Original Research Article

Intra- and inter-Individual Variation in Population of *Lactobacillus* and *Bifidobacterium* in Faeces of Some Healthy Individuals

ABSTRACT

Aims: To evaluate the variation of *Lactobacillus* and *Bifidobacterium* population within and between healthy individuals.
Methodology: Five healthy participants were recruited, and advised not to consume yoghurt, antibiotics, alcohol and steroid during the study. Three (3) fecal samples from each participant obtained at three weeks interval (total of 15 samples a week) for a period of 1 month were examined for each subject bacteria. The samples were collected in sterile specimen jars and immediately taken to the laboratory for analysis. MRS and BIM-25, a selective medium were used for the enumeration of *Lactobacillus sp* and *Bifidobacteria respectively* using plate count method. Target bacteria were confirmed by PCR technique and biochemical tests.

Results: All isolates picked from their respective selective media were confirmed to be lactobacillus and bifidobacteria. The confirmation was carried out using both biochemical tests and PCR. The results of the statistical analysis of the data obtained using SPSS version 16 showed that intra-individual variation of the population of Lactobacillus and Bifidobacteria was significantly lower than inter-individual variation at $P < 0.05$.
Conclusion: This study establishes the fact that the composition level of *Lactobacillus* and *Bifidobacterium* varies within and between healthy human gut at different points in time.

1. INTRODUCTION

Lactobacillus and *Bifidobacterium* constitute two extremely important genus of probiotic bacteria of the gastrointestinal microbiota of man and animals. There is a substantial and growing body of evidence that these microbes provide benefits to the host in which they reside (Walter, 2008, Reuter, 2001). Understanding the roles of these two groups of bacteria in the intestine continues to be a significant challenge. Human gastrointestinal tract (GIT) has over the time been identified as the organ richly endowed with ample numbers of microorganisms (Prosser, 2007). This in effect constitutes a high level of the complexity of the gut microbial communities. Available literature has shown that the bacteria residing in the human gut is estimated to be over 400 species (Wall 2006, Sheil *et al.* 2007, Prosser, 2007) and ranges between 10^{11} - 10^{12} cells per gram of faeces. Among these numbers of species are those that are able to influence significant commercial and clinical interests (Abubakar *et al*, 2009). Research has shown that the varying population distribution of microbial cells 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, thus provides an environment that could either be suitable or unsuitable for individual groups of bacteria (Freter *et al*, 1995). Available literature has also demonstrated that human stomach is colonized by very few microbes majority of which are transient, of those that resides in such a hostile environment noted for its low pH of 3 or 1 are characterized by their exceptional capability to survive acidic pH environment (Hammes and Hertel, 2006).
Non-pathogenic microbial cells usually referred to as “indigenous microbiota”, constitute complex microbial community (CMC). The CMC also constitute non-indigenous microbes that are able to confer

pathogenic activities, and are hence considered as opportunistic pathogens (Freter *et al*, 1995). *Lactobacillus* and *Bifidobacterium* species which are among the indigenous microbiota constitute the two extremely important groups of probiotic bacteria. Probiotics have been generally considered to play significant role in immune modulation, digestive and respiratory functions and are also able to alleviate infectious disease in children (Lebeer *et al*, 2008; FAO/WHO, 2001).

The gastrointestinal microbiota is a very complex community with different habitats constituting the mouth, stomach, small intestine, large intestine and rectum. Literature reports have shown that near stability exist within these habitats. However, stability of the gut microbiota is influenced by Host's individuality. This means that considerable interpersonal or individual variation exists. Thus, individual will have fixed microbiota as far as quantities and qualitative structure of both lactobacilli and bifidobacteria are concerned (Reuter, 1963). There are emerging evidences that shows significant variation of the composition level of *Lactobacillus* and *Bifidobacterium* in human gut, and that the population level of these subject probiotics are peculiar to individuals.

This present study aimed to evaluate the inter- and intra- individual variation of lactobacilli and bifidobacteria among healthy individuals.

2. MATERIALS AND METHODS

Prior to collection of samples, participants were advised not to consume yoghurt, antibiotics, alcohol and steroid three weeks before and during the time of collection of sample in order not alter the microbial population level in their gut, but maintain their usual diet. Fecal samples were collected into a sterile screw-capped plastic container from five (5) healthy volunteers who are within the age bracket of 25 - 35 years old. Written consents were obtained from individual participants. Three sets of the faecal sample were obtained from individual volunteers over the space of 3 weeks, which make up to a total of 15 samples per week.

The reference bacteria 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.

2.1 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 Laboratory. 1 g (wet weight) of the faecal sample obtained from individual volunteers was weighed and collected in a 30 ml sterile universal bottle, another 1gram was weighed and stored in the freezer at -20°C for the PCR analysis. For the microbiological analysis, 9ml of buffered solution was added to fecal sample and then thoroughly homogenized using the vortex machine.

2.1.1 Microbiological enumeration of bacteria

2.1.1.1 Lactobacillus spp.

Using aseptic technique, the well homogenized samples were serially diluted into tubes containing 9ml of peptone water from 10^{-1} to 10^{-6}. 100 µl from each of the diluted suspensions (10^{1} to 10^{6}) were separately inoculated in triplicate on plates of Rogosa agar media (*Lactobacillus* Selecting Agar, pH 5.4 ± 0.2, Oxoid, CM0627). Sterile spreader was used to evenly distribute the inoculums. The inoculated plates were incubated under anaerobic condition using an anaerobic gas jar containing gaspaks (GasPak™ EZ Gas Generating container system, Becton Dickson and Company, USA) at 37°C for 72hrs.

After 72 h of incubation, plates that contains between 30-300 colony forming unit (CFU) of the isolates were selected and the representative colonies which differ in terms of their morphotype (varying sizes, colours and shapes) were counted. Three to four colonies based on their differences in size and shapes were aseptically picked from both culture plate with an inoculating loop and sub-cultured on MRS agar

(de Man Rogosa Sharpe, pH 6.2 ± 0.2, Oxoid, CM0359). After incubation, the isolates were subjected to further characterization.

2.1.1.2 Bifidobacterium spp.

On the other hand, faecal samples were serially diluted in phosphate buffer solution supplemented with L-cysteine up to 10^{-6}. 100 µl aliquots of each of the diluted colony suspensions were inoculated in triplicate onto BIM-25 bifidobacteria selective media. Plates were then incubated anaerobically (85% nitrogen, 10% hydrogen, and 5% carbon dioxide supplied by gas packs in anaerobic jars) at 37℃ for 72 h. Representative isolates with different morphology were aseptically picked and sub-cultured on a Reinforced Clostridial Medium (RCM) agar for further characterization.

2.1.2 Identification of the bacterial isolates

Identification of each isolated colony was further carried out in order to ensure precise quantitation of the target bacteria.

2.1.2.1 Lactobacillus spp.

Various biochemical tests which include Grams reaction, Catalase test, Motility test, Bile aesculine test, Phenol red Glucose broth with gas production test, and API 50 CHL system to determine the sugar fermentation pattern were carried with strict adherence to the standard of the Health protection agency (HPA, 2010) See line 301

2.1.2.2 Bifidobacterium spp.

Colonies that appeared in different morphologies; pale-pink, red, bright pink, and the very small colonies were picked, Gram-stained and observed by optical microscopy at oil emersion magnification to determine their cell morphology and Gram staining reaction. Identification of the selected colonies were further confirmed by detecting the presence of fructose-6-phosphate phosphoketolase (F6PPK) activity.

2.2 PCR Qualitative analysis

Known colonies of *lactobacillus acidophilus* and *Bifidobacterium adolescent* which were used as reference strains were prepared in a suspension and used directly as a template to test the sensitivity of the primers (**see table 1**).

2.2.1 *Lactobacillus* spp.

PCR reaction was carried out in a total volume of 25µl with reaction mixture that contains 2.5 µl of 10x PCR buffer, 2 µl of 2.5 mM dNTP, 2 µl of 25 mM $MgCl_2$, 1 µl of 1 µM primer each of the forward and reverse, 0.2 µl of 5U/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 for disruption of the cell wall and eruption of the cell content, followed by 30 cycles of which each cycles consisted of each step of denaturation at 95°C for 30 s, annealing at 54.4^{o}C for 30 s and extension for 30 s 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.

2.2.2 *Bifidobacterium* spp.

Amplification of the target bacterial DNA was performed with primers g-Bifid-F and g-Bifid-R (table 1) in a PCR iCycler apparatus. The PCR mixture was composed of 1X PCR buffer from 10X reaction buffer with 1.5 mM of $MgCl_2$, 0.2 mM of deoxynucleotide triphosphates (dNTPs) from 20mM, 0.125µl each of forward and reverse primer, and 0.625U of Red hot Thermoscientific *Taq* DNA polymerase from 5U/µl. 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 tested colony suspension. *B. adolecentis* was used as the positive control, while distilled water was used as the negative control. The set up were placed in the PCR machine under the following conditions; Initial denaturation of 95℃ for 5min, followed by 30cycles of which each cycles consisted of each

step of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension for 30 s at 72°C. The final extension was set at 72°C for 5 min and final hold at 4°C.

Table 1. Primers used in this study.

Bacteria	Primers	Sequence	Product size (bp)	Reference
Lactobacillus	LbLMA1-rev	CTCAAAACTAAACAAAGTTTC	250	Dubernet *et al*. (2002)
	R16-1	CTTGTACACACCGCCCGTCA		
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	549-563	Matsuki *et al*. (2002)
	g-Bifid-R	ACATCTATAGCCCTTCTTGTGG		

2.3 Gel Electrophoresis of the PCR products

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 110V in TBE electrophoresis buffer. Size marker of 100bp was used on each gel. Gels were visualized with an UV trans-illuminator.

2.4 STATISTICAL ANALYSIS OF DATA

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 16 to determine the mean, standard deviation and analysis of variance (ANOVA) of the data obtained.

3.0 RESULTS AND DISCUSSION

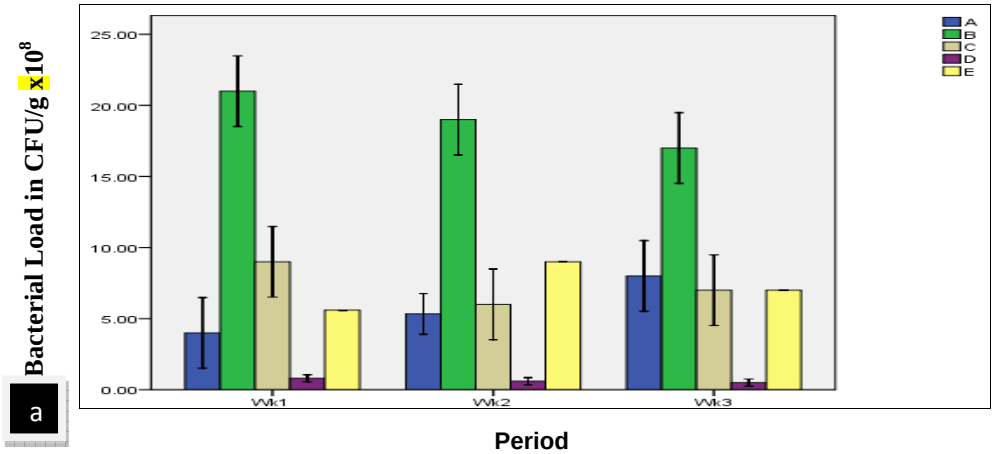
3.1 Bacteria counts

Following incubation, colony counts were made from dilution plates of the two selective media that contained discrete colonies that falls within the range of 30 to 300. The total population of each genus of *lactobacilli* and *Bifidobacterium* cultured on Rogosa and BIM-25 agar respectively was calculated as CFU per gram (wet weight) of feces.

Table 2. *Lactobacillus* sp. and *Bifidobacterium* spp. load isolated from faecal samples of different participants.

	Participants				
Probiotics	**A**	**B**	**C**	**D**	**E**
Lactobacillus	5.67 ± 1.20[a]	19.00 ± 1.15[b]	6.33 ± .88[a]	0.500 ± .17[a]	8.23 ± .53[a]
Bifidobacterium	18. 33 ± 1.45[b]	17.00 ± 1.73[a]	7.97 ± .43[b]	18.33 ± 2.60[b]	7.73 ± .69[a]

Values are means of triplicate readings ± standard deviation. Different letters in a column indicate significant difference (P <0.05)



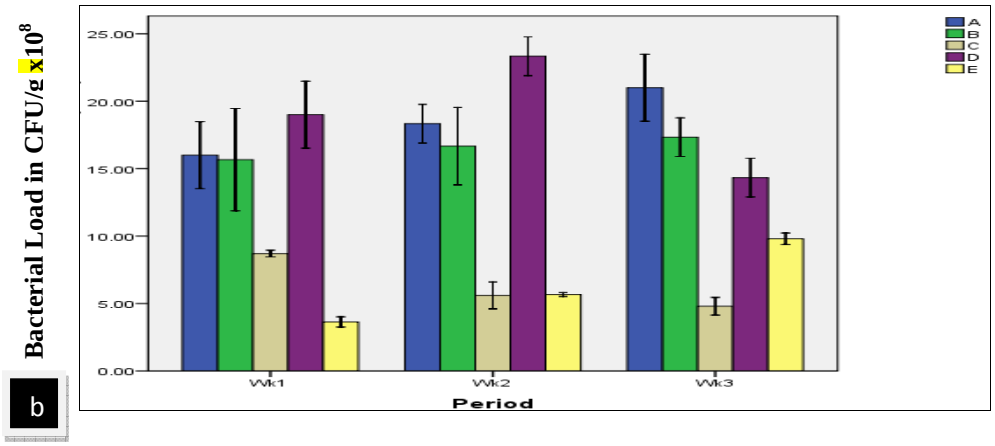


Fig. 1: a). Mean *Lactobacillus* load in CFU/g (x10^8) from faecal samples of participants. **b).** Mean *Bifidobacterium* load CFU/g (x10^8) from faecal samples of participants. Each sample from respective participants was obtained once for each week at 2 weeks interval.
Key: A-E=Participants

The results of the statistical analysis of the data obtained using SPSS version 16 showed that there was significant difference at $P<0.05$ within the participants for both *Lactobacillus* and *Bifidobacteria* respectively. The *Lactobacillus* load for participant **A** increased significantly from 4.5×10^8 CFU/g at week one to 5.33±0.33 ×10^8 CFU/g by week two and 8.00±0.58×10^8 CFU/g by the third week as represented in **Figure 1a**. A different pattern however was observed for participant **B** in which there was decrease in the *Lactobacillus* but an increase in the level of *Bifidobacterium* load from week one through to week three. Participant **C** and **E** had an irregular pattern in which there was significant decrease by week two and an increase by week three for participant **C** and a significant increase by week two but decreased by week three for participant **E** respectively. There was however no significant increase or decrease for the *Lactobacillus* load in participant **D**.

There was significant difference ($P<0.05$) in the level of *Bifidobacterium* load isolate from participants as seen in **Figure 1b**. Participant **A** increased significantly from 16.33±0.33×10^8 CFU/g at week one to 22.57± 0.57×10^8 CFU/g by week three. The same pattern was observed for participant **E**. Participant **B** recorded a constant load without significant difference in their values all through the three weeks of experiment. The result pattern for participants **C** showed a decrease by week two and constant by week three, while participant **D** increased by week two and decreased significantly by week three. Comparatively, there was significant difference at $P<0.05$ for the two bacteria (the *Lactobacillus* and *Bifidobacterium*) in their load among participants **A-E** as recorded in **Table 2**. Participant **B** however maintained high level of both genera.

The result as obtained in this study shows a significant inter-individual variation of the composition level of both *Lactobacillus* and *Bifidobacterium* (**Figure 1**). The result also shows intra-individual variation of the subject bacteria at different time point as demonstrated in **Table 2**. This supports the findings of (Reuter, 2001, Hammes and Hertel, 2006) which demonstrated inter- and intra-individual variation in the fecal microbiota of different healthy human subjects.

The population level of lactobacillus as obtained in this study ranges between 10^6 and 10^8 CFU/g, although with an exception (**Table 2**), is in agreement with Walter, (2008) 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 10^8CFU/g. Previous studies have also reported averages of 10^6 and 10^8 CFU/g (Dal Bello and Hertel. 2006, Dal Bello *et al*. 2003). This accounts for only about 0.01% of the total cultivable counts (Walter 2008). As evidenced in this study, lower levels of lactobacillus have also been observed in previous study on healthy (Hopkins *et al.* 2001).
Kimura *et al*. (1997) 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. The Human Microbiome Project consortium (2012) 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. (Buddington and Sangild, 2011) also in their findings showed that the type and number of microbial species that persist and colonise 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. It is not clear the 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.

Table 3. Result showing the biochemical characteristics of *Lactobacillus* spp. isolates.

P	CIM	GR/CM	BA	PRGB	↥	CT	MT
A	Rhizoid	**+**/Long rod	**+**	**+**	_	_	_
	Circular	**+**/Short rod	**+**	**+**	_	_	_
B	Rhizoid	**+**/Long rod	**+**	**+**	_	_	_
	Circular	**+/**Short rod	_	**+**	_	_	_
C	Circular	**+/**Short rod	**+**	**+**	_	_	_
	Rhizoid	**+/**Long rod	**+**	**+**	_	_	_
D	Circular	**+/**Short rod	**+**	**+**	_	_	_
	Rhizoid	+/Long rod	**+**	**+**	_	_	_
E	Circular	+/Short rod	**+**	**+**	_	_	_

P: Participant, CIM: Colony morphology, GR: Gram's reaction, CM: Cell morphology, BA: Bile aesculin test, PRGB: Phenol red glucose broth, ↥: Gas production, CT: Catalase test, MT: Motility test.

Table 4. API result indicating species identified using the Apiweb™ computer software

Subject	Colony	Identification comment	Specie/strain identified

	Morphology		
A	Rhizoid	Very good	*Lactobacillus acidophilus*1
	Circular 2 mm	good	*Lactobacillus fermentum*
B	Rhizoid	Doubtful	*Lactobacillus acidophilus* 1,2 and 3
	Circular 2 mm	Doubtful	*Lactobacillus fermentum* 1, *lactobacillus acidophilus* 3
C			
	Circular 2 mm	good	*Lactobacillus salivarius*
	Rhizoid	Very good	*Lactobacillus acidophilus* 1
D	Rhizoid	Doubtful	*Lactobacillus acidophilus* 1 and 3
E	Circular 2 mm	Very good	*Lactobacillus plantarum*

Table 5: Gram Staining and F6PPK test for the confirmation of *Bifidobacterium* isolates

Colony type	Gram's reaction and cell morphology	Results of F6PPK TEST
Pale pink colonies with dark centres	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	Slight colour change
Bright pink colonies	Gram positive, cocobacilli, club shape Y and V branching	Positive

All the isolates recovered from selective medium for the respective target bacteria were confirmed to be *Lactobacillus* spp and *Bifidobacterium* spp. respectively (**Table 3, 4 and 5**). Lactobacilli were confirmed using the API CHL kits and identification was done with API-web program. PCR analyses also confirmed the *Lactobacillus* colony. On the other hand, *Bifidobacterium* were identified by the detection of fructose-6-phosphate phosphoketolase (F6PPK) enzyme in cellular extracts as described by Scardovi (1986), and also confirmed by PCR method, see **Figure 3**. The primer sets (**Table 1**) used in this study was adopted from Dubernet *et al.,*(2002) and Matsuki *et al.* (2002), and these corresponds to the flanking sequence of the 16S rRNA gene which is known to be highly conserved among the *genus Lactobacillus* and *Bifidobacterium* respectively.

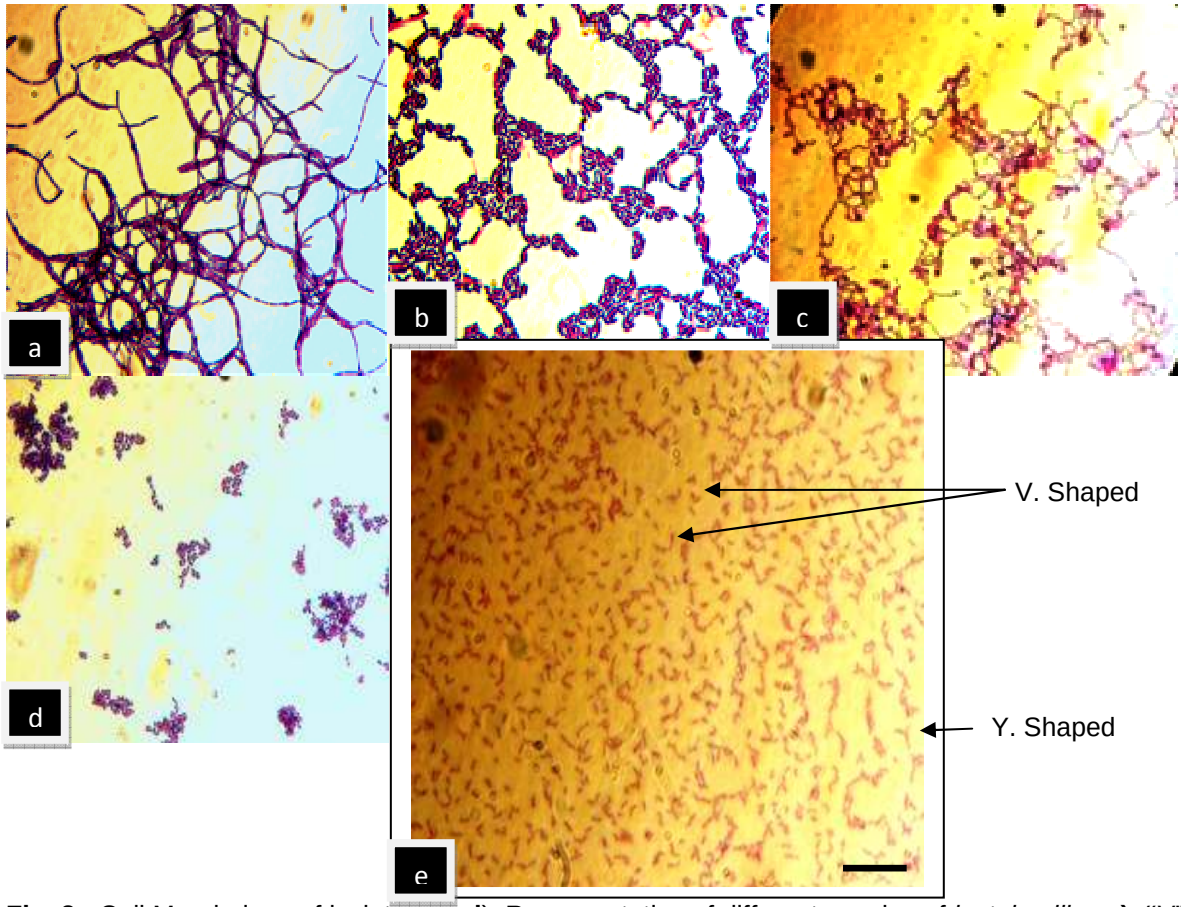


Fig. 2: Cell Morphology of isolates: **a -d**). Representative of different species of *lactobacillus* **e)**. **"**V" and "Y" shaped *Bifidobacterium.* Bar = 10 µm

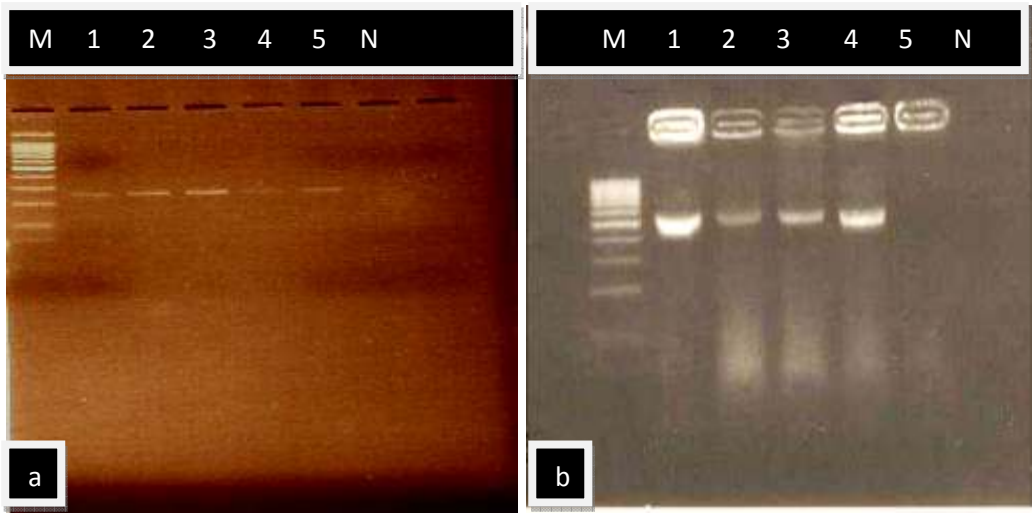


Fig. 3. Electrophoretic detection of amplified DNA of *Lactobacillus* spp. and *Bifidobacterium* spp. **a). Lane 1** shows the known *Lactobacillus acidophilus* used as positive control in this experiment. **Lane 2 – 5** shows the band of Lactobacillus spp. isolated from the individual volunteer isolates (250bp). **b). Lane 1**- positive control, **Lane 2 -4** shows band of *Bifidobacterium* isolated from the individual participants (549 – 563bp), lane 5 shows a failed PCR product. The **N** is negative control and **M** is the DNA size marker of 100bp.

Although the methodology as used in this study did not permit the strict detection of species of both genera to determine which species is numerically predominant in the samples, but *L. acidophilus, L. fermentum, L. salivarious* and *L. plantarum* were the most common isolates, where *L. plantarum* was found repeatedly only in one patient. It is therefore, most likely to have certain species of either genus that is unique to individual subjects. This study suggests the possibility of isolating only a small number of either species of both genera which in effect might not represent the true inhabitants of patients's intestinal tract. Genus *Bifidobacterium* has been discovered to contain up to four or five different species that is commonly isolated from adults (Gavini *et al.* 2001), while previous studies have revealed the following species of lactobacilli that are commonly found in human faeces: *L. paracasei, L. rhamnosus, L. gasseri, L. fermentum, L. plantarum* (Vaughan, 2005).

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 (Reuter, 2001). Literature reports have also shown that human have to some extent a stable autochthonous *Lactobacillus* and *Bifidobacterium* microbiota (Reuter, 2001). 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 (Reuter, 2001). It is also known that autochthonous strains that naturally persist in human patients 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 health (Walter, 2008).

Further studies are necessary to reveal the molecular differences that exist between the allochthonous and autochthonous species of both genera of *Lactobacillus* and *Bifidobacterium* 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 specificities in the composition of *lactobacillus* and *Bifidobacterium* microbiota in human gut is also a desirable factor to look into.

CONCLUSION

The present results highlight that sufficient number of baseline samples when comparing the level of bacterial population among patients are needed. However, these data were in agreement with previous studies which found that population levels of *Lactobacillus* and *Bifidobacterium* varies among patients.

~~Although, the data as obtained suggest a need to have a sufficient number of baseline samples when comparing the level of bacterial population within and between individual subjects. However, this data concord with previous studies which establish the fact that population level of *Lactobacilli* and *Bifidobacteria* varies within and between individuals.~~

CONSENT

All authors declare that 'written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the editorial office/chief editor/editorial board members of this journal.

ETHICAL APPROVALS

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

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