Inability of Probiotic Bacterial Strains Lactobacillus rhamnosus HN001 and Bifidobacterium lactis HN019 To Induce Human Platelet Aggregation In Vitro

J. S. ZHOU,1* K. J. RUTHERFURD,1,2 AND H. S. GILL1,2†

1Institute of Food, Nutrition and Human Health (formerly Milk and Health Research Centre), Massey University, Palmerston North, New Zealand; and 2Fonterra Innovation (formerly New Zealand Dairy Research Institute), Palmerston North, New Zealand

ABSTRACT

Platelet aggregation contributes to the pathogenesis of infective endocarditis, and aggregation of platelets induced by lactobacilli is thought to be an important contributory factor in the development and progression of Lactobacillus endocarditis. The main purpose of this study was to examine the effect of immunity-enhancing probiotic strains Lactobacillus rhamnosus HN001 and Bifidobacterium lactis HN019 on the activation and aggregation of human blood platelets. Whole blood samples from healthy individuals were incubated in vitro with HN001 or HN019 and subsequently labeled with platelet-specific monoclonal antibodies, fluorescein isothiocyanate–conjugated anti-CD41a (expressed on normal platelets), and phycoerythrin–streptavidin–conjugated anti-CD62p (expressed on activated platelets) before analysis by flow cytometry. Platelet-rich plasma was used to assist the gating of the platelet cluster. ADP and epinephrine were used as the physiological platelet activation agonists. Platelet aggregation–inducing strain Streptococcus sanguis 133-79 was used as a positive control strain. The mean fluorescence intensity of phycoerythrin and the percentage of platelets expressing the CD62p marker were used to assess the degree of platelet activation. The percentage of CD62p-positive platelets and the light scatter profiles of the agonist-activated platelets were used to identify the occurrence and degree of platelet aggregation. HN001 and HN019 had no effect on spontaneous platelet activation and aggregation; they also failed to exacerbate the platelet aggregation activity induced by ADP and epinephrine. Therefore, these test probiotic strains HN001 and HN019 are less likely to participate in the pathogenesis of infective endocarditis or other thrombotic disorders with regard to platelet aggregation factors.

The interaction between platelets and bloodborne bacteria is likely involved both in the pathophysiological mechanisms of septicemia and disseminated intravascular coagulation and in bacterial infective endocarditis (IE). IE is one of the most frequently encountered problems associated with opportunistic infections caused by lactic acid bacteria, particularly lactobacilli (3, 8, 25). Platelet activation and aggregation are believed to contribute to the pathogenesis of IE, and aggregation of platelets by lactobacilli is thought to be an important contributory factor in the progression of Lactobacillus–associated endocarditis (2, 9, 13, 17).

Various authors have reported that platelets in plasma activate and aggregate in response to incubation with selected gram-positive bacteria, including lactic acid bacteria strains (15, 16, 21). For example, Herzberg and coworkers (17) reported that intravenous inoculation of New Zealand white rabbits with a bacterial strain capable of causing platelet aggregation in vitro (i.e., Agg+ phenotype, such as Streptococcus sanguis 133-79) consistently caused IE, with significantly more vegetation (the bacterial colonization of a platelet-fibrin clot on the endothelial surface of the heart), more gross lesions in major organs, and greater mortality than observed after inoculation with Agg− strains (i.e., strains incapable of inducing aggregation). Isolates of certain bacteria, including Lactobacillus rhamnosus, from patients with IE have been reported to uniformly induce irreversible platelet aggregation in vitro (13, 18).

Some lactobacilli can produce enzymes (e.g., glycosidases and proteases) that function in the breakdown of human glycoproteins and the synthesis and lysis of human fibrin clots. These processes could aid the colonization and survival of bacteria and the initiation of endocarditis vegetation (14, 24, 25). Most oral lactobacilli are commonly able to induce platelet aggregation in vitro and could thus potentially cause IE in vivo if they came into contact with platelets in blood (2, 13, 27).

One hypothesis is that lactic acid bacteria from the gastrointestinal tract or oral cavity can be introduced into the bloodstream as the result of poor dental hygiene, gastrointestinal lesions, or surgical procedures and thus may contribute to the pathogenesis of IE and other pathological processes associated with platelet aggregation (4). Thus, a lack
of platelet aggregation potential has been suggested as one of the important criteria for selection of bacterial strains for use as probiotics (7, 13, 19). Investigation of the ability of IE-associated enzymes to produce or elicit the aggregation of platelets has been recommended when the intrinsic properties of probiotic strains are being evaluated (12, 20, 23, 25). L. rhamnosus HN001 and Bifidobacterium lactis HN019 are two newly identified probiotic strains (26) with immune system-enhancing properties. For example, enhancement of the phagocytic activity of granulocyte and monocyte populations has been observed in the blood of human volunteers (5) and animals (10) following consumption of B. lactis HN019 or L. rhamnosus HN001. In mice fed a diet containing L. rhamnosus HN001 and B. lactis HN019, the number of B lymphocytes in the Peyer’s patches in the gut increased and the proliferative responses of spleen cells to mitogens was enhanced (10). In the current study, the ability of L. rhamnosus HN001 and B. lactis HN019 to induce or enhance human blood platelet activation and aggregation in vitro was evaluated.

MATERIALS AND METHODS

Preparation of cultures. Stock cultures of test strains L. rhamnosus HN001, B. lactis HN019, and a commercial probiotic strain L. rhamnosus GG (used as a reference strain) were obtained from Fonterra Innovation (formerly New Zealand Dairy Research Institute) Culture Collection (Palmerston North, New Zealand). These strains were propagated three times before being subjected to testing. Bacteria were grown in deMan Rogosa Sharpe (MRS; BBL, Becton Dickinson, Sparks, Md.) broth for 18 h (i.e., to late stationary phase) at 37°C in an anaerobic jar (BBL Gaspack System, Becton Dickinson) with Microbiology Anaerocult A (Merck, Darmstadt, Germany) and BBL Dry Anaerobic Indicator Strip (Becton Dickinson). For B. lactis HN019, MRS broth was supplemented with 0.005% (wt/vol) cysteine hydrochloride (Sigma Chemical Co., St. Louis, Mo.). Streptococcus sanguis 133-79 (Dr. Mark Herzberg, School of Dentistry, University of Minnesota, Minneapolis) is a prototype strain that induces platelet aggregation and was originally isolated from a patient with IE. S. sanguis 133-79 cultures were grown overnight at 37°C in 5% CO2 in Todd-Hewitt broth (Remel, Lenexa, Kans.). The cells were harvested by centrifugation (Megaufuge 1.0R, Heraeus Instruments, Germany; 3,000 × g at 4°C for 15 min) and washed three times in cold phosphate-buffered saline (PBS, pH 7.3). The cells were resuspended in PBS to an optical density of 0.5 ± 0.02 at 610 nm and then concentrated fourfold to give a suspension containing approximately 109 CFU/ml (12, 19).

Chemicals and monoclonal antibodies. ADP, epinephrine reagent (EPN), and prostaglandin E1 (PGE1) were purchased from Sigma. ADP and EPN were used as physiological agonists to induce platelet aggregation and PGE1 was used as an antagonist to block thrombin-induced platelet activation. Working solutions of ADP (0.2 mM/liter), EPN (0.1 mM/liter), and PGE1 (3 μM/liter) were prepared freshly before use.

Fluorescein isothiocyanate (FITC)–conjugated anti-CD41a (mouse IgG1, κ) and phycoerythrin (PE)–streptavidin–conjugated anti-CD62p (mouse IgG1, κ) monoclonal antibodies (MoAbs) were purchased from Pharmingen International (North Ryde, New South Wales, Australia). The anti-CD41a MoAb reacts with a calcium-dependent complex of CD41-CD61 (GPIIb-IIIa) expressed on normal platelets (both resting and activated) and megakaryocytes and therefore was used as a pan marker for platelets in this study. Anti-CD62p reacts with the 140-kDa membrane glycoprotein P-selectin (formerly known as platelet activation–dependent granule membrane protein or GMP-140), which is stored in the a-granules of platelets and the Weibel-Palade bodies of endothelial cells, and is rapidly transported to the plasma membrane upon activation; it was used as a marker for activated platelets in this study.

Tyrode’s buffer (11) (1% bovine serum albumin, 2 mM/liter MgCl2, 137.5 mM/liter NaCl, 12 mM/liter NaHCO3, and 2.6 mM/liter KCl; pH 7.4), ACK lysis buffer (10), and a fixative solution (2% paraformaldehyde in PBS, pH 7.3) were prepared and stored at 4°C.

Collection and preparation of blood samples. Blood samples were collected from six healthy human volunteers (24 to 45 years of age). Two samples from these donors and another blood sample from an additional donor were used in the validation experiment using S. sanguis 133-79. Written consent from these donors was obtained before blood collection. These individuals were nonsmokers, had consumed no alcohol within the previous 48 h, and had not taken any drug during the previous 2 weeks. On the day of blood collection, a light standard breakfast was allowed before blood was collected (with the aid of a light tourniquet) from a forearm vein through a 19-gauge needle. Twenty microliters of blood was collected into a heparin-coated Vacutainer tube. Platelet-rich plasma was prepared by centrifugation of the whole blood sample at room temperature (RT) at 300 × g for 10 min. Twenty-microliter samples of whole blood or platelet-rich plasma were immediately transferred into a set of 2-ml Eppendorf tubes containing Tyrode’s buffer and different reagents or bacterial cells. In these reaction systems, the ratio of bacterial cells to platelets was approximately 1:1. The samples were incubated at RT for 30 min, and then 100 μl of 2% formaldehyde (pH 7.3) was added to each tube to stop the reactions. After 5 min of incubation at RT, 1 ml of ACK lysis buffer was added into each tube (whole blood samples), which were incubated for another 10 min at RT to lyse the red blood cells. The fixed platelets were then harvested by centrifugation (MC 12V, DuPont Sorvall, Newtown, Conn.; 2,000 × g at RT for 10 min). Fifty microliters of Tyrode’s buffer and 5 μl each of FITC-CD41a and PE-CD62p MoAbs or corresponding isotype controls (mouse IgG1, κ) were added into each tube, which were then incubated at RT in the dark for 20 min. After washing in Tyrode’s buffer twice, the platelets were suspended in 200 μl of Tyrode’s buffer and subjected to flow cytometry analysis.

Flow cytometry analysis. Platelet samples treated as above were analyzed by flow cytometry on a FACS Calibur (Becton Dickinson) within 30 min after labeling with MoAbs. Light scatter and fluorescence data were obtained with gain settings (FITC-CD41a: 520 voltage; PE-CD62p: 566 voltage) in the logarithmic mode (22, 29). The platelets were primarily distinguished from red blood cells and leukocytes on the basis of their forward- and side-light scatter profiles and were further confirmed by running a platelet-rich plasma sample prepared following a standard method (6). Because of the much smaller scatter profiles, debris or “machine noise” was excluded from the platelet gate position. Ten thousand cells within the platelet gate were analyzed, and the cells expressing FITC-CD41a (normal pan platelet marker) were selected (or back gated) for further analysis. The expression of PE-CD62p (the marker specific for activated platelets) was displayed in a quadrant dot plot (PE-CD62p fluorescence intensity versus FITC-CD41a fluorescence intensity). The mean fluorescence intensity (MFI) and percentage of PE-CD62p and FITC-
TABLE 1. Platelet activation by probiotic strains

<table>
<thead>
<tr>
<th>Platelet treatment</th>
<th>% CD62p-positive platelets (mean ± SE)</th>
<th>MFI index (mean ± SE)</th>
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<tbody>
<tr>
<td>PGE&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5.185 ± 0.9616 c</td>
<td>101.50 ± 17.820 c</td>
</tr>
<tr>
<td>HN019</td>
<td>8.864 ± 0.9227 c</td>
<td>220.80 ± 35.580 c</td>
</tr>
<tr>
<td>HN001</td>
<td>4.658 ± 0.8046 c</td>
<td>107.70 ± 20.230 c</td>
</tr>
<tr>
<td>GG</td>
<td>5.820 ± 1.2516 c</td>
<td>145.50 ± 37.740 c</td>
</tr>
<tr>
<td>ADP + ENP</td>
<td>64.25 ± 4.2960 A</td>
<td>2,910.3 ± 372.23 A</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>83.7 ± 4.32</td>
<td>9,520 ± 4,198</td>
</tr>
<tr>
<td>HN019 + ADP + ENP</td>
<td>64.27 ± 11.060 A</td>
<td>2,596.3 ± 935.12 AB</td>
</tr>
<tr>
<td>HN001 + ADP + ENP</td>
<td>59.09 ± 10.440 AB</td>
<td>2,249.5 ± 748.64 AB</td>
</tr>
<tr>
<td>GG + ADP + ENP</td>
<td>50.38 ± 11.103 B</td>
<td>1,755.7 ± 593.73 B</td>
</tr>
</tbody>
</table>

*Fresh whole blood samples were incubated with test probiotic strains (HN001, HN019, or GG), the positive control strain (S. sanguis 133-79), or ADP, epinephrine (EPN), or prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) for 30 min and then stained with platelet-specific antibodies CD41a (for normal platelets) and CD62p (for activated platelets). Only CD41a-positive cells were gated and analyzed. The percentage of CD62p-positive cells was gated and analyzed. The percentage of CD62p-positive platelets and the mean fluorescence intensity (MFI) index (MFI multiplied by the percentage of CD62p-positive cells) were calculated. Values are means ± standard errors of a group of six samples (treated with probiotic strain and ADP, EPN, or PGE<sub>1</sub>) or three samples (treated with S. sanguis 133-79). Means followed by the same letter are not significantly different (P > 0.001).

CD41a double-positive cells were used to evaluate the degree of platelet activation or aggregation. The MFI index is the MFI multiplied by the percentage of CD62p-positive cells. The antibody-positive platelets were defined as those cells with fluorescence intensity higher than log 10, based on the fluorescence profiles of nonlabeled and isotype control antibody-labeled platelets in platelet-rich plasma and samples treated with fixative or PGE<sub>1</sub> before labeling with PE-CD62p and FITC-CD41a.

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**Platelet activation.** There was no significant difference in platelet activation between resting platelets and samples incubated with test probiotic strains. The platelets incubated with *L. rhamnosus* HN001, *B. lactis* HN019, or the reference strain *L. rhamnosus* GG exhibited CD62p expression percentage and MFI index similar to those of resting platelets (4.6 to 8.8% and 107 to 220, respectively). However, in platelets treated with ADP and EPN there was a much higher percentage of CD62p-positive platelets (64.25%) and dramatically increased MFI index (2,910.3 ± 372) compared with that of resting platelets (101 ± 17.8) or probiotic-treated platelets (107 to 220 ± 20 to 37) (Table 1 and Fig. 1). The activation of platelets induced by *S. sanguis* 133-79 is shown in Figure 2A and 2B. Similar to the effect of ADP and EPN, strain *S. sanguis* induced dramatic platelet activation under these experimental conditions. More than 91% of CD41a-positive cells were CD62p positive, and they had a very high MFI index (9,520 ± 4,198). Incorporation of probiotic strains *L. rhamnosus* HN001, *B. lactis* HN019, or *L. rhamnosus* GG did not further increase the expression of CD62p in samples treated with ADP and EPN (Fig. 1). Incubation with test probiotic strains actually caused a slight decrease in the percentage and MFI index of CD62p-positive platelets treated with agonists compared with the samples treated with ADP and EPN only, although the only significant difference was for samples treated with *L. rhamnosus* GG (Table 1).
FIGURE 2. S. sanguis–induced platelet activation and aggregation. Fresh blood samples were treated with S. sanguis 133-79 or 2% paraformaldehyde or ADP and epinephrine (EPN) as indicated in the footnote of Table 1. CD41a and CD62p double-positive cells were selected and analyzed. The fluorescence intensities (A and B) and light scatter profiles (C) of these cells from one representative sample of each treatment are displayed.

Platelet aggregation. Resting platelets and platelets treated with test probiotic strains had a much lower forward scatter height than did platelets treated with ADP and EPN or S. sanguis 133-79. On the light scatter versus forward height density plot, the cluster of platelets treated with ADP and EPN (data not shown) or with S. sanguis 133-79 (Fig. 2C) was shifted up and to the right, indicating an increase in the size of platelet particles (aggregates). Blood samples incubated with probiotic strains had light scatter profiles very similar to those of resting platelets. Incorporation of probiotics into the reaction systems of platelets treated with ADP and EPN did not change the light scatter profiles of the activated platelets. These results demonstrate that L. rhamnosus HN001, B. lactis HN019, and L. rhamnosus GG do not induce or exacerbate platelet aggregation.

DISCUSSION

L. rhamnosus HN001 and B. lactis HN019 were isolated from cheese and yogurt, respectively, and have been consumed by humans for centuries with few safety concerns. Our earlier studies (32) revealed that L. rhamnosus HN001 and B. lactis HN019 had no ability to degrade gastric mucin. Arunachalam and coworkers (5) found no obvious adverse effects on the health of elder individuals following 3 weeks of consumption of B. lactis HN019 in low-fat milk. In a more recent study, Tannock et al. (31) reported that consumption of L. rhamnosus HN001 for 6 months is safe for humans. These observations indicate that the probiotic strains tested in this study are unlikely to be pathogenic.

However, bacteria from the gut or oral cavity can be passively introduced into the blood stream by surgical or other invasive procedures; hence, these nonpathogenic bacteria still may have the potential to cause infections or participate in the pathogenesis of some diseases, including IE. Platelet aggregation is the key initiating event in the formation of a thrombus and makes an important contribution to the progression of IE. In light of these concerns, a bacterial strain with platelet-aggregating properties may be more virulent than nonaggregating strains. Thus, strains that do not aggregate platelets are likely to be safer than aggregating strains for human consumption.

L. rhamnosus GG was included as a reference strain because there are more safety data for this strain than for any other commercial probiotic strain (27, 28) and because it is unable to induce platelet aggregation in vitro (20). To
assess the normal functions of tested platelets and observe any potentially exacerabatory effect of test probiotic strains on platelet aggregation under physiological conditions, ADP and EPN were used as physiological triggers of platelet aggregation. Both PGE$_1$ (an antagonist) and fixatives were used to achieve minimum activation of platelets that were used as negative controls. An approximately 1:1 ratio of platelets to bacterial cells, which was reported as the optimum ratio for inducing platelet aggregation, was used in this study (13, 18, 21). S. sanguis 133-79, the strain that induces platelet aggregation (16), was included as a positive control to validate the experimental conditions and induced dramatic platelet activation and aggregation. Flow cytometry using platelet-specific MoAbs is the most sensitive and specific technique for studying platelet function (1, 6) because of the advantages of using whole blood samples instead of platelet-rich plasma and avoiding the processes required in conventional platelet aggregation methods to prepare samples (which may increase the chance of artificially inducing platelet activation). Because interindividual variation in the degree of augmentation of platelet aggregation has been reported (13, 20), six whole blood samples were included in this study to reduce the artificial effects caused by individual variation. In this study, no obvious interindividual variation in the degree of augmentation of platelet activation and aggregation was observed; these differences in findings may be related to the differences in experimental methods used in the current and previous studies.

Under these experimental conditions, S. sanguis 133-79 induced extensive platelet activation and aggregation (Fig. 2) in all tested blood samples, but the test probiotic strains L. rhamnosus HN001, B. lactis HN019, and L. rhamnosus GG failed to induce or enhance any spontaneous (or agonist-induced) platelet activation or aggregation. Because L. rhamnosus HN001 and L. rhamnosus GG belong to the same species, they have some characteristics in common. According to Gasser (9) and Adams et al. (3), the overwhelming majority of reported endocarditis cases caused by nonenterococcal lactic acid bacteria have been caused by Lactobacillus species, particularly L. rhamnosus and Lactobacillus casei or Lactobacillus paracasei. However, the results obtained in this study demonstrated that L. rhamnosus HN001 is unlikely to be involved in the pathogenesis of IE because of its inability to induce platelet aggregation. In a total of 53 cases of endocarditis reported over 55 years (1938 to 1993), no cases were associated with Bifidobacterium spp. (9). Therefore, there are no reports documenting the platelet aggregation properties of bifidobacteria.

According to Kirjavainen et al. (19), the incidence of bacteremia caused by lactic acid bacteria is quite low (0.24%, 8 of 3,317 blood culture isolates), and in most cases patients with cultures positive for lactic acid bacteria had a severe underlying disease that predisposed them to bacteremic complications. Lactobacillus only rarely causes endocarditis (30), with a reported rate of less than one case per year (9). Nevertheless, the ability to aggregate human platelets is a common attribute of the lactobacilli in the oral cavity of normal humans (13). Therefore, strains of lactic acid bacteria with no platelet-aggregating activity might be limited in their ability to participate in the pathogenesis of IE.

The probiotic strains L. rhamnosus HN001 and B. lactis HN019 are unable to induce spontaneous platelet activation and do not enhance agonist-induced platelet aggregation processes. Therefore, they are safe probiotic strains in terms of their potential to participate in the pathogenesis of IE or other thrombotic disorders.

ACKNOWLEDGMENTS

We thank Drs. Howard Katz and Frank Cross for their valuable scientific and editorial reviews, Dr. Mark Herzberg for his generous gift of S. sanguis 133-79, Fonterra Brands (formerly New Zealand Milk) for financial assistance, and all blood donors for their generous support.

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