Investigation of Oligosaccharides for Prebiotic Action on Vaginal Lactobacilli

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ABSTRACT
Objective: Vaginitis is a very common gynaecological problem in women of all age groups resulted in millions of visit to physician. Decrease in vaginal Lactobacilli is one of the reasons of Bacterial vaginitis. Approach that could improve decreased level of Lactobacilli and inhibit growth of pathogenic bacteria can be alternative to antibiotic therapy. Oligosaccharides influence the growth of Lactobacilli species and benefits vaginal ecosystem. The present study was designed to evaluate prebiotic potential of oligosaccharides in vaginitis. Methodology: The potential of oligosaccharides to stimulate the growth of three selected vaginal Lactobacilli strains was studied by optical density, pH, titrable acidity and dry mass after 48 h of incubation. The antimicrobial effect of vaginal Lactobacilli in presence of oligosaccharide against pathogenic bacteria E. coli and C. albicans was studied by agar diffusion method. Vaginal irritation test was studied on female Swiss albino mice. Results and Discussion: Increased lactic acid production and lowering of pH by Lactobacilli strains in presence of oligosaccharides confirmed that oligosaccharide can stimulate the growth of selected Lactobacilli species. Optical density and dry biomass was also increased during 48 h of incubation. Higher zone of inhibition was observed in presence of prebiotic compared to control (without prebiotic). Vaginal irritation study showed no significant changes. Conclusively, oligosaccharides supported the growth of selected strains and could restore bacterial environment.

Key words: Lactobacilli, Fructo-Oligosaccharide (FOS), Galacto-Oligosaccharide (GOS), Prebiotic, Vaginitis.
Key message: The FOS and GOS stimulated the growth of Lactobacilli strain and Suppressed growth of harmful species in the vagina ecosystem.

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INTRODUCTION
Human microbiota is microorganisms colonized in human body. Microbiota generally resides on skin, mouth, gut and vagina.1 Female genital tract is one of the major sites occupied by microbiota, commonly dominated by Doderlein’s bacilli (Lactobacillus species).2 Presence of Lactobacilli is important sign of healthy vagina which inhibits the growth of pathogenic microorganism. Lactobacilli avoid growth, hold and expansion of pathogenic microorganism. Antimicrobial activity of Lactobacilli is owed to H2O2, bacteriocins and biosurfactants production.3 Vaginal Lactobacilli produces lactic acid from anaerobic glycolysis of glycogen that comes from shedding of vaginal epithelium. Lactic acid produced by Lactobacilli in concentration of 110 mM with acidic vaginal milieu pH 3.5 is the reason to prevent attack of pathogenic microorganism in vagina. Another probable reason is competition for the supplements, steric avoidance for the adhesion to the epithelium. It is quite difficult to identify different species of Lactobacilli colonizing vagina with culture dependent methods such as H2O2 production test, catalase reaction and sugar utilization test.4 Whereas culture independent identification methods like sequencing of 16S rRNA of bacterial colonies, terminal restriction fragment length polymorphism (T-RFPL) of 16S rRNA, qPCR and next generation sequencing (NGS) have wide applications in the field of microbiology.5 Common Lactobacilli isolated from the vagina include L. acidophilus, L. fermentum, L. plantarum, L. brevis, L. jensenii, L. casei, L. cellobiosus, L. leichmannii, L. delbrueckii, and L. salivarius. Of all these species, L. acidophilus, L. fermentum and L. casei has been the vaginal Lactobacillus most widely accepted to be predominant.6 Composition of vaginal flora may change due to disturbances in hormone levels, anti-toxin medications, sexual exercises and improper cleanliness.7 Compromise in healthy vaginal flora i.e. decrease in Lactobacilli is the main reason for bacterial vaginitis (BV). If asymptomatic BV is untreated, it can extend to Sexually transmitted infections (STI), including Neisseria gonorrhea, Chlamydia trachomatis, Trichomonas vaginalis, Herpes simplex infection sort 2 (HSV-2), Human papilloma infection (HPV), and in addition HIV, premature birth, post-abortal contamination, unsuccessful labour, and upper contraceptive tract infection. Symptomatic BV is treated with oral metronidazole for 7 days, metronidazole gel for 5 days or clindamycin cream for 7 days. Resistance offered by microorganism to antimicrobials is currently becoming a major concern.8 Antibiotic therapy causes disturbance of normal flora i.e. decrease in Lactobacilli and it is not possible to restore the pre-BV flora after antibiotic treatment. Use of suppositories to treat vaginal infection is also restricted because of constant irritation and noncompliance of working women. Recurrence is common after antibiotic therapy, may be because of resistance of pathogenic microorganism and/or failure to restore vaginal flora to pre BV condition. Recurrence of BV is 30% inside 3 months and 50% within 6 months of antimicrobial treatment.9
Because of recurrence of bacterial vaginosis after antimicrobial therapy, concept of probiotic to restore the vaginal flora came into existence.10 Probiotics can be characterized as “live microorganisms which when regulated in sufficient sums give a medical advantage on the host.”11 Pharmacological action of probiotic formulation depends on colony forming unit of respective microorganism. It is very difficult to maintain stability of probiotic formulation as oxygen, temperature, humidity and pressure may affect probiotic formulation unavourably. To overcome disadvantages of probiotics, it was necessary to explore new option that can be detrimental on pathogenic bacteria and beneficial to useful bacteria.12

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Prebiotic are non-digestible oligosaccharides that are not digested in upper gastrointestinal tract and fermented in colon. Fermentation of oligosaccharides provides energy to beneficial microbial flora of lower intestine. FOS and GOS are qualified as prebiotics.

From the literature review it was observed that prebiotic could help to restore pre-vaginitis environment. Rosseau et al. studied the effect of FOS and GOS on selected endogenous vaginal Lactobacilli strains showing probiotic properties. The aim of the present study was to investigate effect of FOS and GOS on three dominant strains, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus casei, that are considered as predominant Indian women vaginal Lactobacilli.

**MATERIAL AND METHODS**

**MATERIALS**

Lactobacilli acidophilus (ATCC 4796), Lactobacillus fermentum (ATCC 14931), Lactobacillus casei (ATCC 393) were generously provided by Dr. D. Y. Patil Arts, Commerce and Science College, Pimpri, Pune. Fructooligosaccharides (FOS) and Galacto-oligosaccharides (GOS) were purchased from Himedia, Mumbai, India. All other chemicals used were of analytical grade.

**METHOD**

**Effect of Oligosaccharides on the Growth of Selected Lactobacilli Strains:**

**Inoculum Preparation and Cultivation Conditions**

Reconstituted MRS broth supplemented with FOS and GOS medium was used in the study. Reconstituted standard broths (250 mL), containing, tryptone 10 g/L, meat extract 10 g/L, yeast extract 5 g/L, tween 80 1.1 g/L, potassium phosphate dibasic 2 g/L, sodium acetate 5 g/L, ammonium citrate 2 g/L, magnesium sulphate 0.2 g/L and manganese sulphate 0.05 g/L and oligosaccharides (FOS and GOS) were added to the broth at a final concentration of 10 g/L. The experimental media were sterilized at 121°C for 15 min before inoculation. The overnight (18 hours) cultures were used for inoculation of media at 2% (v/v) concentration. Reconstituted media was incubated for 48 hours under anaerobic conditions. Samples were taken at every 4 hours of growth to determine i) % Titratable acidity ii) pH iii) Optical density at 600nm iv) Determination of dry mass / biomass v) Antimicrobial activity against E. coli and C. albicans.

**Determination of Lactic Acid Production**

Titratable acidity (as % lactic acid) of the sample was determined in triplicate using 0.1 M NaOH.

\[
\% \text{ acid} = \frac{NxVxM}{Sx10}
\]

Where, 
- \( N \) = Normality of standard NaOH used for titration.
- \( V \) = volume of standard NaOH used for titration in mL.
- \( M \) = Molecular weight of predominant acid in the sample divided by the number of hydrogen ions in the acid molecules that are titrated.
- \( S \) = Sample size in mL or gm.

**pH**

The pH of each sample was measured by an electronic digital type pH meter (Equip-Tronic, Mumbai, India). The pH meter was standardized using reference pH 4.0 and pH 7.0 buffer solutions.

**Optical Density at 600nm**

The optical density was measured by using UV-Visible spectrophotometer (Shimadzu Analytical Pvt. Ltd, Japan) at 600 nm.

**Determination of Dry Mass/Biomass**

For drymass or biomass, the reconstituted MRS broth sample was centrifuged at 4,000 rpm for 15 min. Antimicrobial assay was performed by agar well diffusion method. MacConkey agar and potato dextrose agar were used for E. coli and C. albicans respectively. After solidification of agar 0.1 mL of overnight cultures of E. coli and C. albicans was spread and 100 µL of cell free supernatant was added in 6 mm wells. Plates were kept in refrigerator to facilitate diffusion for 4 h. Then plates were incubated aerobically at 37°C for 24 hours in case of E. coli and for 48°C in case of C. albicans. Zone of inhibitions were recorded in triplicate.

**Vaginal Irritation Study Using Mice**

In order to study the safety of prebiotic effect (FOS and GOS), vaginal irritation study using female Swiss albino mice was conducted. Fifteen mice were divided into 3 groups. The FOS and GOS gel was prepared using nutrient agar (2% w/v) as a gelling agent. The prepared gel was applied by micropipette to the vagina of mice in quantity of 0.1gm once daily for 10 days. The toxic manifestation, if any, on the vaginal region was assessed by observing the vaginal mucosa at pre-selected time intervals between and after treatment for 10 days. The findings were recorded for each animal.

**RESULT AND DISCUSSION**

**Effect of Oligosaccharides on the Growth of Selected Lactobacilli Strains:**

**Effect of FOS and GOS on Lactic acid production (% titratable acidity ) by Lactobacilli strains.**

The amount of lactic acid produced by the selected strains of Lactobacilli is represented in Table 1. Study showed that the percentage of lactic acid produced by Lactobacillus acidophilus was higher than Lactobacillus casei. Lactobacillus acidophilus uses Embden-Meyerhoff-Parnas (EMP) pathway using NADH as the cofactor and the enzyme lactate dehydrogenase and produces lactic acid and pyruvic acid as main metabolites. Whereas Lactobacillus casei utilizes EMP pathways as well as 6-phosphogluconate/ phosphoketolase pathways and produces CO₂ and ethanol along with lactic acid.

**Changes in pH by Lactobacilli strains containing Fructoligosaccharide (FOS) and Galactooligosaccharide (GOS):**

Figure 1 indicate distinct pH lowering effect of FOS and GOS compared to control (without prebiotics). It proved the stimulating effect of FOS and GOS on Lactobacilli. Reduction in pH showed strong relationship to production of lactic acid which is main metabolic product produced by the Lactobacilli.
Table 1: Effect of FOS And GOS on Lactic Acid Production (% Titratable Acidity) by Lactobacillus acidophilus, Lactobacillus fermentum and Lactobacillus casei.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control (without prebiotic)</th>
<th>FOS (1%w/v)</th>
<th>GOS (1%w/v)</th>
<th>Control (without prebiotic)</th>
<th>FOS (1%w/v)</th>
<th>GOS (1%w/v)</th>
<th>Control (without prebiotic)</th>
<th>FOS (1%w/v)</th>
<th>GOS (1%w/v)</th>
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</thead>
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<tr>
<td>0</td>
<td>0.12±0.03</td>
<td>0.15±0.03</td>
<td>0.15±0.03</td>
<td>0.10±0.06</td>
<td>0.13±0.06</td>
<td>0.11±0.03</td>
<td>0.02±0.05</td>
<td>0.05±0.05</td>
<td>0.05±0.05</td>
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<td>4</td>
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<td>0.23±0.02</td>
<td>0.26±0.01</td>
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<td>0.20±0.09</td>
<td>0.15±0.03</td>
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<tr>
<td>8</td>
<td>0.22±0.01</td>
<td>0.28±0.01</td>
<td>0.35±0.04</td>
<td>0.15±0.03</td>
<td>0.32±0.07</td>
<td>0.26±0.01</td>
<td>0.13±0.03</td>
<td>0.2±0.02</td>
<td>0.2±0.02</td>
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<td>12</td>
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<td>0.32±0.04</td>
<td>0.38±0.02</td>
<td>0.21±0.01</td>
<td>0.31±0.06</td>
<td>0.28±0.01</td>
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<td>0.26±0.01</td>
<td>0.28±0.01</td>
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<td>16</td>
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<td>0.39±0.04</td>
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<td>0.40±0.06</td>
<td>0.40±0.06</td>
<td>0.22±0.01</td>
<td>0.29±0.02</td>
<td>0.33±0.03</td>
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<td>20</td>
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<td>0.55±0.03</td>
<td>0.51±0.03</td>
<td>0.40±0.06</td>
<td>0.50±0.02</td>
<td>0.56±0.02</td>
<td>0.33±0.03</td>
<td>0.42±0.01</td>
<td>0.44±0.01</td>
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<tr>
<td>24</td>
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<td>0.67±0.07</td>
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<td>0.67±0.06</td>
<td>0.62±0.03</td>
<td>0.44±0.01</td>
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<td>0.48±0.01</td>
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<td>28</td>
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<td>0.68±0.02</td>
<td>0.79±0.02</td>
<td>0.62±0.04</td>
<td>0.69±0.02</td>
<td>0.71±0.02</td>
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<td>0.62±0.04</td>
<td>0.53±0.07</td>
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<td>32</td>
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<td>0.71±0.08</td>
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<td>36</td>
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<td>1.07±0.04</td>
<td>0.92±0.02</td>
<td>0.87±0.03</td>
<td>0.91±0.02</td>
<td>0.92±0.02</td>
<td>0.71±0.05</td>
<td>0.81±0.02</td>
<td>0.76±0.06</td>
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<tr>
<td>40</td>
<td>0.92±0.02</td>
<td>1.29±0.05</td>
<td>1.14±0.03</td>
<td>0.90±0.02</td>
<td>1.13±0.02</td>
<td>1.10±0.03</td>
<td>0.83±0.02</td>
<td>1.05±0.02</td>
<td>0.94±0.06</td>
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<td>44</td>
<td>0.90±0.02</td>
<td>1.20±0.04</td>
<td>1.11±0.02</td>
<td>0.81±0.02</td>
<td>1.02±0.05</td>
<td>1.07±0.02</td>
<td>0.79±0.05</td>
<td>0.95±0.03</td>
<td>0.97±0.04</td>
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<tr>
<td>48</td>
<td>0.85±0.03</td>
<td>0.92±0.03</td>
<td>0.92±0.02</td>
<td>0.78±0.02</td>
<td>0.98±0.03</td>
<td>0.96±0.02</td>
<td>0.80±0.06</td>
<td>1.03±0.06</td>
<td>0.78±0.07</td>
</tr>
</tbody>
</table>

Lactic acid and Short Chain fatty acids produced by Lactobacilli contribute to the maintenance of a low vaginal pH which is thought to be an important control mechanism preventing colonisation by pathogens. Acidification of vagina produces unfavourable condition for growth of Salmonella typhimurium, Helicobacter priori, Candida albicans, E. coli and G. vaginalis. The reduction in pH was from 6.5 (t=0) to 4.5 after 48 hours of incubation. The normal pH of vaginal fluid is about 4.5 - 5.5. The resulting low pH is important for preventing non indigenous organisms in the vagina.

Effect of FOS and GOS on growth curve of Lactobacilli strain

Figure 2 showed that optical density of selected Lactobacilli strains was higher in reconstituted broth containing prebiotic than control (without prebiotic) which confirms the ability of oligosaccharide to stimulate the growth of selected Lactobacilli.

The optical densities of all the Lactobacilli strains containing FOS tended to be slightly higher than the optical densities of the GOS and the control. Shorter chain fructo-oligosaccharides containing primarily fructose chains and fructose chain with terminal glucose bound by β (1,2) bond glycocylic linkages could have undergone rapid degradation in highly acidic environment. Rapid degradation of FOS was supporting faster growth of Lactobacilli that was indicated by higher optical density in FOS as compared to GOS. The bacterial growth started to lag phase from early period of inoculation. Lactobacillus acidophilus, Lactobacillus fermentum and Lactobacillus casei growth approached to exponential phase after 8 hours. Then, all bacterial growth reached to stationary phase after 28 hours and turned to be relatively constant for 48 hours.

Determination of dry mass on Lactobacilli strain containing fructooligosaccharide (FOS) and galactooligosaccharide (GOS)

The dry mass of the Lactobacillus acidophilus, Lactobacillus fermentum and Lactobacillus casei, containing FOS, GOS and control are represented in Figure 3. Upon observing the cellular concentration in terms of dry mass of Lactobacilli strains, cultivated in reconstituted MRS medium supplemented with prebiotic as a function of time, a potential increase in dry mass was observed at the end of 48 hours in sample containing prebiotic to that of control. Higher increase in dry mass in FOS could be attributed to transport systems for sucrose in Lactobacilli including the oligosaccharide transporter MsmEFGK and the sucrose phosphotransferase system PtsLBCA. Both the transport systems are responsible for internalization of FOS. Because of these transport systems Lactobacilli could utilize FOS rapidly than GOS as a carbon source giving higher biomass in FOS than GOS.

Antimicrobial activity of Lactobacillus acidophilus containing FOS and GOS against E.Coli and C.albicans

The sensitivity of the pathogenic microorganisms to inhibitory substances produced by selected Lactobacilli is presented in Table 2 and Figure 4. The inhibitory activity was distinct towards Gram –ve E. coli compared to that of control. S. typhimurium, H. priori, C. albicans, E. coli and G. vaginalis. The sensitivity of the pathogenic microorganisms to inhibitory substances present in the control mechanisms was higher in broth containing prebiotic than control (without prebiotic) which could be attributed to transport systems for sucrose in Lactobacilli including the oligosaccharide transporter MsmEFGK and the sucrose phosphotransferase system PtsLBCA. Both the transport systems are responsible for internalization of FOS.
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Table 2: Antimicrobial Activity of Lactobacillus acidophilus, Lactobacillus fermentum and Lactobacillus casei Containing FOS and GOS against E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation time (hrs)</th>
<th>Lactobacillus acidophilus</th>
<th>Lactobacillus fermentum</th>
<th>Lactobacillus casei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(without prebiotic)</td>
<td>Reconstituted MRS broth with FOS</td>
<td>Reconstituted MRS broth with GOS</td>
<td>Reconstituted MRS broth with FOS</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2.25±0.21</td>
<td>5.3±0.28</td>
<td>4.2±0.21</td>
</tr>
<tr>
<td>T&lt;sub&gt;6&lt;/sub&gt;</td>
<td></td>
<td>4.3±0.28</td>
<td>12.3±0.28</td>
<td>10.6±0.21</td>
</tr>
<tr>
<td>T&lt;sub&gt;48&lt;/sub&gt;</td>
<td></td>
<td>5.65±0.28</td>
<td>15.3±0.35</td>
<td>14.4±0.42</td>
</tr>
</tbody>
</table>

*Error bars represent standard deviations of replicates.

Figure 2: Growth of Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus casei in reconstituted MRS broth either with (1% W/V FOS and 1% W/V GOS) or without prebiotic.

Vaginal Irritation Study Using Mice

The Microscopic examination of vaginal tissues were evaluated by scoring system as given by normal (0), mild (1), moderate (2), severe (3). FOS group (Group II) revealed mild focal hyperplasia of squamous epithelium and multifocal mild lymphocytic infiltration at lamina propria (1). GOS group (Group III) revealed mild diffuse lymphocytic infiltration at lamina propria (1). Thus vaginal irritation study showed no significance changes in vagina after application of FOS and GOS (Figure 5).

CONCLUSION

The selected Lactobacilli strains were able to grow on FOS and GOS as indicated by the increase in turbidity i.e. optical density, lactic acid production and dry mass obtained after 48 h of incubation as compared to control (without prebiotic). Prebiotic had a greater influence on the growth of Lactobacilli strain and a greater pH lowering effect in a reconstituted MRS medium containing FOS and GOS. There were significant

to C. albicans, both being common vaginal infection causative agents. No zone of inhibition was observed against C. albicans. The absence of effect on C. albicans growth can be correlated with the resistance of yeasts to acidic conditions and H<sub>2</sub>O<sub>2</sub>.

Significant zone of inhibitions were observed against E. coli. Bacteriocin (antimicrobial peptides or proteins) are produced by almost all genera of lactic acid bacteria. The FOS and GOS stimulate the growth of Lactobacilli strain and suppress growth of potentially harmful species in the vagina ecosystem.

Figure 3: Changes in Dry Mass of Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus casei containing FOS, GOS and Control (Without Prebiotic).

Figure 4: Antimicrobial activity against E. coli in presence of prebiotic (FOS and GOS), Lactobacilli Strains (without prebiotic), control (without Prebiotic and Lactobacilli strains) of; (A) Lactobacillus acidophilus, (B) Lactobacillus fermentum, (C) Lactobacillus casei, (D) Antimicrobial Activity of Lactobacillus acidophilus against C. albicans in presence of prebiotic FOS and GOS.
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- Lactooligosaccharides.

- *Escherichia coli*; *Lactobacillus fermentum*; *Lactobacillus casei*; *Lactobacillus acidophilus*; *Hydrogen peroxide*; *Fructooligosaccharides*; *Galactooligosaccharides*.

The authors are declared no conflict of interest.

**CONFLICTING INTEREST**
The authors are declared no conflict of interest.

**ABBREVIATION USED**

**HIV**: Human immunodeficiency virus; **LA**: *Lactobacillus acidophilus*; **LF**: *Lactobacillus fermentum*; **LC**: *Lactobacillus casei*; **E.Coli**: *Escherichia coli*; **H₂O₂**: Hydrogen peroxide; **FOS**: Fructooligosaccharides; **GOS**: Galactooligosaccharides.

**REFERENCES**


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