

The Effect of Probiotic Lactic Acid Bacteria (LAB) Strains on the Platelet Activation: A Flow Cytometry-Based Study

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Abstract

Platelet-activation and agonist-induced platelet aggregation process to the pathogenesis of Infective Endocarditis (IE), bacteremia symptoms or other thrombotic complications and cardiovascular diseases. Activation of platelets by probiotic lactic acid bacteria strains is considered as thrombotic initiative factor contributing to the development and progression of *Lactobacillus endocarditis*. The main purpose of the current study was to evaluate the immunologic enhancement effect of probiotic strains *L. plantarum*, *L. acidophilus* and *L. rhamnosus* on the activation of blood platelets. Whole fresh blood flow cytometry was used to measure p-selectin expression and fibrinogen binding at basal levels and following stimulation with platelet agonists and probiotic lactic acid bacteria strains. Platelet activation was determined by labelling with FITC-conjugated anti-human fibrinogen and phycoerythrin (PE)-conjugated anti-human CD62p before analysis by flow cytometry. Thrombin Receptor Activator Peptide-6 (TRAP-6) was used as positive control. The percentage of CD62p-positive platelets, FITC-conjugated and the light scatter profiles of the agonist-activated platelets were used to identify the occurrence and degree of platelet activation. Probiotic lactic acid bacteria strains included in this study did not show any effect on spontaneous activation of human blood platelets. These test strains also failed to exacerbate or diminish the platelet activation property when co-incubated with TRAP-6 platelet agonist. Hence, this is the first *in vitro* report showing the safety of a group of probiotic lactic acid bacteria in terms of their potential to contribute to the pathogenesis of infective endocarditis (IE), bacteremia symptoms or other thrombotic disorders and correlated cardiovascular complications by initiating the platelet activation.

Keywords: Probiotic; Lactic acid bacteria; Platelet activation; Vein thrombosis

Introduction

Platelets have a crucial role in hemostasis, and an important role on immune system and host defense in human. Whereas the role of activation of platelet is crucial on innate immunity, excessive activation of platelets may lead to organ malfunction, and increasing risk of cardiovascular diseases e.g., Infective Endocarditis (IE) [1]. A wide range of microorganism has been investigated for their effect on aggregation [2] of blood platelets, including probiotic lactic acid bacteria such as *Lactobacillus* [3,4], *Escherichia coli* [5], *Streptococci* [6,7], *Enterococci* [8], *Staphylococci* [9], *Listeria monocytogenes* [10], *Fusobacterium necrophorum* [11,12], *Yersinia pseudotuberculosis* [13], *Aspergillus fumigates* [14] and *Candida albicans* [15]. Previous scientific works have shown an increased incidence of cardiovascular disorders [16] in patients with a history of Gram-positive bacterial infection [17,18]. Gram-positive microbiota located in the gastrointestinal tract may leak into the blood circulation due to gastrointestinal injuries, surgery or poor dental hygiene, and then contribute to promote septicemia via aggregation of platelets or contributing in the platelet-fibrin clot formation on the endothelial surface. *Lactobacilli* [19] are an example of Gram-positive bacteria that are found in the intestines and in the genital tracts [3].

Probiotic bacteria, are defined as 'live micro-organisms, which when consumed in adequate amounts confer a health benefit on the host

[20,21]. Lactic acid bacteria (LAB) [22] have been used as probiotics for several conditions including renal insufficiency, management of metabolic imbalance, in cancer treatment, and for preventive reasons. Numerous [23] *in vivo* and *in vitro* studies showed beneficial results of some lactic acid bacteria strains in the treatment and prevention of gastrointestinal disorders, cardiovascular disorders (coronary heart diseases, hypertension, hypercholesterolemia) and urogenital infections [23-26].

The physiological ability of platelets to bind fibrinogen can result in unintended aggregation of platelets, and thus to serious cardiovascular complications as a result of vascular thrombosis or infective endocarditis.

Nowadays, lactic acid bacteria, being considered as friendly bacteria, are widely promoted and even used in functional foods and pharmaceutical industry. What still is lacking is evidence that these strains do not induce unintended platelet aggregation, and thus increase the risk of venous and arterial thrombosis. Therefore, the aim of this study was to assess the platelet activation of *Lactobacillus* strains.

Materials and Methods

We used whole blood samples collected from healthy non-smoking blood donors, aged 25 to 40 years to reduce individual variation in the degree of augmentation of platelet aggregation [27].

Preparation of bacterial cultures

Stock cultures of test strains *Lactobacillus acidophilus* (NCCB 47025), *Lactobacillus plantarum* subsp. *plantarum* (NCCB 46042) and *Lactobacillus rhamnosus* (NCCB 98073) were obtained from culture collection of Westerdijk Fungal Biodiversity Institute (formerly known as The Fungal Biodiversity Centre), that is an institute of the Royal Netherlands Academy of Arts and Sciences (KNAW) and situated in Utrecht, the Netherlands. These strains were cultivated three times before being subjected to experiment. For *in vitro* assays *Lactobacillus* strains were cultured in MRS (de ManRogosa Sharpe) broth for 18 hrs (i.e., to late stationary phase) at 37°C, then harvested by centrifugation (Eppendorf Centrifuge 5810R; 3,000Xg at 4°C for 15 min), and washed three times using cold PBS buffer (phosphate-buffered saline), pH-7.3. The probiotic bacteria cells were diluted in PBS buffer to an optical density (OD) of 0.5 ± 0.01 at 610 nm and then concentrated to give a suspension of 10^9 CFU/ml approximately [24].

Chemical and antibodies

Thrombin Receptor Activator Peptide-6 (TRAP-6) was used as physiological agonist to induce platelet activation. A working solution of TRAP-6 (50 μ M/liter) in HBS buffer was prepared freshly before use in the Hematology laboratory at University Medical Center, Utrecht, the Netherlands. Phycoerythrin (PE)-conjugated anti-human CD62p (mouse IgG1, k) monoclonal antibody (MoAb) (clone AK-4) was purchased from BD Pharmingen™ (555524, the Netherlands). This MoAb interacts with P-selectin, the 140-kDa membrane glycoprotein, also known as platelet activation-dependent GMP-140 (granule membrane protein), which is stored in the α -granules of platelets and the Weibel-Palade bodies of endothelial cells, and is rapidly transported to the plasma membrane upon activation. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human fibrinogen antibody was obtained from Agilent Technologies (F011102-2, the Netherlands). This antibody reacts both with natural fibrinogen and the fragments D and E and accordingly was used as a marker for platelet fibrinogen binding in this study. Both antibodies were used as a biomarker for activated platelets in this experiment. HBS buffer [25], Hepes 10 μ M/liter, 1 mM/liter $MgSO_4$, 150 mM/liter NaCl, and 5 mM/liter KCl; pH 7.4, and a fixative buffer containing 0.2% formaldehyde, 154 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2PO_4 , 1.15 mM KH_2PO_4 , 10.2 mM $NaHPO_4$, and 4 mM EDTA; pH 7.4, were prepared freshly and stored at 4°C.

Collection and preparation of blood samples

Blood samples from six healthy human volunteers were collected into 0.105 M tri-sodium citrate tubes (367714, BD Vacutainer). The blood donors were aged between 25 to 40 years, non-smokers, had consumed no alcohol within the previous two days, and had not taken any medicine during the previous 14 days. On the day of blood sampling, a light standard breakfast was permitted before blood collection.

Five-microliters samples of whole blood were immediately transferred into a set of 2 ml Eppendorf tubes containing 25 microliters HBS buffer or agonist (TRAP-6), bacteria or a combination of agonist and bacteria. Subsequently 25 microliters antibody mix (FITC-anti-fibrinogen and PE-anti-CD62P) was added into each test tube. In these experiments, the ratio of bacteria to blood cells was approximately 1:1. The samples were incubated at 37°C for 10 min, and then 500

microliters of fixative solution was added to each test tube to stop the reactions.

Flow cytometry analysis

Blood samples treated as above were diluted twice and analyzed on a FACSVerse flow cytometer (BD-biosciences, San Jose, CA, USA) on the same day of processing. Forward angle light scatter (FSC), Side Scatter (SSC) and fluorescence data were obtained with gain settings in the logarithmic mode [26].

Single platelets were gated on the basis of their forward-and side-light scatter profiles in logarithmic mode excluding debris and machine noise from the platelet gate position. Within the platelet gate ten thousands cells were analyzed for binding of fibrinogen and expression of CD62P. The mean fluorescence (MFL) and the percentage of anti-fibrinogen-FITC and CD62p-PE positive cells were utilized to assess the level of platelet activation. The antibody positive platelets were specified as those platelet cells with higher than \log^{10} fluorescence intensity, based on the fluorescence figure of non-labeled platelets with CD62p-PE and anti-fibrinogen-FITC (Figure 1).

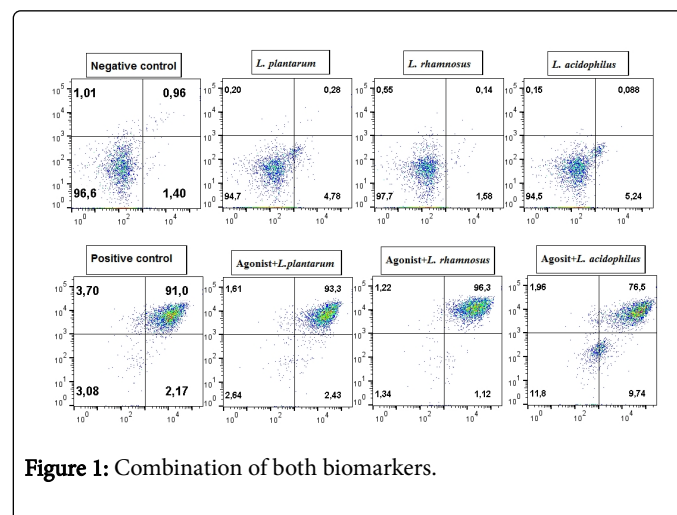


Figure 1: Combination of both biomarkers.

Hematology

Total red blood cell and platelet counts, hemoglobin concentration, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were determined on a CELL-DYN Sapphire (Abbott USA) in the clinical biochemistry laboratory at University Medical Center, Utrecht, the Netherlands.

Statistical analysis

All values are presented as a mean standard error (SE). Where applicable, a 2 tailed Paired Samples t-test was used to compare continuous variable within groups (SPSS software; IBM SPSS Statistics 21). P values < 0.004 was considered significant. AP value of 0.004 (0.05/13) was used as cut-point to correct for multiple testing.

Results

All hematology values for blood samples were in the normal range.

Platelet activation

There was no significant difference in platelet activation between resting platelets and samples incubated with test probiotic strains, $P > 0.004$. The platelets incubated with *L. acidophilus* showed CD62p and FITC expression percentages similar to resting platelets (6.5 and 5.7 vs. 4.8 and 6.0%, respectively). *L. rhamnosus* and *L. plantarum*

exhibited CD62p and FITC expression percentages were twice as high than in resting platelets (4.8 and 6.0% respectively). The CD62p and FITC expression percentage for *L. rhamnosus* was 8.4% and 17.1%, respectively and for *L. plantarum* reached 9.1% and 19.8%, respectively. See also Table 1.

| Platelet Treatment | % CD62p-Positive (means \pm SE) | MFL CD62p (means \pm SE) | % FITC-Positive (means \pm SE) | MFL FITC (means \pm SE) |
|--------------------------------|-----------------------------------|----------------------------|----------------------------------|---------------------------|
| Negative Ctrl | 4.8 \pm 0.8 | 758 \pm 547 | 6.0 \pm 2 | 669 \pm 51 |
| Positive Ctrl | 94.4 \pm 1.4 | 9990 \pm 1014 | 89.5 \pm 1.6 | 21191 \pm 6122 |
| <i>L. plantarum</i> | 9.1 \pm 3.4 | 656 \pm 248 | 19.8 \pm 8.7 | 2017 \pm 721 |
| <i>L. rhamnosus</i> | 8.4 \pm 2.0 | 716 \pm 359 | 17.1 \pm 1 | 2150 \pm 901 |
| <i>L. acidophilus</i> | 6.5 \pm 2.7 | 315 \pm 121 | 5.7 \pm 2.6 | 1414 \pm 554 |
| Agonist+ <i>L. plantarum</i> | 91.7 \pm 2.0 | 9533 \pm 721 | 88.6 \pm 2.1 | 26448 \pm 6064 |
| Agonist+ <i>L. rhamnosus</i> | 94.2 \pm 0.8 | 10059 \pm 738 | 89.0 \pm 3.3 | 30607 \pm 5662 |
| Agonist+ <i>L. acidophilus</i> | 92.0 \pm 0.8 | 8538 \pm 1237 | 64.7 \pm 22.5 | 18080 \pm 10074 |

Table 1: Platelet activation by LAB Probiotic strains.

In contrast, the activation of platelets induced by TRAP-6 dramatically increased, a significant difference in activation of platelets compared to resting platelets (4.8%), $P < 0.004$, and the percentage of CD62p-positive platelets reached up to 94.4%. In FITC-positive platelets activation was 89.5% compared to resting platelets (6.0%, $P < 0.004$). Platelets incubated only with probiotic strains showed similar biomarker expression percentage of both CD62p and FITC to resting platelets, however, platelets treated with TRAP-6 or incorporation of TRAP-6 and bacterial strains demonstrated similar levels of CD62p-positives and FITC-positives.

MFL for the platelets incubated with *L. acidophilus* was less than half of resting platelets (315 to 758 respectively), however, *L. rhamnosus* and *L. plantarum* presented MFL (716 and 656 respectively) similar to those of resting platelets just 758. In platelets treated with TRAP-6, there was significantly higher MFL (9990 \pm 1014) in comparison with those of resting platelets (758 \pm 547) or probiotic-treated platelets *L. plantarum* (656 \pm 248), *L. rhamnosus* (716 \pm 359) and *L. acidophilus* (315 \pm 121), however, similar to platelets incubated with contribution of TRAP-6 and probiotic strains *L. plantarum* (9533 \pm 721), *L. rhamnosus* (10059 \pm 738) and *L. acidophilus* (8538 \pm 1237) (Table 1).

Platelet activation induced by TRAP-6 showed a significant difference, $P < 0.004$, of biomarker expression percentage of CD62p and FITC in comparison with resting platelets; 91% vs. 0.96%, respectively, upper right quadrant Figure 1. In platelets incubated with probiotic strains, there was an activation pattern of biomarker expression similar to resting platelets, in contrast, platelets incubated with both TRAP-6 and probiotic lactic acid bacteria strains exhibited similar biomarker expression of those platelets that incubated with TRAP-6.

Discussion

In the present study, there was no obvious inter-individual variation in the degree of augmentation of platelet function which is in contrast

to previous studies. In our lab, the test probiotic strains *L. plantarum*, *L. rhamnosus* and *L. acidophilus* failed to induce or enhance significantly any spontaneous (or agonist-induced) platelet activation.

Our results are in line with that of Shried et al. [28] and Kirjavainen et al. [24] who reported lactic acid bacteria rarely induce bacteremia, and most likely patients with cultures positive for LAB had severe underlying diseases that predisposed them to bacteremic complications. Our results, however, are in contrast to the PROPATRIA study from Besselink et al. [29] in which patients with severe pancreatitis received a combination of *L. acidophilus* with other lactobacilli and *bifidobacteria* in a randomized controlled trial that needed to be stopped early because of increased mortality in the probiotic-treated group compared to the placebo group. In line with our findings, Kailasapathy and Chin [30] reported no obvious adverse effects on human health following consumption of *L. acidophilus*.

Naruszewicz et al. [31] reported that *L. plantarum* administration led to a reduction in cardiovascular disease risk factors (e.g., lowering levels of blood lipids and blood pressure in those with hypertension) and could be useful as a protective agent in the primary prevention of atherosclerosis. More recently, Linares et al. [32] have reviewed the potential health-promoting effect of LAB on human and indicated that the probiotic lactic acid bacteria strains are unlikely to be pathogenic. In our study probiotic lactic acid bacteria strains did not induce platelet activation, and this is an important finding because platelet activation may be in the causal pathway of development of a thrombotic event. It is important to realize that there are also non-entococcal lactic acid bacteria species and these *Lactobacillus* species, in particular *L. rhamnosus*, *L. caseior* and *L. Paracasei* have been related to inducing endocarditis [4,20,33,34].

In spite of the fact that *Lactobacilli* rarely have been reported to initiate causes of infective endocarditis (IE). Nevertheless [35] the capacity of a few lactic acid bacteria strains to aggregate human platelets is a common contribution of the *Lactobacilli* in the oral cavity

of normal human [4]. Hence, probiotic lactic acid bacteria strains with no platelet-aggregating activity are most likely unable to participate in the pathogenesis of IE. Our results are in line with previous reports by Korpela et al. [36] and Zhou et al. [26] which indicated *L. rhamnosus* GG and *L. rhamnosus* HN001 less likely participate in the pathogenesis with regards to platelet aggregation factors.

Strengths and limitations

Flow cytometry using platelet-specific Abs is the most sensitive and specific technique for studying platelet function [25] because of the advantages of using whole blood samples instead of platelet-rich plasma (P-RP) and avoiding the processes required in conventional platelet aggregation methods to prepare samples (which may increase the chance of artificially inducing platelet activation).

Conclusion

Probiotic strains *L. rhamnosus*, *L. plantarum* and *L. acidophilus* do not initiate spontaneous platelet-activation, and thus very unlikely could contribute to thrombotic disorders by the mechanism of platelet activation.

Ethics Statement

Informed consent was obtained from all subjects, in accordance with the Declaration of Helsinki. Approval from the medical ethics committee of the University Medical Center Utrecht was attained (METC-protocol 07-125/C approved on 1 March 2010).

Conflict Interests

The authors report no conflict of interest.

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Authorship

Contribution: Khalil Azizpour and Kok van Kessel performed the lab experiments and wrote the paper; Khalil Azizpour, Kok van Kessel and Ruud Oudega designed the research, all authors including Frans Rutten wrote and edit the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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