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Original Article

Investigation of Oligosaccharides for Prebitoic 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 prebitoic 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 Doderlen'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 H_2O_2, 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 H_2O_2 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-RFLP) 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. leichmanii, 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 conceptive 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 résistance 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 unfavourably. 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]

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.[13]

From the literature review it was observed that prebiotic could help to restore pre-vaginitis environment. Rosseau *et al.*[14] 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, *Lactobacilli acidophilus, Lactobacillus fermentum, Lactobacillus casei,* that are considered as predominant Indian women vaginal *Lactobacilli.*[15]

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[14]

Reconstituted MRS broth supplemented with FOS and GOS medium was used in the study. Reconstituted standard broths (250 mL), containng, 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[16]

Titratable acidity (as % lactic acid) of the sample was determined in triplicate using 0.1 M NaOH using phenolphthalein as an indicator. The titratable acidity was expressed as a percentage of that of lactic acid.

The % titratable acidity was calculated by using formula:

$$\% \text{ acid} = \frac{N \times V \times M}{S \times 10}$$

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[17] (Equip-Tronic, Mumbai, India). The pH meter was standardized using reference pH 4.0 and pH 7.0 buffer solutions.

Optical Density at 600nm[18]

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

Determination of Dry Mass/Biomass[19]

For drymass or biomasss, the reconstituted MRS broth sample was centrifuged at 4,000 rpm (Cooling Centrifuge, Remi Electro lab, Mumbai) for 20 min. Clear supernant was removed carefully and pellets were collected. Weight of pellets was taken immediately and pellets were dried at 80°C for 24 hours and again weight of dry pellets was taken.

Determination of Antimicrobial Activity against *E. coli* and *C. albicans*[16]

Cell-free supernatant was collected by centrifugation 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 supernant 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[20]

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 preselected 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 EmbdenMeyerhoff-Parnas (EMB) 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 EMB pathways as well as 6-phosphogluconate/ phosphoketolase pathways and produces CO_2 and ethanol along with lactic acid.[21]

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*.

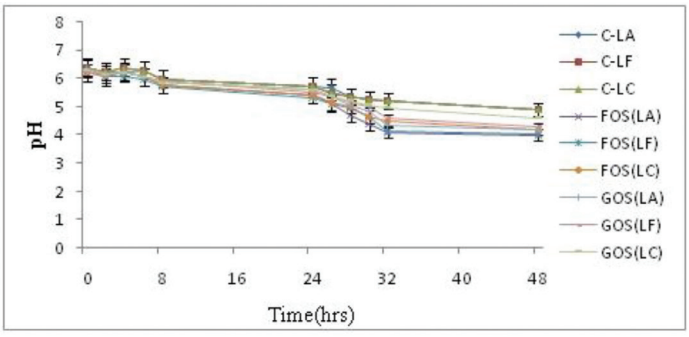
Table 1: Effect of FOS And GOS on Lactic Acid Production (% Titratable Acidity) by *Lactobacillus acidophillus,Lactobacilus fermentum* and *Lactobacillus casei*.

	%Titratable acidity (Lactic acid %)								
	Lactobacillus acidophillus			*Lactobacillus fermentum*			*Lactobacillus casei*		
Time(hrs)	Control (without prebiotic)	FOS (1%w/v)	GOS (1%w/v)	Control (without prebiotic)	FOS (1%w/v)	GOS (1%w/v)	Control (without prebiotic)	FOS (1%w/v)	GOS (1%w/v)
0	0.12±0.03	0.15±0.03	0.15±0.03	0.10±0.06	0.13±0.06	0.11±0.03	0.02±0.05	0.05±0.05	0.05±0.05
4	0.15±0.03	0.23±0.02	0.26±0.01	0.12±0.03	0.20 ±0.09	0.15±0.03	0.05±0.05	0.15±0.03	0.15±0.03
8	0.22±0.01	0.28±0.01	0.35±0.04	0.15±0.03	0.3±0.07	0.26±0.01	0.13±0.03	0.2±0.02	0.2±0.02
12	0.35±0.04	0.32±0.04	0.38±0.02	0.21±0.01	0.31±0.06	0.28±0.01	0.2±0.02	0.26±0.01	0.28±0.01
16	0.34±0.02	0.39±0.04	0.405±0.06	0.28±0.01	0.40±0.06	0.40±0.06	0.22±0.01	0.29±0.02	0.33±0.03
20	0.40±0.06	0.55±0.03	0.51±0.03	0.40±0.06	0.50±0.02	0.56±0.02	0.33±0.03	0.42±0.01	0.44±0.01
24	0.51±0.03	0.67±0.07	0.71±0.06	0.56±0.02	0.67±0.06	0.62±0.03	0.44±0.01	0.52±0.07	0.48±0.01
28	0.71±0.05	0.68±0.02	0.79±0.02	0.62±0.04	0.69±0.02	0.71±0.02	0.48±0.01	0.62±0.04	0.53±0.07
32	0.74±0.03	0.91±0.05	0.87±0.03	0.74±0.04	0.71±0.08	0.83±0.02	0.58±0.06	0.72±0.02	0.67±0.06
36	0.83±0.02	1.07±0.04	0.92±0.02	0.87±0.03	0.91±0.02	0.92±0.02	0.71±0.05	0.81±0.02	0.76±0.06
40	0.92±0.02	1.29±0.05	1.14±0.03	0.90±0.02	1.13±0.02	1.10±0.03	0.83±0.02	1.05±0.02	0.94±0.06
44	0.90±0.02	1.20±0.04	1.11±0.02	0.81±0.02	1.02±0.05	1.07±0.02	0.79±0.05	0.95±0.03	0.97±0.04
48	0.85±0.03	0.92±0.03	0.92±0.02	0.78±0.02	0.98±0.03	0.96±0.02	0.80±0.06	1.03±0.06	0.78±0.07

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*.[22] The reduction in pH was from 6-6.5 (t=0) to 4 - 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*.



*Error bars represent standard deviations of replicates.

Figure 1: Changes in pH by *Lactobacillius acidophillus, Lactobacillus fermentum, Lactobacillus casei* in Presence of FOS, GOS and Control (without prebiotic)

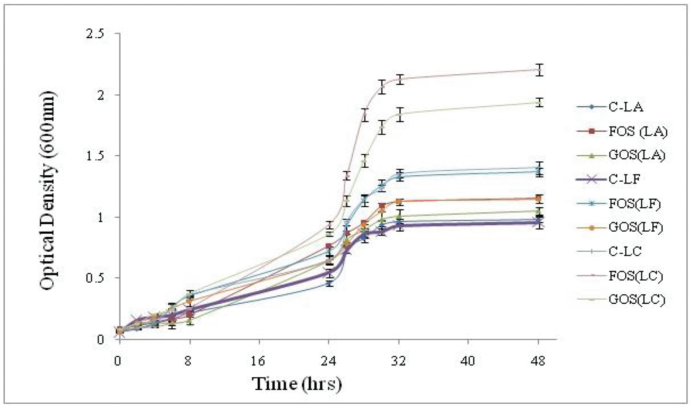
The optical densities of all the *Lactobacilli* strains containing FOS tended to be slightly higher than the optical densities of the GOS and the control. Shorter chain fructo-oligosaccharides containing primarily fructose chains and fructose chain with terminal glucose bound by β (1,2) bond glycocidic 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.[23] 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 fructoligosaccharide (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 Pts1BCA. Both the transport systems are responsible for internalization of FOS.[24] Because of these transport systems *Lactobacilli* could utilize FOS rapidly than GOS as a carbon source giving higher biomass in FOS than GOS.

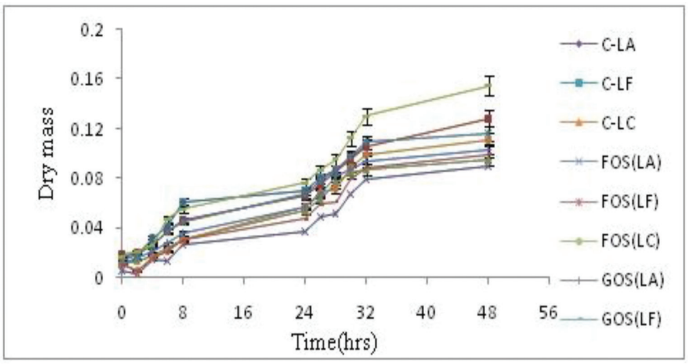
Antimicrobial activity of *Lactobacillus acidophilus* containing FOS and GOS against *E.Coli* and *C.albicans*

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



*Error bars represent standard deviations of replicates.

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



*Error bars represent standard deviations of replicates

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

Table 2: Antimicrobial Activity of *Lactobacillus acidophillus, Lactobacilus fermentum* and *Lactobacillus casei* Containing FOS and GOS against *E. coli*

		Inhibition zone(mm)								
		Lactobacillus acidophillus			Lactobacilus fermentum			Lactobacillus casei		
Strain	Incubation time(hrs)	(without prebiotic)	Reconstituted MRS broth with FOS	Reconstituted MRS broth withGOS	(without prebiotic)	Reconstituted MRS broth with FOS	Reconstituted MRS broth withGOS	(without prebiotic)	Reconstituted MRS broth with FOS	Reconstituted MRS broth withGOS
E. coli	Control	–	–	–	–	–	–	–	–	–
	T_6	2.25±0.21	5.3±0.28	4.2±0.21	2.55±0.35	6.3±0.28	5.6±0.28	3.8±0.14	12.7±0.21	10.7±0.21
	T_{24}	4.3±0.28	12.3±0.28	10.6±0.21	5.6 ±0.28	13.6±0.14	11.3±0.21	8.55±0.35	15.3±0.28	13.4±0.35
	T48	5.65±0.28	15.3±0.35	14.4±0.42	8.2±0.42	18.4±0.42	16.6±0.28	10.5±0.42	20.3±0.35	18.6±0.42

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_2O_2.[25]

Significant zone of inhibitions were observed against *E. coli*. Bacteriocin (antimicrobial peptides or proteins) are produced by almost all genera of lactic acid bacteria.[26] The FOS and GOS stimulate the growth of *Lactobacilli* strain and suppress growth of potentially harmful species in the vagina ecosystem.

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).

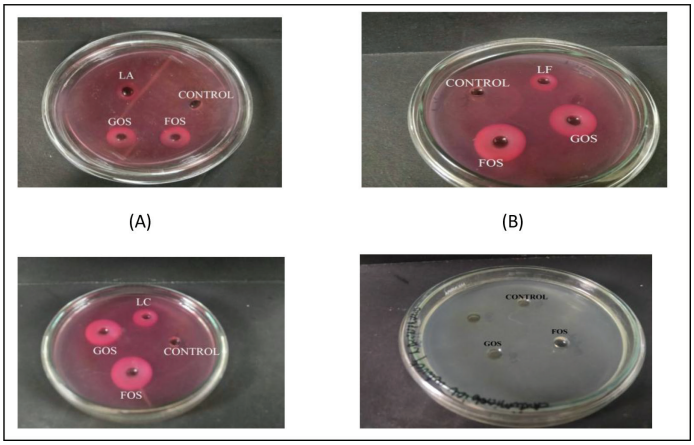


Figure 4: Antimicrobial activity against *E. coli* in presence of prebiotic (FOS and GOS), *Lactobacilus* Strains (without prebiotic), control (without Prebiotic and *Lactobacilli* strains) of; (A) *Lactobacillius acidophillus,* (B)*Lactobacillius fermentum*, (C) *Lactobacillius casei,* (D) Antimicrobial Activity of *Lactobacillius acidophillus a*gainst *C. albicans* in presence of prebiotic FOS and GOS.

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 increase in the inhibition activity of *Lactobacilli* strains in presence of FOS and GOS as compared to control. Therefore from the results of all the parameters, it was concluded that prebiotics promoted the growth of *Lactobacilli* that generated lactic acid to lower the vaginal pH and secreted antibacterial substances that inhibited the adhesion and replication of

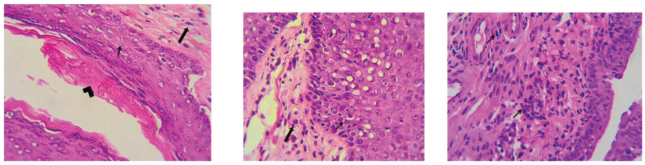


Figure 5: Microscopic examination of vaginal tissues of mice (A) control group (B) FOS group (C) GOS group.

the pathogenic bacteria. FOS and GOS showed potential prebiotic property for selected *Lactobacilli* strains.

CONFLICTING INTEREST

The authors are declared no conflict of interest.

ABBREVIATION USED

HIV: Human immunodeficiency virus; ***LA:*** *Lactobacillus acidophilus;* ***LF:*** *Lactobacillus fermentum;* ***LC:*** *Lactobacillus case;* ***E.Coli:*** *Escherichia coli;* **H_2O_2:** Hydrogen peroxide**; FOS:** Fructooligosaccharides; **GOS:** Galactooligosaccharides.

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