

## Diversity and abundance of lactobacilli during ulcerative colitis in North Indian patients: a case control study

Reena Kumari<sup>1</sup>, Nirmal Verma<sup>1</sup>, Anil Kumar Verma<sup>1</sup>, Vineet Ahuja<sup>2</sup> and Jaishree Paul<sup>1\*</sup>

<sup>1</sup>School of life sciences, Jawaharlal nehru university, New delhi, India; <sup>2</sup>Department of gastroenterology, All India institute of medical sciences, New delhi, India

This article was published in the following Scient Open Access Journal:

Clinical Microbiology & Case Reports

Received December 10, 2014; Accepted January 13, 2015; Published January 17, 2015

### Abstract

Lactic acid bacteria execute beneficial health effects by adhering to the intestinal epithelial layer. We compared the abundance and diversity of genus *Lactobacillus* in the fecal sample of ulcerative colitis (UC) patients vs. healthy control. About 40 samples belonging to moderate, severe, remission category and controls were subjected to fluorescent in situ hybridization (FISH) and quantitative Polymerase Chain Reaction (qPCR) analysis to study the abundance. To check the diversity, same samples were subjected to denaturing polyacrylamide gel electrophoresis (PAGE) fingerprinting. The FISH ( $p=0.0001$ ) and qPCR ( $p=0.0432$ ) analysis shows that the population of *Lactobacilli* was higher in severe category of samples. This was further supported by increase in lactate level by Gas chromatography (GC) ( $p=0.032$ ). We observed high degree of diversity in moderate ( $p=0.002$ ) and severe ( $p=0.003$ ) category of patient samples as compared to controls. The profile in remission category exhibited similarities with the controls. Real time PCR analysis showed decreased expression of MUC9 (Mucin gene) ( $p=0.011$ ) in biopsy samples of patients compared to controls. Summary-We speculate that increased fecal *Lactobacilli* population during active condition is due to loss of mucin necessary for their adherence to the intestinal epithelial cell lining. This is the first report analyzing the fluctuation of *Lactobacilli* during ulcerative colitis in North Indian individuals.

**Keywords:** Ulcerative colitis, *Lactobacilli*, Lactate, FISH flow cytometry, qPCR, Urea PAGE

### Introduction

Dysbiosis in intestinal commensal bacteria is regarded as an important factor in the initiation and progression of ulcerative colitis (UC). The role of an altered bacterial ecosystem has been reported in a variety of clinical and experimental studies [1]. Lactic acid bacteria (LAB) are characterized by the formation of lactic acid as sole or main end product of carbohydrate metabolism from different food substrates [2,3]. Several strains of lactic acid bacteria (LAB) including *Enterococcus faecalis* are known for their role in priming the immune system [4-6]. The *Enterococci* are known to ferment sugars and produce lactate as major product [7]. *Lactobacilli* are considered to be beneficial group of bacteria to the host, and many studies have demonstrated that some strains of *Lactobacilli* can reduce the severity and maintain the remission of UC [8]. Their adhesion to the intestinal mucosa is considered one of the important factors for the beneficial health effects. Specific lactic acid bacteria help to fight pathogens, transient colonization, modulation of the immune system, and enhanced healing of damaged intestinal mucosa [9]. We earlier observed significant decrease of *Lactobacillus* population in the mucosa of UC patients by qPCR analysis [10]. We focused on *Lactobacilli* as a subgroup because of their known potential probiotic effects in the human gastro intestinal tract (GIT). Lactic acid bacteria have ability to inhibit the growth of various pathogenic Gram-positive or Gram-negative bacteria. This inhibition may be due to the secretion of organic acids such as lactic acid, acetic acid, bacteriocins, bacteriocin-like substances and possibly biosurfactants, which are active against certain pathogens [11].

The extent of fluctuation in the composition of *Lactobacillus* genus in fecal microbiota due to disease activity has not been studied so far in North Indian population. In this study we applied qPCR and FISH flow cytometry techniques to compare the abundance of *Lactobacilli* in fecal microbiota of healthy individuals vs. UC patients. We have also compared and quantified the fecal lactate level between healthy and UC patients. In addition to this we also compared the diversity of *Lactobacilli* during disease condition using urea polyacrylamide gel electrophoresis (PAGE) fingerprinting technique that has

\*Corresponding author: Dr. Jaishree Paul, School of life sciences, Jawaharlal nehru university, New delhi, India 110067, Tel: 91-11- 26704156, Fax: 91-11-26742558, Email: jpaul33@hotmail.com

been employed earlier to study microbial community structure [12,13]. Lactobacilli are known to adhere to the mucin layer of the intestinal epithelium [14]. In order to assess the loss of mucin layer during disease condition, we checked the expression of a member of mucin gene family, MUC9 that is expressed in epithelial cells [15].

## Methodology

### Fecal sample collection and processing

Fecal samples were collected from 14 control individuals and 26 UC patients under three categories (severe-12, moderate-6, and remission-8). Subjects were recruited by the attending physician from the Gastroenterology Department, All India Institute of Medical Sciences, New Delhi with necessary permissions from the patients. Disease activity in UC patients was determined by simple clinical colitis activity index [16] and total index score, 7-10, >10 and 0-2 was referred for moderate, severe and remission category of UC respectively [17]. Clinical score defines the severity of the disease and calculated on the basis of stool frequency, rectal bleeding, mucosal appearance, physician's rating of disease activity, and each having a defined score ranges [17]. Patients who had hemorrhoids only and showed no evidence of small and large intestinal disease were enrolled as control individuals in this study. Subjects under any antibiotic or probiotic or prebiotic treatment past 3 months of sample collection were excluded from this study. The fecal samples were collected in sterile stool specimen plastic container, transported at 4°C and stored at -80°C within 3 hrs of sample collection until processed further. The fecal sample details were published previously by Kumari et al. [18].

### Biopsy sample collection

We collected biopsy samples from few controls (n=10) (without any ulcerative colitis or Crohn's disease) and ulcerative colitis patients (n=12). All biopsy samples were collected under the supervision of the attending doctors at the Gastroenterology Department, All India Institute of Medical Sciences, New Delhi. The biopsies were taken from involved areas in the intestine of patients with UC and from normal sigmoid mucosa in control group. The control population consisted of patients who were taken up for sigmoidoscopy for evaluation of anal hemorrhoids. The biopsy samples, with associated mucus, were collected aseptically in a RNA later solution and processed immediately for RNA extraction. At the time of the study, patients were receiving steroids, 5-aminosalicylic acid, and azathioprine, which have not yet been reported to alter gut microbiota. Histopathological changes of biopsy samples were evaluated (data not shown). The biopsy sample details are represented in the Table S1.

### Ethics statement

Ethical clearance for the study was obtained from the Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi. Written consent was obtained from all the participants.

### Fluorescent probes and oligonucleotides

All the probes used in the study target the 16S rRNA gene. Probe EUB338 was used as a positive control, the sequence of which was conserved within bacteria domain [19] Conversely, NONEUB338 (reverse of EUB) was used as negative control probe

[20]. The positive control probe (EUB338) was covalently labeled with fluorescein isothiocyanate (FITC) at both 5' and 3' end [21] and negative control probe (NONEUB338) was covalently labeled with FITC at 5' end and Cy5 at 3' end. The probe Lab158 [22] specifically targeting *Lactobacillus/Enterococcus* genus was labeled at 5' end with Cy5. Table S2 represents the details of probes used in the present study.

### Analysis of fecal samples by fluorescent in situ hybridization (FISH)

1 gm of fecal sample was suspended in 9 ml of PBS (Phosphate Buffer Saline) and vortex with 10-15 glass beads for 5 min to homogenize the sample. This suspension was centrifuged at 101xg for 1 min to pellet down the debris and the supernatant was collected. To fix the cells, 1 ml of this supernatant was incubated with 4% Paraformaldehyde (1:3 ratio) at 4°C, overnight [18].

### FISH-microscopy

For microscopy, PFA- fixed cells were washed and spotted on gelatin coated slides and dried for 20 min at 45°C. The cells were dehydrated in 96% ethanol for 3 min. 20 µl of hybridization buffer (900 mMNaCl, 20 mMTris-HCl, pH 8, 0.01% Sodium Dodecyl Sulfate, pH 7.2) containing 2ng of Cy5 labeled probe was added and incubated at 50°C for 2 hrs in humid chamber [23]. To the same slide 25 µl of (5 µg/ml) Hoechst 33342 (Invitrogen, India) was added to stain the DNA and the incubation was continued for further 1 hr. After hybridization, slides were washed with low salt washing buffer (65 mM NaCl, 20 mMTris-HCl, 5 mM EDTA, 0.01% Sodium Dodecyl Sulfate, pH 7.2) for 20 min at same temperature. The slides were further rinsed in water, dried immediately and mounted in Glycerol and sealed. Digital images were viewed with confocal microscope (Olympus FluoView™FV1000, USA).

### Analysis of fecal samples by FISH flow cytometry

The PFA fixed cells were washed twice with PBS and incubated in ethanol with PBS (1:1 ratio) at -20°C for 2 hr. The suspension was again washed twice with PBS and the cells were resuspended in 50µl hybridization buffer (as used above). For each hybridization reaction, 60 µl of fixed cells were used. Hybridization was performed in dark at 50°C, 40 hrs, in the hybridization solution containing 4 ng/µl each of Lab158 and EUB338 probe. After hybridization, 150 µl of hybridization solution without probe was added to stop the reaction and cells were pelleted at 1610xg for 10 min. Hybridized cells were resuspended in prewarmed washing buffer (as used above) and incubated at 50°C for 20 min to remove non-specific binding of the probe. Cells were finally pelleted at 1610xg for 10 min and suspended in 200 µl PBS. An aliquot of 100 µl was added to 0.5 ml of flow sheath solution (Becton Dickinson, USA) for flow cytometry analysis. Data acquisition was performed with FACS calibur flow cytometer (Becton Dickinson, USA) as explained by Kumari et al. [18].

### Enumeration of Lactobacillus/Enterococcus in fecal samples

The abundance of lactobacilli was estimated in the form of Lab158- Cy5 probe hybridized cells as a proportion of total bacteria hybridized with EUB338 FITC probe. This proportion of hybridized bacteria was corrected by subtracting the background fluorescence obtained, when only negative control probe NONEUB 338 was hybridized.

## Quantitative PCR (qPCR) Analysis

Total DNA from the fecal samples (220 mg) was extracted (Qiagen stool DNA kit) and eluted in 50 µl of Tris-EDTA (TE) buffer. The concentration and integrity of DNA was checked by nanodrop and electrophoresis. About 20 ng of this DNA from each sample was used to analyze the bacterial population. Primer sets for lactobacilli genus are represented in Table S2 [10]. The amplified product was cloned and sequenced and sequences were deposited in the EMBL database and accession number (AM042701) was obtained. These 16S rRNA gene fragments containing plasmids were used as reference strains for subsequent experiments. The reaction was carried out in 7500 real time PCR system (Applied Biosystems, USA) using SYBR green method. The standard curves were constructed by serial dilutions of each reference clone prepared from 0.05 to 50,000 pg/tube, corresponding to  $1 \times 10^1$  to  $1 \times 10^7$  copy numbers. The standard curve (Figure S1a) of the reference clones was used to extrapolate the numbers of bacteria in the fecal samples. The dissociation curves for targeted bacteria are shown in Figure S1b. With the molecular mass of the plasmid and insert known, the copy number was calculated as earlier mentioned by Verma et al. [24].

## Urea PAGE fingerprinting

We used genus specific forward primer designed from the end of 16S rRNA gene of lactobacilli and universal reverse primer targeting 23S rRNA gene. This would amplify variable length of intergenic spacer region of different *Lactobacillus* species. Equal amount of fecal DNA from each sample within a category was pooled and mixed to minimize the sequencing reactions. This pooled DNA was used as a template to amplify the 16S rRNA and 23S rRNA gene encompassing the intergenic spacer region (ISR) of lactobacilli (Table S2). The amplified product was resolved on 6% urea PAGE revealing unique band profile in different categories of samples. This experiment was performed in triplicate to see if any technical or biological variation exists in band profile during different experiments. In total 15 DNA bands of interest were excised from gel and DNA was eluted, cloned and sequenced. These sequences were deposited to EMBL database to obtain the accession numbers (1). Gel images were processed with gel doc photo imager (alpha imager). Cluster analysis was performed using unweighted pair-group method with arithmetic

mean (UPGMA) and thus dendrogram showing relationship between band profiles in different category was constructed with Pyelph 1.4. Phylogenetic tree was constructed out of the sequences obtained from the cloned products. First of all a multiple-sequence alignment was performed using MUSCLE and the alignment was cured with Gblocks and finally the phylogenetic tree was constructed using maximum likelihood software phymml and visualized in treedyn format (<http://phylogeny.lirmm.fr/phylo.cgi/index.cgi>).

## Gas chromatography (GC) analysis of fecal lactate

Fecal Lactate was analyzed using GC flame-ionization-detection (GC-FID). An aliquot of fecal content (250 mg) was extracted with 1ml of extraction buffer (0.1% (w/v)  $\text{HgCl}_2$  and 1% (v/v)  $\text{H}_3\text{PO}_4$ ) supplemented with 0.045 mg/ml 2,2-dimethylbutyrate (as internal standard). The resulting slurry was centrifuged for 30 min at 5000 g at 4°C, and the supernatant was filtered through 0.2 µm filters. The supernatant collected were analyzed using a GC (Shimadzu-2010, USA) equipped with flame ionization detector (FID) and a stabilwax column (Restek, USA) of 30m length, 530 µm diameter and 1 µm film thickness. The system was run with nitrogen as carrier gas at an inlet constant pressure of 18.1 kpa. Samples were run at an initial temperature of 120°C for 0.5 min, and then with 8°C/min change in temperature till it reached 220°C and hold at 220°C for 8 min with total program time 20.5 min. Lactate was identified using external standards (Sigma, India) and the concentration was calculated using area percentage method [18].

## Real time PCR analysis of MUC 9 gene

Real time reaction was carried out in two steps. In the first step, total RNA was isolated from biopsy samples by Trizol method. Concentration and purity of isolated RNA was determined by nanodrop. Reverse transcription of total RNA was performed by means of the Revert Aid First Strand cDNA Synthesis kit (Fermentas, St. Leon Rot, Germany) using 1 µg of total RNA per sample in a final volume of 20 µl. Quality of cDNA was checked by normal PCR. In the second step, reverse-transcribed products were utilized for quantitative real-time PCR using MUC9 gene specific primer (Table S2). The reaction was carried out in 7500 real time PCR system (Applied Biosystems,

OTU	Match	Control	Moderate	Severe	Remission	Accession no. obtained
L15	<i>L. rogosae</i>	+	-	-	-	KC505222
L11	<i>L. minutes</i>	-	+	-	-	KC505219
L12	<i>L. rhamnosus</i>	-	+	-	-	KC505225
L13	<i>L. amylovorus</i>	-	+	-	-	KC505224
L14	<i>L. plantarum</i>	-	+	-	-	KC505223
L1	<i>L. sakei</i>	+	-	+	-	KC535088
L2	<i>L. vini</i>	-	-	+	-	KC505229
L3	<i>L. apodemi</i>	-	+	+	-	KC505220
L4	<i>L. rimae</i>	-	-	+	-	KC505221
L5	<i>L. agilis</i>	-	+	+	-	KC505227
L6	<i>L. curvatus</i>	-	+	+	-	KC505226
L7	<i>L. delbrueckii</i>	-	-	+	-	KC505228
L8	<i>Enterococcus faecalis</i>	+	+	+	+	KC879284
L9	<i>L. gasseri</i>	+	+	+	+	KC702842
L10	<i>L. casei</i>	+	+	+	-	KC702843

Table 1. Details of OTU obtained with 16S rRNA gene analysis of lactobacilli from human fecal samples (Control and UC).

USA) using Sybr green universal PCR master mixture from Applied Biosystems, USA as per the instructions of the supplier. Prior to each quantitative real-time PCR, cDNA was diluted as required. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as an internal standard and sample values were normalized against GAPDH. The normalized values ( $\Delta CT$ ) of the target genes in patient samples were expressed relative to the normalized values of the target gene in control samples ( $\Delta\Delta CT$ ). The CT values of the gene ranged from 20 to 30. To quantify gene expression the comparative threshold cycle method for relative quantification ( $2^{-\Delta\Delta CT} = n$  fold) was used [25].

### Statistical analysis

Student's t test was applied to compare significant changes in *Lactobacillus* population observed by FISH and qPCR studies. Same test was applied to differentiate the number of bands observed in each category of samples during urea PAGE fingerprinting. Similarities between band profiles were compared between any two group using Pearson coefficient of correlation [26]. In order to see any association of lactobacillus diversity with disease severity, we have performed multiple linear regression analysis-ordinary least squares using multiple regression (vs1.0.29) in free statistics software (v1.1.23-r7) [27]. All experiments were performed in triplicate to find any technical or biological variation.

## Results

### Enumeration of genus *Lactobacillus*

Validation of *Lactobacillus* genus specific probe was done by FISH-microscopy (Figure 1) where hybridization of the probe was clearly seen with members of *Lactobacillus/Enterococcus* group. We observed significant increase in population of *Lactobacillus/Enterococcus* ( $p=0.004$ ) in severe category of UC patients samples compared to controls (Figure 1). *Lactobacillus* (long rods) and *Enterococcus* (coccoid) were distinguished on the basis of morphological feature.

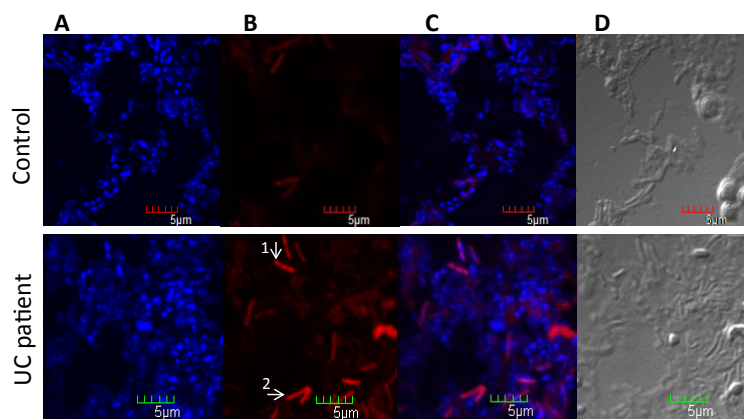
Bacteria belonging to the genus *Lactobacillus/Enterococcus* vs. total bacteria present in a representative control sample was enumerated by flow cytometry analysis as represented in Figure

S2. Similar method was followed for analyzing other samples. Flow cytometry data revealed that the members belonging to the above genus exhibited significant increase in samples of severe ( $p=0.0001$ ) and moderate ( $p=0.0001$ ) category as compared to control. During remission stage of the disease, we observed that *Lactobacillus/Enterococcus* significantly reverted back to normal level, close to controls when compared with samples in severe ( $p=0.009$ ) and moderate ( $p=0.001$ ) conditions of the disease (Figure 2a).

During qPCR analysis, increase of lactobacilli population was observed by 0.8 ( $p=0.05$ ) fold in moderate category, 2.1 ( $p=0.04$ ) fold in severe category and further reduced to 1.79 fold during remission stage in comparison to controls. However, in contrast to flow cytometry data, the value in qPCR analysis did not attain significance in remission category of samples (Figure 2b).

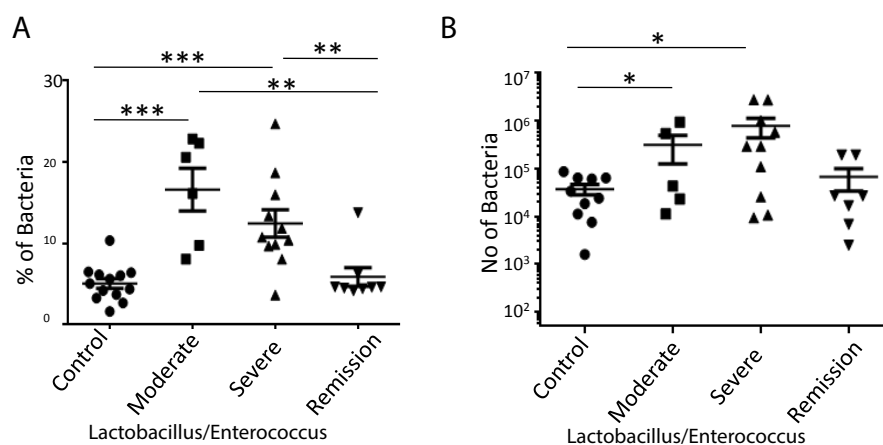
### Diversity in lactobacilli

Urea PAGE (6%) fingerprinting revealed increased number of bands in lanes representing active disease state compared to control. The profile revealed high degree of diversity (on the basis of number of bands) in moderate ( $p=0.002$ ) and severe ( $p=0.003$ ) category of samples as compared to controls (see bottom of Figure 3A). However the profile in remission category was similar to control category ( $p=0.13$ ). Upon comparison of band patterns, Pearson coefficients ( $R^2$ ) values were found to be 0.59 in control versus moderate category; 0.65 for controls vs. severe category; 0.75 for severe vs. moderate category and 0.89 for control versus remission category (Figure 3A). On analyzing the sequenced data from Figure 4, we observed that out of the total [15] bands excised out of the gels, two bands were present in all the categories of samples and upon sequencing represented *Enterococcus faecalis*, *L. gasseri* respectively. These species indicates stable *Lactobacillus sp.* that represents autochthonous flora. *Lactobacillus rogosae* was observed only in control samples however *L. sakei* was detected in both control and severe category of samples. *L. minutes*, *L. rhamnosus*, *L. amylovorus* and *L. plantarum* were detected only in moderate category. *L. apodemi*, *L. agilis* and *L. curvatus* were observed in both moderate and severe category of the fecal samples. Other than the above species *L. vini*, *L. rimae* and *L. delbrueckii* were found only in severe category

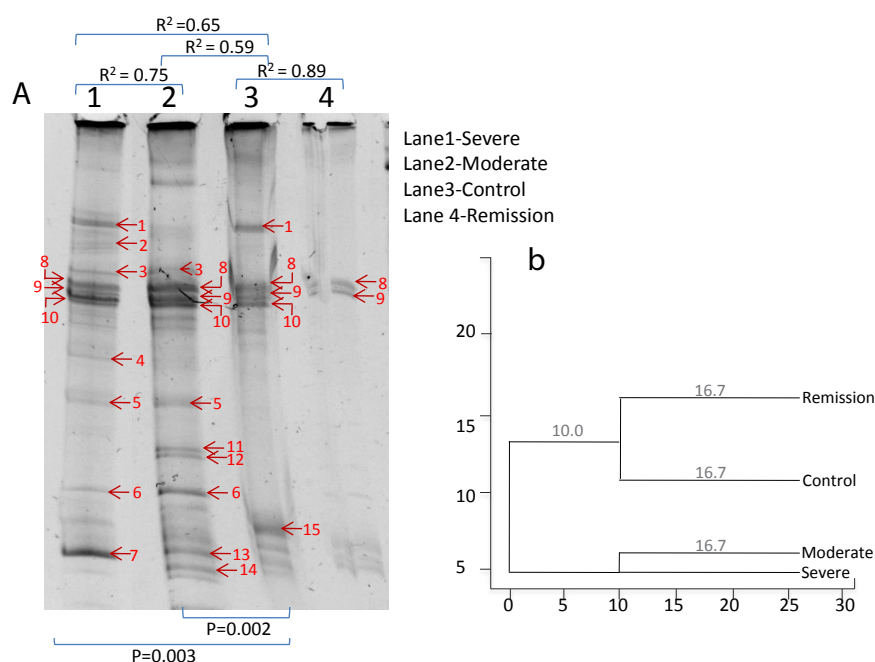


**Figure 1.** FISH (fluorescent in situ hybridization) carried out in control vs patient fecal samples using Lab-158 ((lactic acid bacteria)) labeled Probe. (A) Total cell counts with Hoechst 3342 (B) Lab 158-Cy5 (C) Merge (D) DIC. Arrow 1 and 2 in Fig. b shows the *Enterococcus* and *Lactobacillus* respectively. *Lactobacilli* and *Enterococci* were distinguished on the basis of morphological feature (rod vs. coccoid shape).





**Figure 2.** Enumeration of lactobacilli in fecal samples of UC (ulcerative colitis) patients vs. controls. (A) FISH flow cytometry data expressed as the mean % group specific probe/EUB338+SE. (B) q-PCR data showing the numbers of lactobacilli. The y-axis represents bacteria count and x-axis represents the sample category. Horizontal bars with asterisks represent comparisons between the UC and control.  $P < 0.05$ , \*;  $P < 0.005$ , \*\*;  $P < 0.0005$ , \*\*\*.



**Figure 3.** Representative gel picture of denaturing PAGE showing diversity of lactobacilli. (A) 16S rRNA gene with ISR region of lactobacilli was PCR amplified and resolved on a denaturing 6% polyacrylamide gel. Differences (p value for band numbers and pearsons of coefficient) between respective gel lanes are represented above and below the figure. DNA from the fragments indicated by numbered arrows were cloned, sequenced and compared to known sequences in gene bank as illustrated in phylogenetic tree (Fig 4). (B) The dendrogram showing relationship between band patterns obtained from different disease condition. It was constructed with Pyelph1.4 using unweighted pair-group method with arithmetic mean (UPGMA). Distance between two sample categories is shown.

of UC samples and were absent in other categories (Figures 3 and 4). An OTU (operational taxonomic unit) was defined as a cluster of reads with 97% similarity, and their detail is given in Table 1. Multiple linear regression analysis-ordinary least squares showed a non linear association between the diversity of lactobacillus with the disease conditions depicting R-squared = 0.36 (adjusted R-squared = 0.29) and p-value = 0.007.

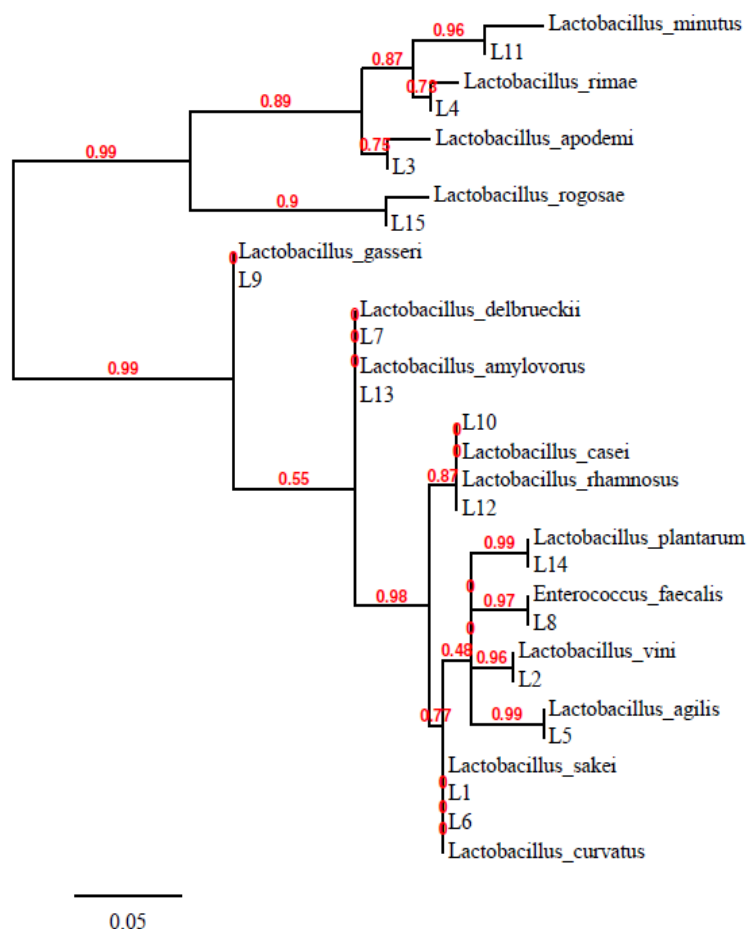
#### Measurement of fecal lactate level

We further quantified the change in concentration of fecal

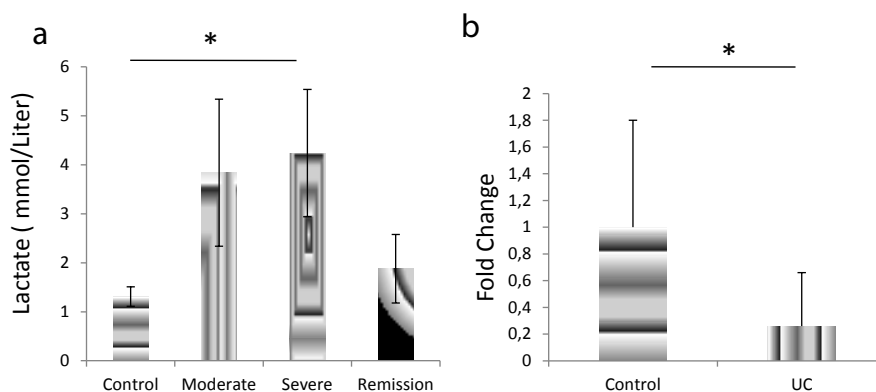
lactate level in control vs. UC fecal samples by Gas chromatography. Concentration of lactate ( $p = 0.032$ ) was significantly increased in severe UC samples when compared with control samples (Figure 5A). However lactate level did not show significant increase in moderate category of samples compared to control. The level further reverted back to normal level in remission category of samples (Figure 5A).

#### Expression of MUC9 gene

The intrinsic capacity of the microbiota to colonize the



**Figure 4.** Phylogenetic tree constructed from the sequences obtained from bands isolated from urea polyacrylamide gel electrophoresis (PAGE) fingerprinting. Phylogenetic tree was constructed using MUSCLE, Gblocks and finally the maximum likelihood software phym. Phylogenetic tree was visualized in treeDyn format. Bootstrap values are shown at branch points; values of 97% or more were considered significant. L1 to L6 represent bands obtained from severe category of samples; L13-L14; L11-L12; L8-10 represents samples obtained from moderate category and L15 represent a band picked up from control sample. Band L8 and 9 were found common in all categories. Band L10 were found common in control, moderate, severe category. L2, L4 and L7 were present only in severe category. L15 was observed only in control. L11 to L14 were detected only in moderate category.



**Figure 5.** Abundance of Lactate  $\pm$  SE (mmol/Liter) and MUC9 (mucin) gene expression. A) Abundance of Lactate  $\pm$  SE (mmol/Liter) in fecal samples of UC patients vs. Control subjects analyzed by gas chromatography. The y-axis represents concentration of lactate and x-axis represents the sample category. B) Expression of MUC9 in biopsy samples through RT-PCR. Horizontal bar with asterisks represent comparisons between the UC and control. Horizontal bars with asterisks represent comparison between the UC and control.  $P < 0.05$ ,\*.

mucus is influenced by host factors. Members of *Lactobacillus* are known to adhere to the mucin layer of gut. In order to check the status of mucin layer during UC, we targeted the MUC9 gene expression. MUC9 mRNA expression was significantly decreased by 4.54 fold in colonic mucosa of active UC compared to control samples ( $p=0.011$ ) as shown in Figure 5B. This result indicates the probable role of MUC9 gene in the pathogenesis of UC.

## Discussion

Increased counts of total facultative anaerobes including *Lactobacillus* and *Enterococcus* as observed in our study, have also been reported in feces of UC patients [28,29]. Members of *Lactobacillus/Enterococcus* group reported in our population represented 5.09 +0.5% of total bacteria whereas other studies reported only about 0.01% of the total counts [30,31]. Greater number and diversity of enterococci was observed earlier in feces of UC patients compared to healthy controls [32]. Our probe includes the members of *Lactobacillus/Enterococcus* group, therefore, it may be assumed that the higher representation as observed by FISH flow cytometry and qPCR analysis probably represents contribution of both the groups.

Urea PAGE analysis revealed some new species of lactobacilli like *L. minutes*, *L. vini*, *L. apodemi*, *L. rimae*, *L. agilis* in human feces which have not been reported earlier. These species can be designated as allochthonous to the human intestinal tract. Details of species obtained in fecal samples are given in Table 1. Beneficial properties of *Lactobacillus* species for e.g. *L. amylovorus* and *L. brevis*, *L. plantarum*, *L. reuteri* [33-36] are well known in potentially suppressing other pathogenic populations within the intestinal microbiota. The limitation of our study includes, restricted sample size due to criteria followed for sample collection. Only a limited number of DGGE bands were sequenced and faecal samples were pooled for diversity analysis. Higher concentration of lactic acid in fecal samples of UC patients than that of control subjects further supported the increase in fecal lactobacilli population. Increased lactate level in IBD fecal samples was detected earlier [37] and has been found to be associated with higher risk of diarrhea and mucosal inflammation [38]. Lactic acid can be further metabolized by propionibacterium to propionic acid and acetic acid or to butyric acid by butyrate producers through the acetyl-CoA pathway [39,40]. Thus the reduction in population of butyrate producing members observed earlier by our group in the same UC patient samples [18] may partially contribute to increase in lactate concentration.

Members of the genus *Lactobacillus* have the ability to adhere to intestinal mucus and extracellular matrix through various putative adherence factors possessing mucin binding domain [14]. Our finding of increased lactobacilli population in UC fecal samples was in contrast to significant decrease that was observed earlier in our laboratory as well as by others in mucosal biopsy samples of UC patients [25] and pouchitis patients [41]. We here hypothesize that the reduction in lactobacilli in biopsy samples observed earlier may be due to altered mucus layer during active UC, which may be less conducive for lactobacilli adherence. These alterations in GIT (gastrointestinal tract) milieu may lead to detachment of lactobacilli from the intestinal epithelium and lead to subsequent passage through the GIT. Various studies have shown that the mucus layer is structurally

altered and become thin in ulcerative colitis patients [42]. Buisine et al. (2001) reported no change in expression of MUC2 and MUC3, however reduced expression of MUC1, MUC4 with no expression of MUC5AC, MUC5B, MUC6 in CD patients [43]. Our result confirmed a significant reduction in the expression of MUC9 gene in the biopsy samples of active UC patients with a simultaneous increase in lactobacilli population during disease condition. This expands our knowledge on the role of mucin in the pathogenesis of UC as also observed earlier. The change in MUC 9 gene expression as also observed earlier in UC patients [15] may lead to defect in the mucus layer and alter the physical barrier. However, the stringent criteria followed for sample collection yielded limited number of samples for our analysis. We have been able to sequence only a limited number of bands from urea PAGE fingerprint.

## Conclusion

Our observation reveals the status of lactobacilli in Indian patients during different stages of UC which has not been explored so far in detail. The increase in number of *Enterococcus/Lactobacillus* in control as well as in disease condition was confirmed by FISH, FISH flow cytometry, qPCR and GC analysis with simultaneous decrease in mucin. Increase in diversity of this group was confirmed by urea PAGE fingerprinting method. We speculate the release of many lactobacilli spp. during active condition of the disease is perhaps due to loss of mucin layer necessary for these bacteria to adhere to intestinal epithelial cell lining. The increased excretion of lactobacilli with feces may be either a cause or a consequence of the disease.

## Acknowledgements

We gratefully acknowledge the subjects who participated in this study. JP is grateful to CSIR, New Delhi, for the research grant (37(1387)/09/EMR-II) obtained to conduct this study. RK is thankful to CSIR for the research fellowship. We also acknowledge Advanced Instrumentation Research Facility (AIRF) of the University for providing the confocal microscope facility.

## Conflict of Interest

There are no conflicts of interest among the authors.

## References

1. Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroenterol*. 2008;134(2):577-594.
2. De Vos WM. Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek*. 1996;70(2-4):223-242.
3. Walter J. Ecological role of lactobacilli in the gastrointestinal tract: Implications for Fundamental and Biomedical Research. *Appl Environ Microbiol*. 2008;74:4985-4996.
4. Cross ML, Gill HS. Can immune regulatory lactic acid bacteria be used as dietary supplements to limit allergies? *Int Arch Allergy Immunol*. 2001;125:112-119.
5. Marteau PR. Probiotics in clinical conditions. *Clin Rev Allergy Immunol*; 2002;22(3):255-273.
6. Wang S, Ng LH, Chow WL, Lee YK. Infant intestinal *Enterococcus faecalis* down-regulates inflammatory responses in human intestinal cell lines. *World J Gastroenterol*. 2008;14(7):1067-1076.
7. Lablanc DJ (2006) *Enterococcus*, The Prokaryotes: a book on the biology of Bacteria: Firmicutes, Cyanobacteria. Ed: Dworkin M, Falkow S, Schliefer KH. 4: 175-204.

8. Zhang M, Liu B, Zhang Y, et al. Structural Shifts of Mucosa-Associated Lactobacilli and *Clostridium leptum* Subgroup in Patients with Ulcerative Colitis. *J Clin Microbiol*. 2007;45(2):496-500.
9. Ouwehand AC, Salminen S, Roberts PJ. Disease-dependent adhesion of lactic acid bacteria to the human intestinal mucosa. *Clin Diagn Lab Immunol*. 2003;10(4):643-646.
10. Verma R, Verma AK, Ahuja V, Paul J. Real-time analysis of mucosal flora in patients with inflammatory bowel disease in India. *J Clin Microbiol*. 2003;48(11):4279-4282.
11. Fernández MF, Boris S, Barbe's C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *J Appl Microbiol*. 2003;94(3):449-455.
12. García-Martínez J, Acinas SG, Antón AI, Rodríguez-Valera F. Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. *J Microbiol Methods*. 1999;36(1-2):55-64.
13. Arias CR, Abernathy JW, Liu Z. Combined use of 16S ribosomal DNA and automated ribosomal intergenic spacer analysis (ARISA) to study the bacterial community in catfish ponds. *Lett Appl Microbiol*. 2006;43(3):287-292.
14. Velez MP, De Keersmaecker SC, Vanderleyden J. Adherence factors of *Lactobacillus* in the human gastrointestinal tract. *FEMS Microbiol Lett*. 2007;276(2):140-148.
15. Yamamoto-Furusho JK, Mendivil-Rangel EJ, Fonseca-Camarillo BSG. Reduced expression of Mucin 9 (MUC9) in patients with ulcerative colitis. *Inflamm Bowel Dis*. 2012;18(3):E601.
16. Walmsley RS, Ayres RC, Pounder RE. A simple clinical colitis activity index. *Gut*. 1998;43(1):29-32.
17. Tursi A, Brandimarte G, Papa A, et al. Treatment of relapsing Ulcerative Colitis with the probiotic VSL # 3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol*. 2010;105(10):2218-2227.
18. Kumari R, Ahuja V, Paul J. Fluctuations in butyrate producing bacteria in ulcerative colitis patients of North India. *World J Gastroenterol*. 2013;19(22):3404-3414.
19. Amann RL, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol*. 1990;172(2):762-770.
20. Wallner G, Amann R, Beisker W. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry*. 1993;14(2):136-143.
21. Kilian S, Christiane D, Holger D, Wagner M. Double-labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. *Appl Environ Microbiol*. 2010;76(3):922-926.
22. Harmsen HJM, Elfferich P, Schut F, Welling GW. A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in fecal samples by fluorescent in situ hybridization. *MicroEcol Health Dis*. 1999;11(1):3-12.
23. Zoetendal EG, Ben-Amor K, Harmsen HJM, et al. Quantification of uncultured *Ruminococcus obeum*-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted Probes. *Appl Environ Microbiol*. 2002;68(9):4225-4232.
24. Verma AK, Verma R, Ahuja V, Paul J. Real-time analysis of gut flora in *Entamoeba histolytica* infected patients of Northern India. *BMC Microbiol*. 2012;12:1-11.
25. Verma N, Ahuja V, Paul J. Profiling of ABC transporters during active ulcerative colitis and in vitro effect of inflammatory modulators. *Dig Dis Sci*. 2013;58(8):2282-2292.
26. Sawa O, Fujisawa T, Pukall R. *Lactobacillus apodemi* sp. nov., a tannase producing species isolated from wild mouse faeces. *Int J SystEvolMicrobiol*. 2006;56(Pt 7):1693-1696.
27. Wessa P (2013) Multiple Regression (v1.0.29) in Free Statistics Software (v1.1.23-r7), Office for Research Development and Education.
28. Takaishi H, Matsuki T, Nakazawa A, et al. Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. *Int J Med Microbiol*. 2008;298(5-6):463-472.
29. Andoh A, Imaeda H, Aomatsu T, et al. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol*. 2011;46(4):479-486.
30. Sghir A, Gramet G, Suau A, et al. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol*. 2000;66(5):2263-2266.
31. Walter J, Hertel C, Tannock GW, et al. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol*. 2001;67(6):2578-2585.
32. Vander Wiel –Korstanje JA, Winkler KC. The faecal flora in ulcerative colitis. *J Med Microbiol*. 1975;8(4):491-501.
33. Kant R, Paulin L, Alatalo E, de Vos WM, Palva A. Genome Sequence of *Lactobacillus amylovorus* GRL1118, Isolated from Pig Ileum. *J Bacteriol*. 2011;193(12):3147-3148.
34. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C.  $\gamma$ -Aminobutyric acid production by culturable bacteria from the human intestine. *J Appl Microbiol*. 2012;113(2):411-417.
35. Paolillo R, Carratelli CR, Sorrentino S, Mazzola N, Rizzo A. Immuno modulatory effects of *Lactobacillus plantarum* on human colon cancer cells. *IntImmunopharmacol*. 2009;9(11):1265-1271.
36. Konstantinov SR, Awati A, Smidt H, et al. Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the guts of weaning piglets. *Appl Environ Microbiol*. 2004;70(7):3821-3830.
37. Huda-Faujan N, Abdulmir AS, Fatimah AB, et al. The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects. *Open Biochem*. 2010;4:53-58.
38. Cummings JH, Macfarlane GT, Englyst HN. Prebiotic digestion and fermentation. *Am J Clin Nutr*. 2001;73(2 Suppl):415S-420S.
39. Macfarlane GT, Cummings JH. In *The Large Intestine: Physiology, Pathophysiology, and Disease*. Philips, S.F. Ed. Raven Press, Ltd: London 1997;51-52.
40. Bourriaud C, Robins RJ, Martin L, et al. Lactate is mainly fermented to butyrate by human intestinal microflora but inter-individual variation is evident. *J Appl Microbiol*. 2005;99(1):201-212.
41. Fabia R, ArRajab A, Johansson ML, et al. Impairment of bacterial flora in human ulcerative colitis and experimental colitis in the rat. *Digestion*. 1993;54(4):248-255.
42. Rhodes JM. Colonic mucus and ulcerative colitis. *Gut*. 1997;40(6):807-808.
43. Buisine MP, Desreumaux P, Leteurtre E, et al. Mucin gene expression in intestinal epithelial cells in Crohn's disease. *Gut*. 2001;49(4):544-551.



# Supplementary Tables

Feature	UC (n=12)	Control(n=10)
Sex (F/M) (%)	3 (25)/9 (75)	2 (20)/8(80)
Age at diagnosis mean $\pm$ SD (years)	34.33 $\pm$ 15.64	36.7 $\pm$ 16.3
<b>Disease extent, n (%)</b>		
Proctitis	2 (16.66)	-
Left sided colitis	2 (16.66)	-
Pancolitis	5 (41.66)	-
None of the above	1 (8.33)	-
<b>Smoking history</b>		
Yes	2 (16.66)	1 (10)
No	10 (83.33)	9 (90)
Appendectomy Y/N (%)	2(16.66)/10(83.33)	3 (30)/7 (70)
Family history Y/N (%)	1(8.33)/11 (91.66)	0/14

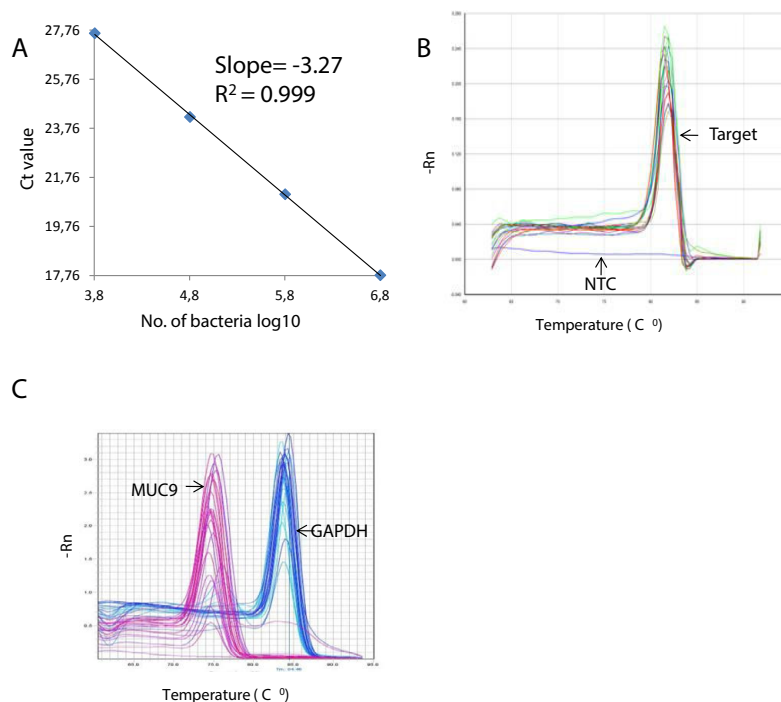
**Table S1.** Clinical and demographic features of biopsy samples.

Primer or probe	Target	Sequence (5'-3')	Used as ( <sup>a</sup> FISH, <sup>b</sup> qPCR, <sup>c</sup> PAGE, <sup>d</sup> RT-PCR)	Reference
NON EUB 338	No bactéria	ACATCCTACGGGAGGC	Probe <sup>a</sup>	[20]
EUB 338	MostBacteria	GCTGCCTCCCGTAGGAGT	Probe <sup>a</sup>	[19]
Lab158	<i>Lactobacillus/ Enterococcus</i>	GGTATTAGCAYCTGTTTCCA	Probe <sup>a</sup>	[22]
LacFP	<i>Lactobacillus-FP</i>	Forward Primer GCAGCAGTAGGGAATCTTCCA	Primer <sup>b,c</sup>	[10]
LacRP	<i>Lactobacillus</i>	Reverse Primer GCATTYACCGCTACACATG	Primer <sup>b</sup>	[10]
23S RP	23S rRNA Universal	Reverse Primer GGGTTBCCCCATTCRG	Primer <sup>c</sup>	[43]
MUC-FP	Mucin9	CCGATGGACAGTAGTGTTCCTCA	Primer <sup>d</sup>	[15]
MUC-RP	Mucin9	AATTCTCTACCCAGAGTTCAACAAA	Primer <sup>d</sup>	[15]
GAPDH-FP	GAPDH	GCTCCTCCTGTTCGACAGTCA	Primer <sup>d</sup>	[25]
GAPDH-RP	GAPDH	GCAACAATATCCACTTTACCAG	Primer <sup>d</sup>	[25]

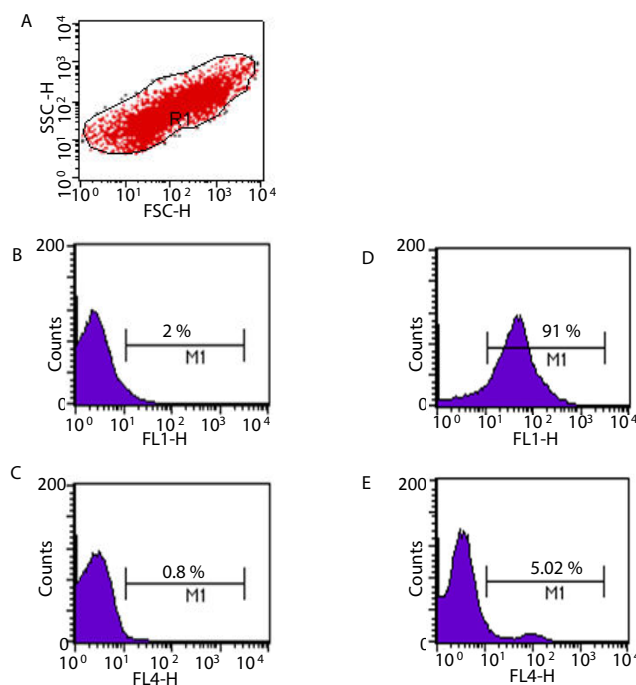
Superscript <sup>a,b,c,d</sup>represents oligonucleotides used in FISH, qPCR, Urea PAGE and RT-PCR respectively.

**Table S2.** Probes and oligonucleotides used in this study.

## Supplementary figures



**Figure S1** Curves obtained from PCR analysis. (A) Standard curve. (B) Dissociation curve obtained from q PCR of *Lactobacillus* bacteria. (C) Dissociation curve for the MUC-9 gene analysis by RT PCR.



**Figure S2** Flow cytometric analysis of fecal microbiota using 16S rRNA targeted probes. (A) The region R1 corresponding in relative size (FSC) - granularity (SSC) of the bacteria was delineated. This region R1 was gated for further dot plots and histograms. Flow cytometry histograms were obtained when the cell suspension was hybridized with (B, C) NONEUB338 probe (D, E) combination of EUB338 probe and Lab 158-Cy5. When the Fixed cell suspension was hybridized with the negative control probe NONEUB 338, auto fluorescence level in FL1 and FL4 ranged between 0 and 10<sup>1</sup>, and the region M1 was defined. A shift in fluorescence to higher intensities was obtained upon hybridization with labeled probe. FL1 histogram shows green fluorescence intensities conferred by EUB 338-FITC probe (Fig C and F). FL4 histogram shows the red fluorescence intensities conferred by Lab 158-Cy5 probe (Figs D and G). The events under M1 represent the proportion (%) of lactobacilli cells hybridized with the Lab 158-Cy5 probe within the total bacterial cells hybridized with the domain bacteria probe EUB 338 (Fig E). The proportion of cells was corrected by subtracting the background fluorescence, which was measured using the negative control NON-EUB 338 probe.