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Adherence properties of lactic acid bacteria as probiotic candidates isolated from breast milk

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Abstract

The ability of bacteria to adhere to intestinal cells has been considered as one of selection criteria for probiotic strains. The aim of this study was to evaluate the adherence properties of nine lactobacilli isolated from breast milk. Evaluation included hydrophobicity, autoaggregation, adhesion to the surface of rat intestine and evaluation of adhesion competition between lactobacilli and Enteropathogenic *E. coli* (EPEC). All lactobacilli tested show hydrophilic properties and low autoaggregation ability. Exposure of rat intestine to suspension of lactobacilli raised the total number of lactic acid bacteria indicating the attachment of lactobacilli isolates on the surface of rat intestine while reducing the total number of indigenous *E. coli*. This result indicates that the lactobacilli isolates were able to displace indigenous *E. coli* on the surface of rat intestine. The most adhesive lactobacilli were *Lactobacillus* A27 followed by *Lactobacillus rhamnosus* B16, R14, and R23. The ability of lactobacilli to compete for adhesion on the surface of rat intestine was strain dependent. The lactobacilli were able to compete with EPEC for adhesion at the dose of lactobacilli higher than EPEC, i.e. 10^8 cfu/ml vs 10^6 cfu/ml. The higher the number of lactobacilli exposed, the higher the number of adhering cells. The exclusion and displacement test using R23 and B16 showed that the lactobacilli tested were likely able to attach well on the surface of rat intestine, however, they were unable to displace the attached EPEC.

Keywords: probiotic, hydrophobicity, autoaggregation, LAB, competition, Indonesia.

Introduction

Probiotic is defined as life microorganism when ingested in sufficient amount will confer beneficial effect to the host [1]. Several criteria have to be met for selecting probiotic strains. These include acid and bile tolerance, survival through the gastrointestinal tract, ability to adhere to intestinal *As.*

surfaces, exhibiting antimicrobial activity against potential pathogenic bacteria and good technological properties [2]. Other functional properties of probiotics include hypocholesterolemic activity by lowering plasma cholesterol [3], preventing and treatment of diarrhoea [4, 5] and altering immune system [6, 7, 8, 9].

Adherence bacteria to mucosal surfaces are prerequisite for transient colonization and one of the probiotic criteria able to control the balance of intestinal microbiota [10]. Adhesion ability in lactobacilli is species-specific or strain dependent [11, 12] and there is positive correlation between adhesion and competitive exclusion ability of lactobacilli [12]. Lactobacilli were able to compete with, exclude and displace pathogenic bacteria when there were incubated together [11].

The microbial adhesion process of lactic acid bacteria includes passive forces, electrostatic interactions, hydrophobic, steric forces, lipoteichoic acids and specific structures [13]. The ability to adhere to epithelial cell has been shown to correlate with hydrophobicity [14]. The major mechanisms for the adhesion of bacteria to gastrointestinal surfaces have been suggested as involving specific adhesin–receptor interactions and nonspecific hydrophobic group interactions [15]. The ability of bacteria to survive and persist in the gastrointestinal track correlates with their ability to aggregate [16, 17]. The cell surface physicochemical properties of lactobacilli are particular to the bacterial species, hence adherence properties are strain dependent [18].

Human breast milk has been reported as a source of lactic acid bacteria with potential as probiotics. *B. longum* was the most widely found in breast milk, followed by B. *animalis, B. bifidum, B. catenulatum* [19]. Occurrence of lactobacilli in human breast milk was also reported such as *L. gasseri, L. fermentum, L. salivarius* [20]. They showed that the lactobacilli isolates had potency as probiotics, at least similar to that of the strains commonly used in commercial probiotic products. Study done in our laboratory showed that most of lactobacilli and bifidobacteria isolated from 28 lactating mothers in Bogor area showed good survival in pH2 and bile salt, and showed antimicrobial activities against pathogenic bacteria [21]. To further explore the potency of these lactic acid bacteria as probiotic candidates, the adherence properties including hydrophobicity, autoagregation, adhesion and ability to compete for adhesion with pathogenic bacteria, exclusion and displacement were evaluated using intestinal surface of rats as a model.

Materials and Methods

Bacterial culture

Nine lactobacilli i.e. *L. rhamnosus* A15, *Lactobacillus sp.* A27, *L. rhamnosus* A29, *L. rhamnosus* B10, *L. rhamnosus* B13, *L. rhamnosus* B16, *L. rhamnosus* R14, *L. rhamnosus* R23 and *L. rhamnosus* R26, previously isolated from breast milk were studied. Enteropathogenic *Escherichia coli* (EPEC) K1.1 used for competition study for adhesion was obtained from Biotechnology Inter University Centre, Bogor Agricultural University. All cultures were maintained in stock culture kept in 20% glycerol at -20^{o}C. When required the lactobacilli were grown in MRS broth while EPEC in NB at 37^{o}C.

Cell surface hydrophobicity [modification of 22]

Hydrophobicity of cell surface was assessed based on MATS (*Microbial Adhesion to Solvent*). Lactobacilli were harvested after growth for 16-18 h at 37^{o}C by centrifugation for 15 min at 5000 rpm, then washed twice in PUM buffer (composition (g/l): $K_2HPO_4.3H_2O$: 22.2, KH_2PO_4: 7.26, urea: 1.8, $MgSO_4.7H_2O$: 0.2, pH 7.1) and finally suspended in the same buffer at the level of 10^8 cfu/ml. The absorbance of the suspension was measured at 600 nm (A). Five millilitres of cell suspension in PUM buffer was taken into clean and dry round bottom test tubes. Then, 1 ml of

different hydrocarbon (xylene, ethyl acetate and chloroform) was added and mixed by vortexing at high speed for 1 min. The tubes were left undisturbed for 1 h at 37^{o}C to allow the phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and final absorbance (A_0) was recorded at 600 nm. Cell surface hydrophobicity (H%), calculated using following equation:

$$H\% = (A-A_0)/A \times 100.$$

Autoaggregation properties *[23]*
The culture was grown in MRSB for 18 h at 37^{o}C. The pellets were washed twice in phosphate-buffered saline (PBS) and re-suspended in similar solution to reach number of cells of 10^8 cfu/ml. Autoaggregation was determined by measuring absorbency at 0 h (A_0) and after 5 h (A_t) incubation at room temperature. Measurement of absorbance was done by taking 0.1 ml of upper aqueous phase and diluted with 3.9 ml PBS. The absorbance was measured at 600 nm. Percentage of autoaggregation was calculated using the following formula:

$$\text{Autoagregation } (\%) = 1- (A_t/A_0) \times 100.$$

Adhesion of lactobacilli isolates to the surface of rats intestine
Rat intestine was taken from 8 week old rats *(Sprague Dawley*). The intestine was cut for about 10 cm long, opened and washed three times with PBS. To evaluate the adhesion, the lactobacilli isolates were grown for 24 h in MRSB, then centrifuged at 5000 rpm for 15 min. The cell pellet was re-suspended in PBS to reach the number of LAB of 10^6 cfu/ml. A piece of rat intestine was placed in Petri dish and 10 ml of LAB cell suspension was added. The suspension was then incubated at room temperature for 60 min. At the end of incubation period, the rat intestine was washed twice with PBS. To calculate the number of lactic acid bacteria (LAB) and *E. coli* on the surface of rat intestine, 1 cm^2 of rat intestine surface was swabbed using sterile cotton bud and then the cells were released in 0.85% NaCl and subsequent dilution was done. Enumeration of LAB was performed in MRSA while *E. coli* in EMBA. The evaluation was done for 3 replicates. A set of experiments using PBS as exposure media was used as control. The number of total LAB and number of *E. coli* after incubation were enumerated using swab methods in a similar way as above. The increase in total LAB indicated the number of attached lactobacilli, while the decrease in *E. coli* indicates the number of displaced indigenous *E. coli*. The figures were calculated as the difference between LAB or *E. coli* count of control and the count after exposure of rat intestine to the lactobacilli cell suspension.

Competition for adhesion between lactobacilli isolates and EPEC K1.1
The experiment was done in three sets of intestine. The first set was exposed to Lactobacilli cells suspension as control lactobacilli, the second set was exposed to EPEC as control EPEC and the third set was exposed to a mixture of Lactobacilli and EPEC. Each set of experiments used three pieces of rat intestine. The number of bacterial cells in exposure media was 10^6 cfu/ml. Incubation was done for 60 min at room temperature. Following incubation, the numbers of LAB and/or *E. coli* were enumerated as described above. To evaluate the effect of dose on the ability to adhere, the rat intestine was exposed to the higher number of lactobacilli (10^8 cfu/ml) and compared to the lower number of lactobacilli (10^6 cfu/ml). Number of cells attached was calculated as the change of number of respective bacteria after exposure compared to its control.

Exclusion and displacement
For evaluation of exclusion, the lactobacilli were allowed to adhere to the surface of intestine first and then EPEC was added subsequently. Meanwhile for displacement study, EPEC was allowed to

adhere first and then lactobacilli was added. Four sets of rat intestine were used for this experiment. The first set was exposed to lactobacilli as control LAB. The second set was exposed to EPEC as control EPEC. The third set was exposed to lactobacilli and subsequently to EPEC. The fourth set was exposed to EPEC and subsequently to lactobacilli. The exposure dose of lactobacilli was 10^8 cfu/ml, while that of EPEC was 10^6 cfu/ml. Incubation for each exposure was 60 min. For exclusion and displacement study, prior to exposure to the second bacteria, the intestine was washed twice with PBS to wash un-attached the first bacterial cells from the surface. Enumeration of LAB and/or *E. coli* was done similarly with the adhesion study described above. Number of cells excluded or displaced was calculated by deducting the number of cells in its control by the number of the cells after exclusion or displacement.

Results and Discussion

Hydrophobicity of lactobacilli isolated from breast milk

Three different solvents were used to evaluate hydrophobic/hydrophilic cell surface properties and acidic-basic character. The results (Figure 1) revealed that most isolates showed negative affinity to xylene. Low affinity to xylene indicates hydrophilic properties of cell surface. While *L. rhamnosus* A15 and R23 indicated being slightly hydrophobic as they have positive affinity to xylene i.e., 15.24% and 9.43% respectively. The present finding is in accordance with a previous study [18] which showed that of 8 *Lactobacillus* (*L. casei, L. paracasei,* and *L. rhamnosus*), all are hydrophilic as shown by low affinity (2.7 – 26.5%) on non-polar solvent.

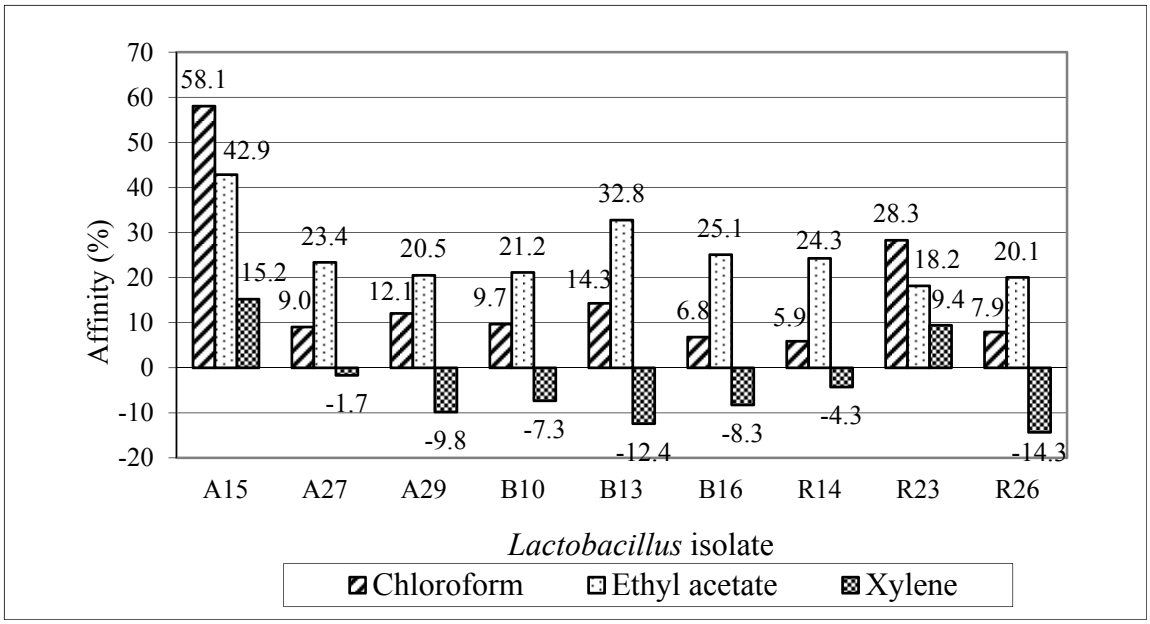

Figure 1. Affinity of Lactobacilli isolates to chloroform, ethyl acetate and xylene.

Affinity to chloroform shows the character of cell surface as electron donor and basic character, while affinity to ethyl acetate shows the characters as electron acceptor and acid character [18]. *L. rhamnosus* A15 and R23 show higher affinity to chloroform as compared to xylene and ethyl acetate indicating that the cell surface of A15 and R23 act as electron donors and have basic character. Meanwhile, lactobacilli A27, A29, B10, B13, B16, R14 and R26 show higher affinity to ethyl acetate indicating the cell surface character of most lactobacilli isolates being electron

acceptor and acidic. Only *L. rhamnosus* A15 and R23 were in accordance with findings of previous studies [18, 24] showing that at neutral pH, the microbial surface has character as electron donor. The character of electron acceptor was observed on low pH due to deprotonation.

Autoaggregation properties of lactobacilli isolated from breast milk

The results revealed that autoaggregation ability varied between strains, i.e., ranged between 4.13% - 39.10%. The highest autoaggreation ability was shown by *L. rhamnosus* R23 (39.10%), followed by isolate B13 (31.8%), B16 (29.42%), B10 (28.04%), R14 (26%) and A15 (14.96%). The lowest autoaggregation ability was shown by A29 (4.13%) and R26 (4.93%). Low autoaggregation properties of the lactobacilli isolate was in accordance with their cell surface properties showing hydrophilic properties. Strains with high autoaggregation properties generally show hydrophobic properties of cell surface [17].

Adhesion of lactobacilli isolates to the surface of rat intestine

Figure 2 shows that the highest adhesion ability was shown by *Lactobacillus sp* A27 followed by *L. rhamnosus* B16, R14 and R23. Exposure of rat intestine to the lactobacilli cell suspension decreased the number of indigenous *E. coli*, except for isolate B10 (Figure 3). This indicates the LAB isolated from breast milk were able to displace indigenous *E. coli*. All lactobacilli were able to adhere to the surface of rat intestine regardless of their hydrophilic properties. The higher adhesion was observed for *Lactobacillus sp.* A27 and *L. rhamnosus* B16, which had hydrophilic properties. In contrast, adhesion ability of *L. rhamnosus* A15 and R23 with higher affinity to xylene was lower than *Lactobacillus sp.* A27 and *L. rhamnosus* B16.

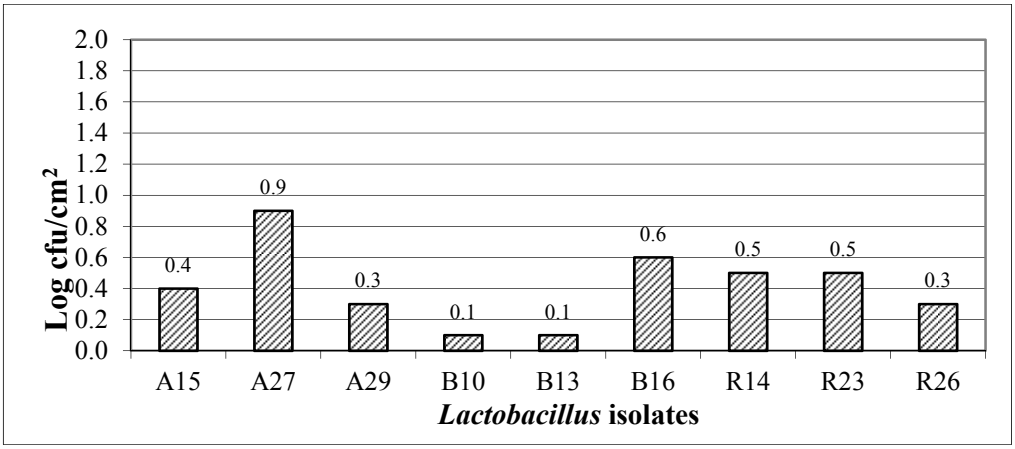

Figure 2. Change in LAB count on the surface of rat intestine after exposure to lactobacilli suspension for 60 min.

The present study showed no correlation between hydrophobicity of cell surface and adhesion ability. These results were in agreement with a previous study [25] showing no correlation between hydrophobicity of cell surface and adhesion ability. Research done by other researchers [26] also showed no correlation between aggregation ability of 4 strains of commercial probiotics (*L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* 99, and *P. freudenreichii* ssp. *shermanii* JS) and their attachment on human intestinal mucosa. *L. rhamnosus* LC705 had the highest aggregation ability but it attachment on mucosa was low (1.2%).

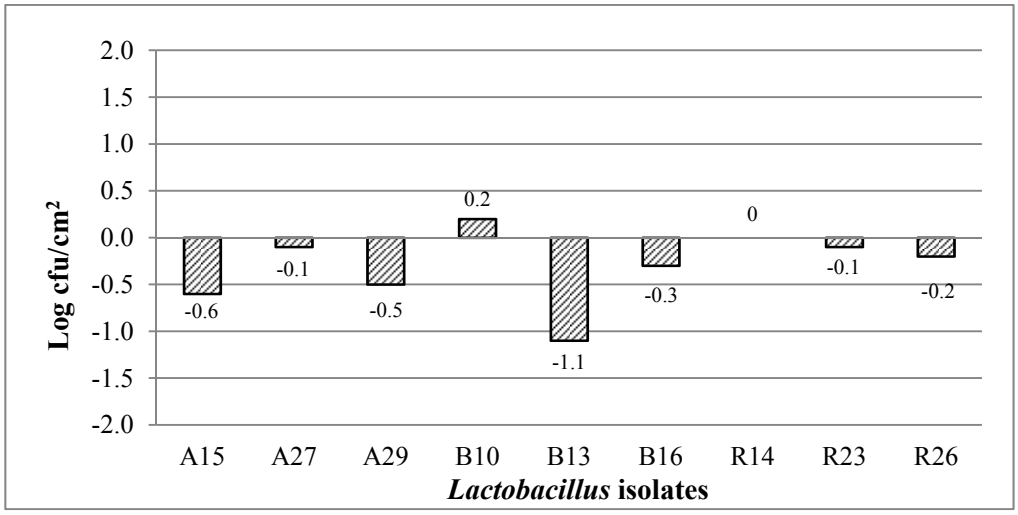

Figure 3. Change in *E. coli* count on the surface of rat intestine after exposure to lactobacilli suspension for 60 min.

Competition for adhesion between lactobacilli isolates and EPEC K1.1

This study used four lactobacilli isolates (A27, B16, R14, and R23), while EPEC K1.1. was used as competitor. The results (Figures 4 and 5) shows that when the surface of intestine was exposed to the mixture of lactobacilli and EPEC, both lactobacilli isolates (Figure 4) and EPEC (Figure 5) were able to adhere to the surface of rat intestine. When the surface of rat intestine was exposed to lactobacilli alone, the number of *E. coli* decreased, indicating that the lactobacilli isolates were able to exclude indigenous *E. coli*. However, when the surface of rat intestine was exposed to the mixture of lactobacilli and EPEC, *Lactobacillus* sp A27, *L. rhamnosus* R14 and R23 were unable to decrease the number of EPEC attaching to the surface of rat intestine. Only *L. rhamnosus* B16 could slightly reduce the number of attaching EPEC (0.6 log cfu/cm^2), as compared to the exposure on EPEC alone. In contrast, on the competition with EPEC, the number of LAB attached to the surface decreased as compared to control (exposed to lactobacilli alone), except for *Lactobacillus sp.* A27. This finding also confirmed that competition ability for adhesion is strain-dependent. The degree of competition was strain-dependent and determined by the affinity of adhesins (binding substances) on respective bacterial surfaces for stereo-specific receptors [27]. Another study showed that each *Lactobacillus* could only compete with a limited range of gastrointestinal bacteria for adhesion site [11].

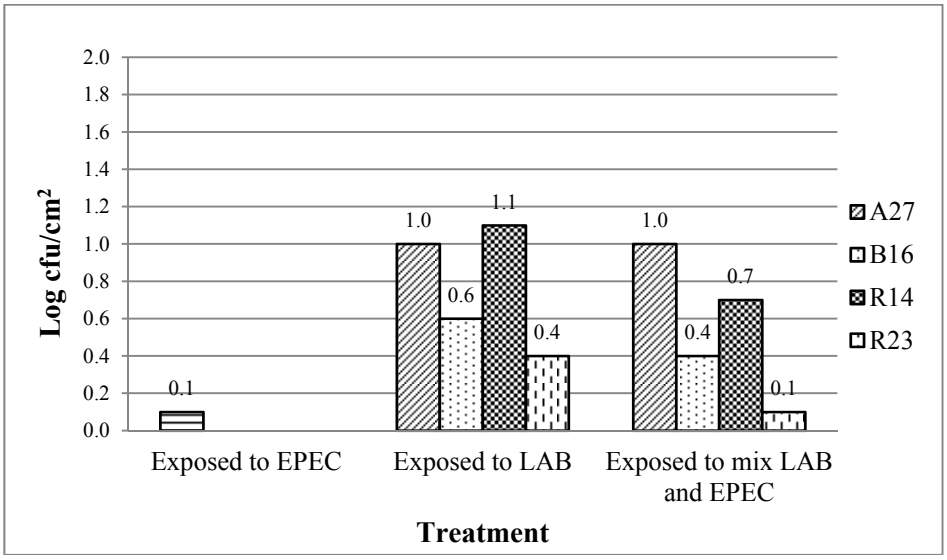

Figure 4. Change in LAB count on the surface of rat intestine after exposure to EPEC, LAB, and mixture of LAB and EPEC.

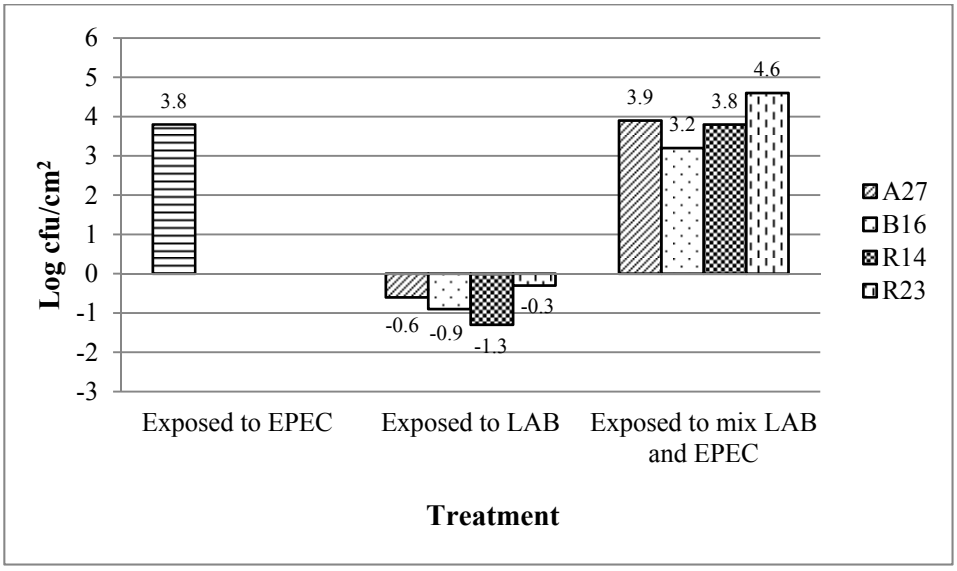

Figure 5. Change in *E. coli* count on the surface of rat intestine after exposure to EPEC, LAB, and mixture of LAB and EPEC.

To evaluate the effect of dose on the ability of lactobacilli to attach and to compete with EPEC, two isolates i.e. *L. rhamnosus* B16 and R23 were used as models. The results (Tables 1 and 2) show that increasing number of lactobacilli cells exposed to the surface of rat intestine from 10^6 cfu/ml to 10^8 cfu/ml increased the reduction of indigenous *E. coli* while increasing the number of attached LAB cells. Increasing number of lactobacilli exposed to the surface of rat intestine (10^8 cfu/ml) also improved the competition ability as shown by the decrease in attached *E. coli* as compared to that of 10^6 cfu/ml. The observation was in agreement with the results of a previous study [28] evaluating adhesion of 12 strains of *Lactobacillus* to Caco-2 cells that highly depend on lactobacilli cell concentration. The higher the number of lactobacilli, the higher the number of cells attached.

Table 2 shows that *L. rhamnosus* B16 was a better competitor to EPEC as compared to *L. rhamnosus* R23, as shown by the decrease in number of attaching EPEC in competition with *L. rhamnosus* B16. Increasing the dose of *L. rhamnosus* R23 cells exposed to the surface of rat intestine only increased reduction of indigenous *E. coli* and increased the number of LAB attached, but did not decrease the number of EPEC when exposure was done to the mixture of cells of R23 and EPEC. The results revealed that increasing the dose of isolate R23 only increased attachment but did not improve competition ability toward EPEC. This finding also confirmed that competition ability for adhesion is strain-dependent.

Table 1. Effect of doses on the number of *Lactobacillus* isolates attached to the surface of rat intestine after exposure to *Lactobacillus* alone and in competition with EPEC.

Treatment	Number of LAB attached (log cfu/cm^2)			
	Lactobacillus B16		*Lactobacillus* R23	
	Dose of 10^6 cfu/ml	Dose of 10^8 cfu/ml	Dose of 10^6 cfu/ml	Dose of 10^8 cfu/ml
Exposed to LAB alone	0.4	2.6	0.6	1.4
Exposed to mixture of LAB and EPEC cells suspension (Competition)	0.1	2.8	0.4	1.6

Table 2. Effect of doses on the number of *E. coli* attached to the surface of rat intestine after exposure to *Lactobacillus* alone and in competition with EPEC.

Treatment	Number of *E. coli* attached (log cfu/cm^2)			
	Competition with *Lactobacillus* B16		Competition with *Lactobacillus* R23	
	Dose of 10^6 cfu/ml	Dose of 10^8 cfu/ml	Dose of 10^6 cfu/ml	Dose of 10^8 cfu/ml
Exposed to LAB alone	-0.3	-1.1	-0.9	-1.4
Exposed to mixture of LAB and EPEC cells suspension (Competition)	4.6	3.0	3.2	3.5

Exclusion and displacement

Exclusion study was to evaluate if the attached LAB could be replaced by EPEC, while displacement study was to evaluate the ability of LAB to displace attached EPEC on the surface of rat intestine. The change of LAB and *E. coli* count after exclusion and displacement are presented in Figures 6 and 7. Only a small amount of the attached *L. rhamnosus* B16 (0.2 log cfu/cm^2) was excluded by EPEC as compared to control (1.4 log cfu/cm^2 as control and 1.2 log cfu.cm^2 after exclusion). Similarly, only a small amount of EPEC (0.2 log cfu/cm^2) attached to the surface of rat intestine could be displaced by *L. rhamnosus* B16 (3.2 log cfu/cm2 as control and 3 log cfu/cm^2 after displacement). Evaluation on *L. rhamnosus* R23 (Figure 7) shows that the number of *L. rhamnosus* R23 attached on the surface of rat intestine did not change (the change remained 1.1 log cfu/cm^2) following exposure to cell suspension of EPEC indicating no exclusion of *L. rhamnosus* R23 by EPEC. There was also no displacement of EPEC by *L. rhamnosus* R23. The number of *E. coli* attached was even higher than control. The present results indicate that both lactobacilli and EPEC were able to attach to the surface of rat intestine and they did not displace each other. This suggests that the adhesin on respective bacterial surface may differ; hence they did not compete for similar receptors. *Lactobacillus* GG (LGG) was unable to displace bacterial cells attached to Caco-2 cells (human colon adenocarcinoma cell-line) unless the bacterial cell detaches from the receptor and the binding of LGG hinder the re-attachment of bacterial cells to the receptor [11].

Theindigenous bacteria attached to the surface of rat intestine may also affect the ability of lactobacilli to displace EPEC. The number of indigenous LAB present on the surface of rat intestine was 10^4-10^5 cfu/cm^2. In contrast, the number of indigenous *E. coli* present on the surface of rat intestine was low (about 10^2 cfu/cm^2), hence the space for adherence of *E. coli* was still available while for LAB it might be saturated.

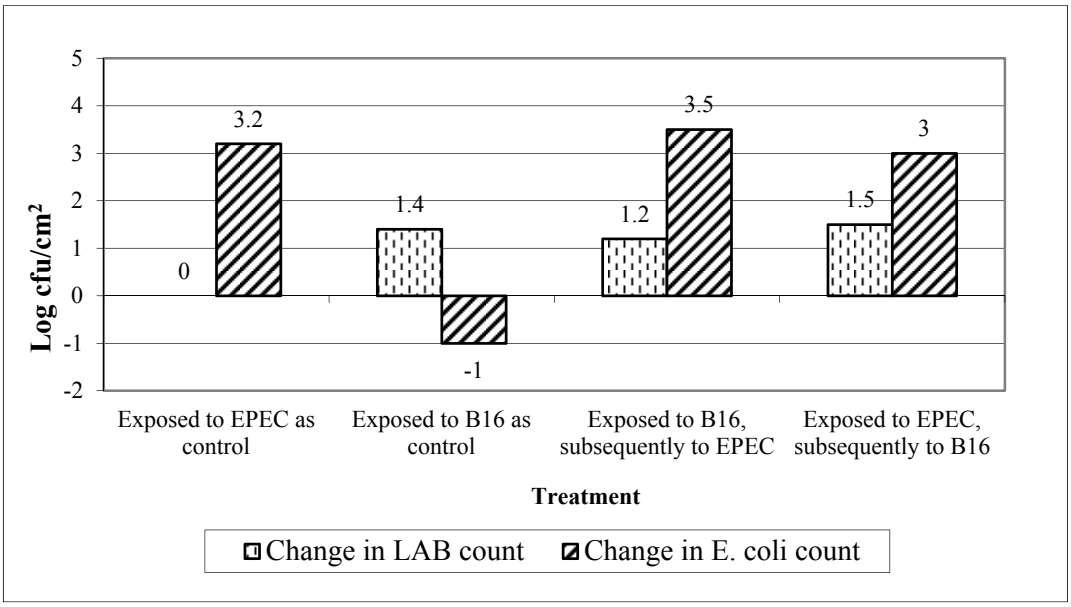

Figure 6. Change in LAB and *E. coli* count on the surface of rat intestine after exposure to EPEC, *L. rhamnosus* B16, subsequent exposure to *L. rhamnosus* B16 and EPEC and subsequent exposure to EPEC and *L. rhamnosus* B16.

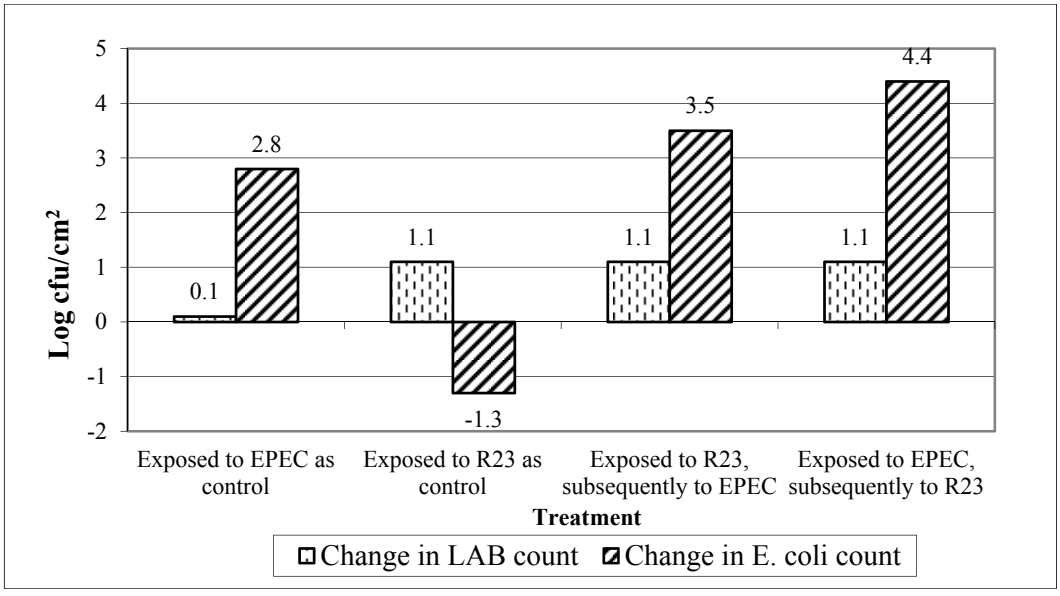

Figure 7. Change in LAB and *E. coli* count on the surface rat intestine after exposure to EPEC, *L. rhamnosus* R23, subsequent exposure to *L. rhamnosus* R23 and EPEC and subsequent exposure to EPEC and *L. rhamnosus* R23.

Correlation between adherence characteristic and the functional properties of LAB as probiotics still needs further study. Several strains with low adherence properties both *in vitro* and *in vivo* showed

positive effect on the host [29]. One study employed three *Lactobacillus* isolates used in the present study i.e. R14, R23 and B16 showed that they were able to prevent diarrhoea in rats due to infection of EPEC K1.1. when the lactic acid bacteria was regularly introduced prior to infection [30]. Isolate R23 showed the best capabilities for preventing diarrhoea in rats compared to two other isolates. *L. acidophilus* LA1, *L. casei* Shirota and *Lactobacillus* GG have proved to deliver beneficial health effects although their ability to attach to Caco-2 cells were low, i.e., LA1 (12.6%), Shirota (3.2%), and LGG (9.7%) [28].

Conclusion

The ability of lactobacilli isolated from breast milk to attach to the surface of rat intestine varied between strains. All isolates show more hydrophilic properties indicating that adhesion ability of lactobacilli isolates was not hydrophobic-dependent. When the added lactobacilli was attaching to the surface of rat intestine, some indigenous *E. coli* was displaced. The number of lactobacilli attached and the number of indigenous *E. coli* displaced was affected by the number of lactobacilli exposed. The higher the number of lactobacilli, the higher the number of cells attached. However, the ability to compete for adhesion site is strain dependent. Both lactobacilli and EPEC were able to attach to the surface of rat intestine; however they were unable to displace each other. The present finding suggests that dose should be considered when developing probiotic products using these lactobacilli isolates.

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