

# Comparative Evaluation of *Lactobacilli* and *Bifidobacteria* in Healthy Individuals

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## ABSTRACT

*Lactobacillus* and *Bifidobacterium spp.* are known as the major group that constitutes probiotic bacteria that form part of the normal biota of humans. The ecological significance of probiotic bacteria in the human gut cannot be exaggerated as studies have revealed many of the health benefits they offer. However, much is yet to be known about its population variation between and within individuals. Five apparently healthy volunteers were recruited and advised not to consume yogurt, antibiotics, alcohol and steroid three weeks before and during the study. Three (3) fecal samples from each participant obtained at two weeks interval (total of 15 samples a week) for six weeks were examined for each subject bacteria. Samples were collected in sterile specimen jars and immediately taken to the laboratory for analysis. Rogosa and BIM-25 selective media were used for the enumeration of *Lactobacillus spp.* and *Bifidobacterium spp.* respectively using plate count method. *Lactobacilli* were classified at the specie level using API 50 CHL kit, while *Bifidobacteria* isolates were identified at the genus level by detecting the presence of Fructose-6-phosphate phosphoketolase (F6PPK) activity. Both groups of bacteria were also identified at the genus level using a genus-specific primer set. All selected isolates obtained from the respective culture media were confirmed to be *Lactobacilli* and *Bifidobacteria*. The data obtained showed that intra-individual variation of the population of *Lactobacillus* and *Bifidobacterium spp.* was significantly lower than inter-individual variation at  $P < 0.05$ . This study establishes the fact that the composition level of *Lactobacillus* and *Bifidobacterium spp.* varies between healthy individuals, but with little or no variation within healthy individuals.

**Keywords:** *Lactobacilli*; *Bifidobacteria*; Intra-individual; Inter-individual; Variation; Population; Gut

## INTRODUCTION

The human Gastrointestinal Tract (GIT) is known to be richly endowed with large numbers of microorganisms [1]. This in effect constitutes a high level of the complexity of the gut microbial communities. Available literature shows varying number of bacterial species present in the human gut, but it has been generally speculated that the number of species of bacteria residing in the human is approximately 500 to 1000 and ranges between 10<sup>11</sup>-10<sup>12</sup> cells per gram of faeces [2-5]. Among these numbers of species are those that are able to influence significant commercial and clinical interests [6,7]. The idea of probiotics as defined by Guarner and Schaafsma as "live microorganisms, which when administered in adequate amounts, confer a health effect on the host" has been on for about a century [8]. Yet its understanding of the impact on human overall health is still an emerging concept. However, there is a substantial and growing body of evidence that shows that these microbes provide enormous benefits on the host in which they reside [9-11].

*Lactobacilli* and *Bifidobacteria* have been reported to constitute two extremely important groups of probiotic bacteria of the

gastrointestinal microbiota of man and animals [10]. These two important probiotics are usually added to several fermented dairy products and have been shown to confer an inhibitory growth effect against a wide range of intestinal pathogens in humans and animals [12,13]. In recent years, studies on understanding the roles of these two groups of bacteria in the intestine have continue to receive considerable attention.

Research has shown that the varying population distribution of this group of probiotic bacteria across various region of the gut is influenced by a number of factors such as biochemical and physiological requirements which are peculiar to certain region of the gut [14]. Available literature has also shown that each individual's intestinal ecosystem possesses its unique characteristics and these characteristics are not uniform over time [15].

There are emerging evidences that shows significant variation of the composition level of *Lactobacilli* and *Bifidobacteria* in human gut, and that the population level of these subject probiotics are peculiar to individuals. That is, individual will have fixed microbiota as far as quantities and qualitative structure of strains of *Lactobacillus spp.* and *Bifidobacterium spp.* are concerned [16]. However, much is yet

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to be known of the comparative population of *Lactobacillus spp.* and *Bifidobacterium spp.* in apparently healthy individuals.

This present study was aimed at evaluating inter and intra individual variation of *Lactobacilli* and *Bifidobacteria* among healthy individuals.

## MATERIALS AND METHODS

Prior to collection of samples, participants were advised not to consume yogurt, antibiotics, alcohol and steroid, but maintain their usual diet three weeks before and during the time of collection of samples in order not to alter the microbial population level in their gut. Fecal samples were collected into a sterile screw-capped plastic container from five apparently healthy (i.e without underlying conditions) volunteers who were within the age bracket of 25-35 years old. Written consents were obtained from individual participants living within North West London in England. The participants include 2 males and 3 females. Three sets of the fecal samples were obtained from individual volunteers at two weeks interval for six weeks.

The reference bacterial cells for identification of the target bacteria were generously donated by Middlesex University's Microbiology Laboratory. These strains were also used for the optimization of PCR conditions as positive control for the genus-specific primers.

### Isolation, characterization and quantification of *Lactobacillus spp.* and *Bifidobacterium spp.*

Collected faecal samples were immediately processed upon arrival to the Microbiology Laboratory of Middlesex University Hendon campus. One gram (wet weight) of the fecal sample obtained from individual volunteers was weighed and collected in a 30 ml sterile universal bottle, another 1 gram was weighed and stored in the freezer at -20°C for the PCR analysis.

**Lactobacilli:** An aliquot of 100 µl from each of the serially diluted suspensions (10<sup>1</sup> to 10<sup>6</sup>) were inoculated in triplicate on plates of Rogosa agar media (MRS, pH 5.4 ± 0.2, Oxoid CM0627). The inoculated plates were incubated under anaerobic condition (GasPak™ EZ Gas Generating container system, Becton Dickinson and Company, USA) at 37°C for 72 hrs. After 72 hrs of incubation, four colonies based on their differences in size and shapes were picked and sub-cultured on MRS agar (de Man Rogosa Sharpe, pH 6.2 ± 0.2, Oxoid, CM0359) for further characterization.

**Bifidobacteria:** To isolate *Bifidobacterium spp.*, faecal samples were serially diluted up to 10<sup>-6</sup> in phosphate buffer solution supplemented with L-cysteine. The volume 100 µl of each of the diluted colony suspensions were inoculated in triplicate onto BIM-25 *Bifidobacteria* selective media. The BIM-25 was prepared as previously described by Munoa and Pares, which constitutes the following in grams/liter: Reinforced clostridial agar 51; nalidixic acid, 0.02; polymyxin B sulfate, 0.0085; kanamycin sulfate, 0.05; iodoacetic acid, 0.025; and 2,3,5-triphenyltetrazolium chloride, 0.025 [17]. The Plates were then incubated anaerobically (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) at 37°C for 72 hours. Representative isolates with different morphology were

aseptically picked and sub-cultured on a Reinforced Clostridial Medium (BD, Germany) for further analysis.

To phenotype *Lactobacillus spp.* various biochemical tests including catalase test, bile aesculine, and phenol red glucose broth with gas production test were used. Fermentation of carbohydrates was determined using API 50 CHL (API® BioMérieux, Durham, NC, USA), a standardized system, consisting of 50 biochemical tests for the study of carbohydrate metabolism by microorganisms. The procedure for the API test was carried out according to the manufacturer's instruction.

For *Bifidobacterium spp.*, colonies that appeared with different morphology on the selective media were further identified by detecting the presence of Fructose-6-phosphate Phosphoketolase (F6PPK) activity as previously described by Scardovi [18].

### PCR qualitative analysis

Suspension of known colonies of *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* were used directly as a DNA template to test the sensitivity of the primers. To genotype *Lactobacillus spp.*, PCR reaction was carried out in a total volume of 25 µl with reaction mixture that contains 2.5 µl of 10X PCR buffer, 2 µl of 2.5 mM dNTP, 2 µl of 25 mM MgCl<sub>2</sub>, 1 µl of 1 µM primer each of the forward and reverse, 0.2 µl of 5 U/µl of Red hot Taq DNA, 15.3 µl sterile water and 1 µl of suspension of colony template. Colony of *Lactobacillus acidophilus* was used as the DNA template under the following PCR conditions; initial denaturation temperature of 96°C for 5 min to facilitate disruption of the cell wall and eruption of the cell content, followed by 30 cycles of which each cycle consisted of each step of denaturation at 95°C for 30 sec, annealing at 54.4°C for 30 sec and extension for 30 sec at 72°C. The final extension was set at 72°C for 7 min and final hold at 4°C. Sterile water was used as negative control in all the experiments (Table 1). For *Bifidobacteria spp.*, amplification of the target bacterial DNA was performed with primers g-Bifid-F and g-Bifid-R in a PCR iCycler apparatus (Table 1). The PCR mixture was composed of 1X PCR buffer from 10X reaction buffer with 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of deoxynucleotide triphosphates (dNTPs) from 20 mM, 0.125 µl each of forward and reverse primer, and 0.625 U of Red hot Thermoscientific Taq DNA polymerase from 5 U µl<sup>-1</sup>. Colony PCR was performed to identify the different colonies obtained from the BIM-25 *Bifidobacteria* selective media. A reaction volume of 24 µl was inoculated with 1 µl of the test colony suspension. *B. adolescentis* was used as the positive control, while distilled water was used as the negative control. The set up were subjected to PCR process under the following conditions; Initial denaturation of 95°C for 5 min, followed by 30 cycles of which each cycle consisted of each step of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension for 30 sec at 72°C. The final extension was set at 72°C for 5 min and final hold at 4°C. The primer sets used in this study was adopted from Dubernet et al. and Matsuki et al., and these corresponds to the flanking sequence of the 16S rRNA gene which is known to be highly conserved among the genus *Lactobacillus* and *Bifidobacteria* respectively (Table 1) [19,20].

Bacteria	Primers	Sequence	Product size (bp)
<i>Lactobacillus</i>	LbLMA1-rev	CTCAAAACTAAA- CAAAGTTTC	250
	R16-1	CTTGTACACACCGCCC- GTCA	
<i>Bifidobacterium</i>	g-Bifid-F	CTCCTGGAAACGGGTGG	549-563
	g-Bifid-R	ACATCTATAGCCCTTCTT- GTGG	

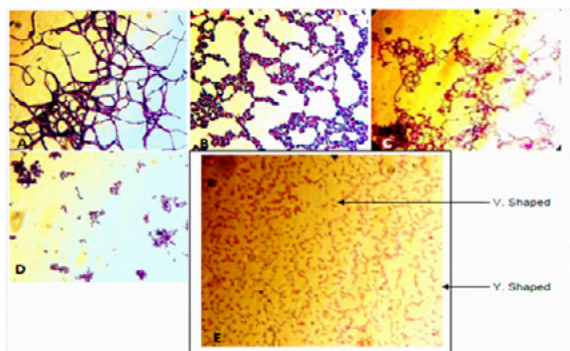
All the PCR products obtained after amplification were checked for the expected size on 2% (w/v) agarose gel stained with ethidium bromide. Gels were run for 45 min at 110 V in TBE electrophoresis buffer. Size marker of 100 bp was used on each gel. Gels were visualized with an UV trans-illuminator.

**Statistical analysis of data**

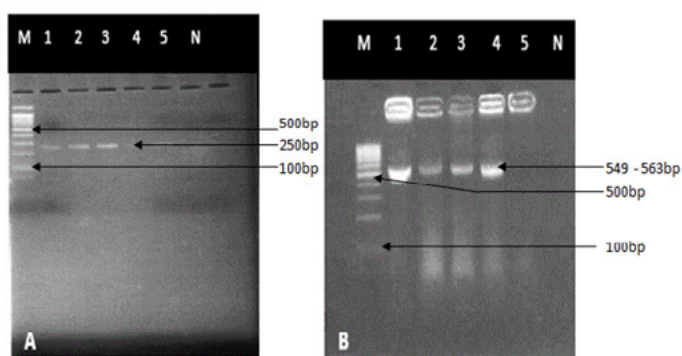
Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 21 to determine the mean, standard deviation and Analysis of Variance (ANOVA) of the data obtained.

**RESULTS AND DISCUSSION**

All the isolates recovered from the respective selective media for the target bacteria were confirmed to be species of *Lactobacillus* and *Bifidobacterium* respectively (Tables 2 and 3). The statistical analysis of the data obtained using SPSS version 21 showed that there was significant difference at  $P < 0.05$  between the participants for both *Lactobacilli* and *Bifidobacteria* respectively (Figures 1 and 2). The population level of *Lactobacilli* in participant A, C and D showed no significant difference (at  $P < 0.05$ ) throughout the duration of the study.



**Figure 1:** Cell Morphology of isolates: a-d) Representative of different species of *Lactobacillus* e) “V” and “Y” shaped *Bifidobacterium*. Bar=10 µm.



**Figure 2:** PCR amplification A) M. 100bp size marker, 1. *Lactobacillus acidophilus*, 2-5. *Lactobacillus spp.* (250bp) B) M. 100bp size marker, 1 *Bifidobacterium spp.* (549-563bp).

However, a significant difference was recorded in the level of *Bifidobacteria* load isolated from participants A, B, D and E. Participant B shows a higher load of *Bifidobacteria* in the first and third sample collection. The result pattern for participants C showed a low population of both genera. Comparatively, the population level of the two group of bacteria shows a significant difference at  $P < 0.05$  between participants A-E. Participant B however maintained high level of both genera.

The result obtained in this study shows a significant inter-individual variation of the composition level of both *Lactobacilli* and *Bifidobacteria*. This suggests that considerable interpersonal or inter-individual variation exists (Table 2).

**Table 2:** API result of identified species using the apiweb™ computer software.

Subject	Colony morphology	Comment	Specie/Strain identified
A	Rhizoid	Very good	<i>Lactobacillus acidophilus</i> 1
	Circular 2 mm	Good	<i>Lactobacillus fermentum</i>
B	Rhizoid	Doubtful	<i>Lactobacillus acidophilus</i> 1,2 and 3
	Circular 2 mm	Good	<i>Lactobacillus fermentum</i> 1
C	Circular 2 mm	Good	<i>Lactobacillus salivarius</i>
	Rhizoid	Very good	<i>Lactobacillus acidophilus</i> 1
D	Circular 2 mm	Good	<i>Lactobacillus fermentum</i>
	Rhizoid	Very good	<i>Lactobacillus acidophilus</i> 1
E	Circular 2 mm	Very good	<i>Lactobacillus plantarum</i>

**Table 3:** Gram Staining and F6PPK test for the confirmation of *Bifidobacteria* isolates.

Colony type	Gram’s reaction and cell morphology	F6PPK test
Pale pink colonies with dark center	Gram positive, cocobacilli, club shape, Y and V branching Diplo & strep	Positive
Red colonies	Gram positive, cocobacilli, club shape, Y and V branching	Positive
Very small colonies	Gram positive, cocobacilli, club shape, Y and V branching	Positive
Bright pink colonies	Gram positive, cocobacilli, club shape, Y and V branching	Positive

It also evidenced in this study that intra-individual variation of the subject bacteria at different time point exists (Table 2). Although, there is an exception for volunteer A (*Lactobacillus spp.*), C (*Lactobacillus spp.* and *Bifidobacterium spp.*) and D (*Lactobacillus spp.*) whose population level shows no significant difference throughout the period of the study. This might be attributable to a number of factors such as lifestyle and food consumption habit, although, participants were advised not to consume Yogurt, antibiotics and steroids, but were told to continue with their usual diet. Previous study has shown that diet constitutes one of the major factors that influence the population level of probiotics [21-23]. Intake of probiotic growth promoting factors like prebiotics has been shown

to contribute to the stimulation of the growth of *Bifidobacteria* and *Lactobacilli* in human intestine [24].

The population level of *Lactobacillus* as obtained in this study ranges between 106 and 108 cfu/g (Table 4). This is in agreement with Walter who explained that, when total anaerobic culturing techniques are used, *Lactobacilli* form a very small proportion of the cultivable human fecal microbiota and can rarely be cultured at population levels exceeding 108 CFU/g [25]. Previous studies have also reported similar averages of 106 and 108 CFU/g of *Lactobacillus spp.* in human faecal samples [26-28]. Lines of evidences have shown that the population of *Lactobacillus spp.* accounts for only about 0.01% of the total cultivable counts in human faeces [25]. As evidenced in this study, Participant C and D who happens to be female showed lower levels of *Lactobacillus spp.* this have also been observed in previous study on apparently healthy female [29]. A recent study has attributed the low population level of gut microbiota in women to a number of factors among which is the change in hormonal conditions [30,31]. Moreover, Subject-to-subject variation has been reported to be significant, and *Lactobacillus spp.* is not detectable in around 25% of human fecal samples [32]. The Human Microbiome Project consortium showed that intra-individual fluctuations of microbial composition occur at different stages of life due to a number of factors including but not limited to environmental changes, lifestyle and pathological

events [33]. Buddington et al. also in their findings showed that the type and number of microbial species that persist and colonize the GI is not determined by chance, but by a combination of factors including inflammatory state of the host, diet, host genetics and environmental factors [3,34]. It is not clear what factors that modulate the composition of the intestinal microbial populations of individual humans, although it has been concluded by some that the genetic characteristics of the host are important [35]. Although, the methodology as used in this study did not allow for the detection of specie composition of both group of bacteria to determine which specie is numerically predominant in the samples. However, *L. acidophilus*, *L. fermentum*, *L. salivarius* and *L. plantarum* were the common isolates in this study, where *L. plantarum* was found repeatedly only in one participant (E). Previous studies have revealed the following species of *Lactobacillus* that are commonly found in human faeces: *L. paracasei*, *L. rhamnosus*, *L. gasseri*, *L. fermentum*, *L. plantarum* [36]. It is therefore, most likely to have certain species of either genus that is unique to individual subjects. The genus *Bifidobacterium* has been discovered to contain up to four or five different species that is commonly found in the colon of adults [37,38]. Evidence in this study thus suggests the possibility of isolating only a small number of specie of both genera which in effect might not represent the true inhabitants of the participant's gastrointestinal tract.

Isolates/Week	Participants				
	A	B	C	D	E
<i>Lactobacillus spp.</i>					
1	4.00 ± 0.58b	21.00 ± 0.58d	0.09 ± 0.01a	0.01 ± 0.00a	9.33 ± 0.67c
2	5.17 ± 0.44b	19.00 ± 0.58c	0.06 ± 0.01a	0.002 ± 0.00a	0.13 ± 0.04a
3	8.17 ± 0.44b	17.00 ± 1.16c	0.90 ± 0.06a	0.70 ± 0.06a	9.00 ± 0.58b
<i>Lactobacillus spp.</i>					
1	1.60 ± 0.06ab	14.00 ± 0.58d	0.85 ± 0.04a	1.83 ± 0.09b	3.60 ± 0.15c
2	1.80 ± 0.06b	7.30 ± 0.21e	0.55 ± 0.05a	2.33 ± 0.15c	5.60 ± 0.12d
3	0.50 ± 0.58a	15.67 ± 0.67c	0.18 ± 0.01a	14.00 ± 1.16c	9.70 ± 0.36b

**Note:** Values are means of triplicate readings ± standard deviation. Values with no common superscripts differ (P<0.05).

**Table 4:** *Lactobacillus spp.* and *Bifidobacterium spp.* load isolated from faecal samples of different participants.

It is possible that the isolates in this study might have been transient bacteria as previous studies have shown that most of the probiotic bacteria present in faeces are allochthonous members derived from fermented food, oral cavity, or at the proximal parts of the colon [23]. Literature reports have also shown that human have to some extent a stable autochthonous *Lactobacilli* and *Bifidobacteria* [9,39]. A clear characteristic of autochthonous intestinal inhabitants is shown by the stable population of strains over several months in the intestinal tract without having significant upstream populations [9]. It is also known that autochthonous strains that naturally persist in human subjects over long periods are tested by nature for their functionality in the gut, and they are thought to likely possess adaptive traits to benefit their human host [25,40]. Kimura et al. in their analysis of *Bifidobacterial* and *Lactobacilli* populations present in the feces of 10 human subjects showed that each subject harboured numerically predominant strains that were characteristic of the particular human host which suggests host-specificity [41]. Further study is necessary to reveal the molecular differences that exist between the allochthonous and autochthonous species of both *Lactobacilli* and *Bifidobacteria* found in human feces. This is important in order to ascertain the true indigenous probiotic bacteria and their functional role in bowel health. Geographical

distribution with possibility of structural and functional specificities in the composition of *Lactobacilli* and *Bifidobacteria* in human gut is also a desirable subject to look into.

## CONCLUSION

In conclusion, the present results suggest that sufficient number of baseline samples when comparing the level of bacterial population among patients is required. However, these data were in agreement with previous studies which found that population levels of the species of *Lactobacillus* and *Bifidobacterium* varies between individuals and thus suggests that the population level could be influenced by Host's individuality.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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