initial BW, kg</td>
<td>47.5</td>
<td>46.3</td>
<td>46.4</td>
<td>.64</td>
</tr>
<tr>
<td>56-d BW, kg</td>
<td>79.3</td>
<td>77.2</td>
<td>71.8</td>
<td>1.31</td>
</tr>
<tr>
<td>BW Gain, kg</td>
<td>31.8*</td>
<td>30.9*</td>
<td>25.4</td>
<td>.97</td>
</tr>
<tr>
<td>Feed intake, kg of DM</td>
<td>65.5</td>
<td>63.5</td>
<td>58.6</td>
<td>1.34</td>
</tr>
<tr>
<td>Feed conversion, kg DM/kg</td>
<td>2.1*</td>
<td>2.07*</td>
<td>2.37</td>
<td>.044</td>
</tr>
<tr>
<td>Fecal score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7–21 d of age</td>
<td>.29</td>
<td>.27</td>
<td>.34</td>
<td>.039</td>
</tr>
<tr>
<td>21–35 d of age</td>
<td>.10</td>
<td>.04</td>
<td>.11</td>
<td>.040</td>
</tr>
<tr>
<td>7–35 d of age</td>
<td>.19</td>
<td>.16</td>
<td>.23</td>
<td>.034</td>
</tr>
</tbody>
</table>

1Feed intake was total DM of feed fed to calves, including milk replacer, starter, and grass.
2Kilograms of DM per kilogram of BW gain.
3Recorded daily the first 28 d of the experiment (0 = normal, 1 = soft, and 2 = scouring).
*Difference between probiotics and control (P < .05).

The mean BW gain of calves at 56 d of age was 31.8 kg for the group fed bifidobacteria, 30.9 kg for the group fed lactobacilli, and 25.4 kg for the control group. The BW gain was different (P < .05) between each treated group and the control group, but not between the groups fed bifidobacteria and lactobacilli. Feed intake of calves was calculated from the weight of the milk replacer, starter, and dried grass that was fed to the calves. The mean feed intake during the trial was 65.5 and 63.5 kg for the groups fed bifidobacteria and lactobacilli, respectively, which was higher than the 58.6 kg for the control group. Feed conversion, however, was 2.10 kg of DM/kg of BW gain for the group fed bifidobacteria, 2.07 kg/kg for the group fed lactobacilli, and 2.37 kg/kg for the control group; feed conversion of the groups fed bifidobacteria or lactobacilli was superior to that for the control group. No difference was significant between the groups fed probiotics. The feces of all calves were observed every day for the initial 28 d of the test. The fecal score for each group fed probiotics was lower than that of the control group and was lowest in the group fed lactobacilli in every period. Also, the fecal score was higher during the early period (7 to 21 d of age) than during the later period (14 to 28 d of age) in all experiments. However, fecal score was not different (P > .05) among the groups in this experiment, possibly because of the protection from pathogenic bacteria resulting from the effect of antibiotics in the milk replacer or starter. Therefore, we investigated the effect of probiotics on the growth and health condition of calves under feeding conditions without antibiotics.

Trial 2

The results of trial 2 are shown in Table 2. The BW gain and health of newborn Holstein calves in a group fed probiotics and a control group were compared under feeding conditions without antibiotics. Initial mean BW (control group, 37.2 kg; probiotic group, 40.0 kg) and health were examined, and no significant difference existed between groups. The mean

TABLE 2. Performance of calves fed probiotics without antibiotics (trial 2).

<table>
<thead>
<tr>
<th>Item</th>
<th>Probiotics</th>
<th>Control</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW, kg</td>
<td>40.0</td>
<td>37.2</td>
<td>1.13</td>
</tr>
<tr>
<td>56-d BW, kg</td>
<td>79.9</td>
<td>73.4</td>
<td>1.85</td>
</tr>
<tr>
<td>BW Gain, kg</td>
<td>40.5</td>
<td>36.2</td>
<td>1.39</td>
</tr>
<tr>
<td>Feed conversion, kg DM/kg</td>
<td>1.59</td>
<td>1.64</td>
<td>.028</td>
</tr>
<tr>
<td>Diarrhea, cases</td>
<td>1*</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

1Kilograms of DM per kilogram of BW gain.
2Recorded the first 28 d of the experiment.
*Difference between probiotics and control (P < .05).
BW gain of the group fed probiotics tended to be greater than that of the control group, as observed in trial 1; however, no significant difference existed between groups. The feces of all calves were observed every day for the initial 28 d. Seven of 9 control calves had diarrhea, but, in the group fed probiotics, only 1 of 10 calves had diarrhea. These results were in agreement with those of trial 1, indicating that the calves that were administered probiotics were more resistant to diarrhea than were the untreated calves.

**Trial 3**

The effect of probiotics was observed on growth and feed conversion of newborn piglets raised under commercial conditions and fed milk replacer containing colistin sulfate, tylosin phosphate, and morantel citrate. The mean initial BW of piglets were 1.46 kg for the group fed bifidobacteria, 1.59 kg for the group fed lactobacilli, and 1.60 kg for the control group; no differences among groups were significant. Figure 1 shows the mean BW gain of animals in each experiment (1 to 3) and the overall mean during the suckling (0 to 28 d), weaning (28 to 56 d), and total trial periods (0 to 56 d). Similar results were observed across replications. The BW gains of piglets in the groups fed bifidobacteria or lactobacilli were greater than that of the control group in all test periods. At the end of the feeding period, the difference in BW gain between each group fed probiotics and the control group was significant ($P < .005$). The administration of probiotics during the suckling period was more effective in promoting BW gain than administration during the weaning period. The effect of probiotics on feed conversion in the weaning period was also examined, and the results are shown in Figure 2. Results were similar for the three replications. Administration of *Bifidobacterium* was more effective (1.98 kg of DM/kg of BW gain) than no administration (2.16 kg/kg), and *L. acidophilus* had no effect on feed conversion (2.21 kg/kg). Feed conversion between the group fed bifidobacteria and the control group was different ($P < .05$).

**Trial 4**

The effect of administered probiotics on the growth and health of newborn piglets was...
investigated for conditions of feeding without antibiotics. Initial mean BW and health of piglets in the test and control groups were examined, and no significant differences were found. The mean BW gain of piglets in the group fed probiotics was greater than that of the control group, as observed in trial 3 (data not shown). Moreover, survival rate was significantly better for the group fed probiotics than for the control group (Figure 3). For the control group, death of some piglets occurred after about 14 d, and the survival rate was about 75% for the 28-d period. However, for the group fed probiotic, survival rate (95%) was much higher throughout the feeding period.

**Measurement of MIC**

The resistance of the probiotic strains to the antibiotics present in the milk replacer that was used in this study was measured by the paper disk method. The MIC of *B. pseudolongum* M-602 and *L. acidophilus* LAC-300 and the maximum concentration of antibiotics in the milk replacer are shown in Table 3. The tentative criterion for judgment of antibiotics resistance was whether the MIC was higher than the concentration of antibiotic in the feed. Both strains tested showed resistance against colistin sulfate, morantel citrate, and zinc bacitracin. Against tylosin phosphate, both strains had weak resistance. However, both strains tested were sensitive to chlortetracycline. The MIC of the other strains, *B. thermophilum* S-501 and *E. faecium* FA-5, to each antibiotic were similar to the MIC of *B. pseudolongum* M-602 and *L. acidophilus* LAC-300 (data not shown).

**DISCUSSION**

Antibiotics are commonly used in feed supplements for livestock. One of the main reasons for the use of antibiotics in feed is their protective effect against infections caused by pathogenic bacteria. Recently, probiotics for livestock have attracted the attention of many people. Use of probiotics is expected to improve BW gain, feed conversion, and health of livestock because probiotics promote the estab-
TABLE 3. The minimum inhibitory concentration (MIC) of antibiotics present in feed against \textit{Bifidobacterium pseudolongum} M-602 and \textit{Lactobacillus acidophilus} LAC-300.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration in feed (µg/g)</th>
<th>B. pseudolongum M-602 (µg/ml)</th>
<th>L. acidophilus LAC-300 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colistin sulfate</td>
<td>40</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Morantel citrate</td>
<td>30</td>
<td>&gt;38.4</td>
<td>&gt;38.4</td>
</tr>
<tr>
<td>Tylosin phosphate</td>
<td>44</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>Zinc bacitracin, units/g</td>
<td>4.2</td>
<td>6.3</td>
<td>50</td>
</tr>
<tr>
<td>Chlorotetracycline</td>
<td>44.4</td>
<td>1.6</td>
<td>6.3</td>
</tr>
</tbody>
</table>

1The MIC of several antibiotics against \textit{B. pseudolongum} and \textit{L. acidophilus} were assayed by the paper disk method.

lishment of a beneficial gut flora and inhibit the growth of pathogenic bacteria in the intestine. Many researchers have studied the use of lactobacilli and streptococci as probiotics (1), especially \textit{L. acidophilus} probiotics (15, 17, 20, 21, 22).

In this paper, the effects of administration of bifidobacteria or lactic acid bacteria to newborn livestock were investigated. \textit{Bifidobacterium pseudolongum} has been isolated from many kinds of animals, including calves, piglets, chickens, dogs, and others, suggesting that this bacterial species has a wide host specificity and might be commercially useful as a probiotic (13, 23). When administered to calves (trial 1), the effects of these bacteria on both BW gain and feed conversion (Table 1) were significant, indicating that the strains tested here are useful as probiotics. The beneficial effects are thought to result from improvement of intestinal conditions because the fecal score was lower for the group fed probiotics than for the control group. Without antibiotics, the effect of these probiotics on the incidence of diarrhea was especially remarkable (Table 2). Administration of probiotics was as effective as administration of antibiotics in protecting against diarrhea caused by pathogenic bacteria. Gilliland et al. (2) reported that administration of \textit{L. acidophilus} to calves caused a decrease of coliforms in the intestine and feces. Our results tend to be consistent with that observation.

When the probiotics were administered to piglets (trial 3), BW gain was increased significantly, and feed conversion was improved over that of the control group (Figures 1 and 2). The BW gain during the suckling period was higher than during the weaning period, suggesting that these probiotics should be administered as soon as possible after birth to be most effective. At birth, there are no bacteria in the intestine; therefore, administration of probiotics orally at this time is most effective to promote their colonization in the intestine. In our investigation, the improved BW gain was thought to result from favorable growth of useful bacteria that colonized the intestine more quickly than pathogenic bacteria. Without antibiotics, the survival rate of the group fed probiotics was improved greatly over that of the control group (Figure 3). This result shows that beneficial bacterial flora in the intestine provides protection to host animals from infection by pathogenic bacteria. One reason for these results could be that the bacteria that induce ill effects in the host were not able to colonized on the intestinal surface because administered bacteria were already colonized thickly on that surface. Also, Pollmann et al. (22) observed that the number of leukocytes in blood was increased by administration of \textit{L. acidophilus} to piglets. Namikawa et al. (16) and Yamazaki et al. (26) have reported that administration of \textit{Bifidobacterium} elevated immunity in animals. In our study, one more reason for the decreased incidence of diarrhea and death rate of piglets administered probiotics could be a stimulation of immunity in host animals.

In our study, \textit{B. pseudolongum} and \textit{L. acidophilus} were administered to livestock. The effects of these bacteria as probiotics were similar, except for feed conversion of piglets
acidophilus is unknown, because a specific interaction piglets have the same effect on calves or chick-
tures of several bacterial strains to livestock be effective in the intestine of each host spe-
cies. For this reason, administration of mix-
tures of several bacterial strains to livestock may be most useful as probiotics, because some of the strains in the mixture would likely be effective in the intestine of each host spe-
cies.

Conversely, administration of lactobacilli or their fermented products to calves was not effective to increase BW gain or improve fecal score. Schwab et al. (24) reported that administration of nonviable Lactobacillus bulgaricus fermented product had no apparent effect on calves; and Morrill et al. (14) reported that milk fermented by L. acidophilus and Lactobacillus lactis did not significantly affect BW gain or frequency of diarrhea in calves. However, in the trial with L. bulgaricus (24), livestock were not given viable cells, and L. bulgaricus was thought to be unable to colonize on the intestine because that species was usually not isolated from animal feces. Also, in the study by Morrill et al. (14), calves might not have received enough viable cells of L. acidophilus or L. lactis to demonstrate an effect because the viable cells counts in the fermented milk administered was not clear and L. lactis was usually not isolated from animal feces. Therefore, those results cannot be com-
pared with our results. For administration of probiotics to have an effect on BW gain or fecal score of livestock, probiotics should be selected for their abilities to colonize on intesti-
tine and to activate effectively the immunity of the host. No significant effect of probiotics on BW gain of calves was observed in our trial 2, but a tendency for BW gain was observed. We surmised that the probiotics were effective on the host animal but that the effects were slower and milder than antibiotics. Therefore, many experiments or many test animals may be needed to observe the effect clearly.

The sensitivities to antibiotics present in the milk replacer of the bacteria tested here were examined (Table 3); no significant difference existed between the strains employed. Both strains were almost resistant to all tested an-
tibiotics except chlortetracycline. Nonetheless, even when feeds contained chlortetracycline, the probiotics improved BW gain and feed conversion of livestock in this study. The probiotics administered orally were thought to have lived in the intestine because the intesti-
nal concentration of antibiotics taken with feed was much lower because of dilution by absorp-
tion and water and by diffusion.

In our study, B. pseudolongum was as effective in promoting the growth of livestock as L. acidophilus. We conclude that probiotics should be administered as soon as possible after birth. Many unknown factors are involved in bacterial colonization of the intestine, and further studies of these factors are required to support the commercial use of probiotics.

ACKNOWLEDGMENTS

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REFERENCES

3 Reference deleted in proof.
4 Reference deleted in proof.