We are IntechOpen,
the world's leading publisher of Open Access books
Built by scientists, for scientists

6,900
Open access books available

186,000
International authors and editors

200M
Downloads

154
Countries delivered to

Our authors are among the

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Bifidobacterium in Human GI Tract: Screening, Isolation, Survival and Growth Kinetics in Simulated Gastrointestinal Conditions

Nditange Shigwedha and Li Jia

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/50457

1. Introduction

Many species of lactic acid bacteria (LAB), *Bacillus*, and fungi such as *Saccharomyces* and *Aspergillus* have been used over the years in the food industry. A few have gained the probiotic status – defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host (Joint FAO/WHO, 2002) – and most of this belong to *Lactobacillus* (e.g., *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. johnsonii*, *L. reuteri*, etc.), *Streptococcus* (e.g., *S. thermophilus*, etc.), and *Bifidobacterium* (e.g., *B. bifidum*, *B. longum*, *B. breve*, *B. infantis*) genera. Bifidobacteria is the predominant species of bacteria in the normal intestinal flora of healthy breast-fed newborns where they constitute more than 95% of the total population (Yildirim & Johnson, 1998). Numerous *Bifidobacterium* strains have gained recognition as probiotics because of their various therapeutic health benefits, including resistance to enteric pathogens (*Clostridium spp., Salmonella spp., Candida spp., Escherichia coli spp. and Listeria monocytogenes*), aid in lactose digestion and/or help to regulate digestion, anti–colon cancer effect, the immune system modulation, anti-allergy, and hepatic encephalopathy (Jia *et al.*, 2010), and also for having a protective effect against acute diarrhoea (Liepke *et al.*, 2002). The food industry recognized the market potential of the numerous strain-specific positive health benefits of the bifidobacteria cultures, namely in beverages. Bifidobacteria can also be administered as capsules or tablets or incorporated into food as dietary adjuncts and into baby foods (Lourens-Hattingh & Viljoen, 2001; Patrignani *et al.*, 2006). In addition, bifidobacteria lower inositol phosphate content during bread making (Palacios *et al.*, 2008).

Several investigators have speculated that the survival of most bifidobacteria is not exceptionally high in most dairy products due to low pH and/or exposure to oxygen.

Nevertheless, problems may arise as a consequence of the difficulties of isolation and cultivation of bifidobacteria. Only a few studies have been published concerning the isolation and characterization of plasmids from bifidobacteria. The human gastrointestinal (GI) tract is the largest tube, running through the body and which include mouth and/or oral cavity, oesophagus, stomach, small intestine and large intestine. (Figure 1).
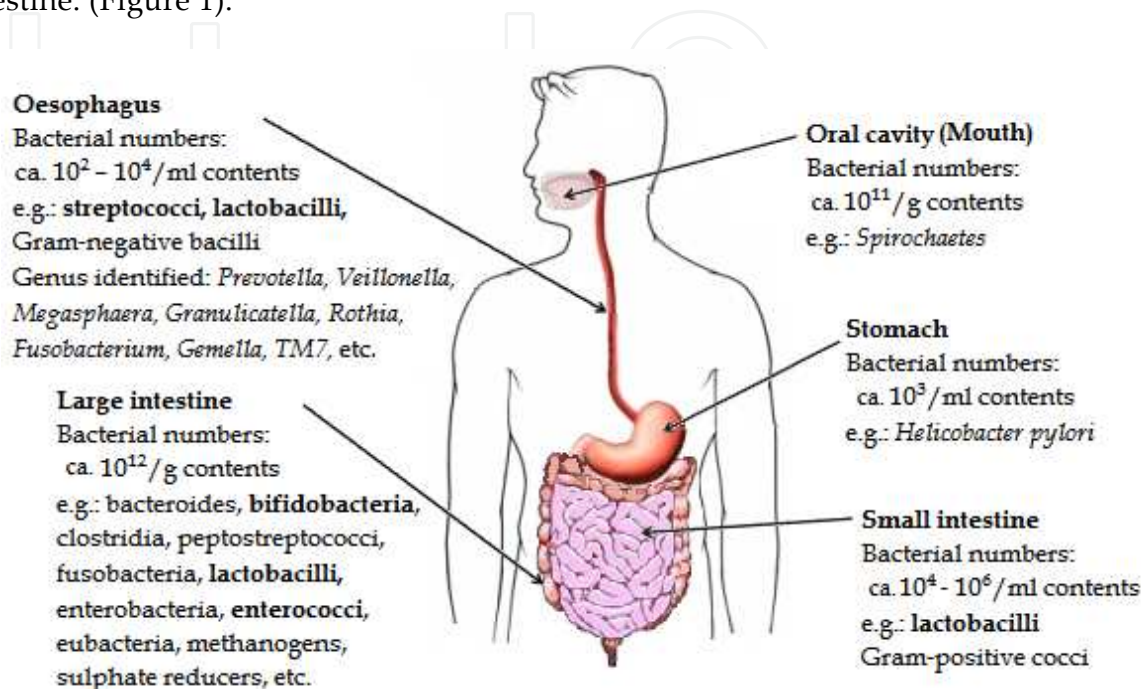

Oesophagus
Bacterial numbers:
ca. $10^2 - 10^4$/ml contents
e.g.: **streptococci, lactobacilli,**
Gram-negative bacilli
Genus identified: *Prevotella, Veillonella, Megasphaera, Granulicatella, Rothia, Fusobacterium, Gemella, TM7,* etc.

Large intestine
Bacterial numbers:
ca. 10^{12}/g contents
e.g.: bacteroides, **bifidobacteria,**
clostridia, peptostreptococci,
fusobacteria, **lactobacilli,**
enterobacteria, **enterococci,**
eubacteria, methanogens,
sulphate reducers, etc.

Oral cavity (Mouth)
Bacterial numbers:
ca. 10^{11}/g contents
e.g.: *Spirochaetes*

Stomach
Bacterial numbers:
ca. 10^3/ml contents
e.g.: *Helicobacter pylori*

Small intestine
Bacterial numbers:
ca. $10^4 - 10^6$/ml contents
e.g.: **lactobacilli**
Gram-positive cocci

Figure 1. The human gastrointestinal tract and its microbiota.

1.1. The oral cavity

Ingested foodstuff first comes into contact with the oral cavity, which is composed of different niches of microbial population. In the oral cavity, bacteria are the main group of microorganisms, although viruses and yeasts can also be found. The main ecological habitants of the mouth are the mucosa of lips, cheeks and palate, the tongue, the tooth surface, the saliva, and the tonsillar area. The population of microorganisms in each section is mainly dependent on the presence of oxygen and nutrients as well as the flow rate of the saliva (see Figure 2). The major species in the oral cavity are lactic acid bacteria of the genera *Streptococcus*, *Lactobacillus* and *Bifidobacterium*. In dental plaque and oral infections, many anaerobic species have been isolated, mainly *Prevotella* and *Porphyromonas* species, as well as *Eubacterium*, *Actinomyces* and *Veillonella* (Hartemink, 1999).

The main source of nutrients and energy for oral bacteria is the ingested food, especially carbohydrates, which are rapidly metabolized to lactic and acetic acids by the predominant LAB, leading to a rapid drop in the pH of the saliva after ingestion of carbohydrates. The surplus carbohydrates can be incorporated into exopolysaccharides by a large number of bacteria and be used as energy storage compounds, or as attachment factors (Hartemink, 1999).
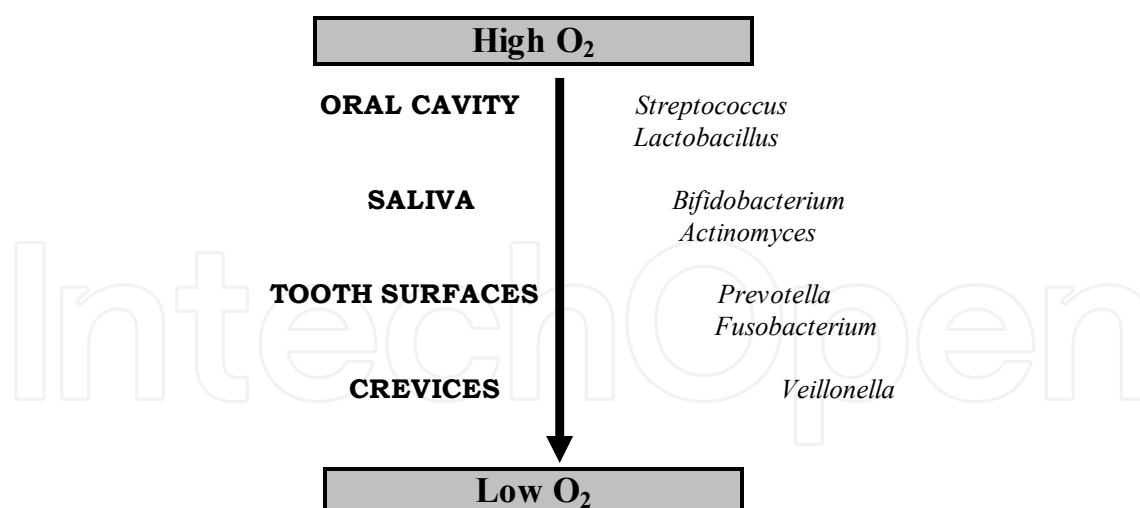
Figure 2. Relationship between bacterial species, oxygen tension and habitat in the oral cavity.

1.2. The oesophagus

In quantitative terms, the oesophagus and stomach carry the lightest microbial loads in the human GI tract. The predominant culturable bacteria are facultative anaerobes, originating in the oral cavity, such as streptococci and lactobacilli, which occur in relatively small numbers (*ca.* $10^2 - 10^3$ cm^{-2} or ml^{-1} of the mucosal surface or lumenal aspirate, respectively) (Macfarlane & Dillon, 2007). The majority of oesophageal bacteria (including the largely ⊘-haemolytic *Streptococcus* species) are cultivable and are almost 10^4 bacteria per mm^2 mucosal surface of the distal oesophagus (Pei *et al.*, 2004). While the bacterial biota in the distal oesophagus is likely to be similar to that of the oropharynx (Kazor *et al.*, 2003), many other species of *Pseudomonas tolaasii*, *Pseudomonas influorensces*, *Pseudomonas syringae*, *Pseudomonas putida*, uncultured *Duganella*, *Stenotrophomonas maltophilia*, *Janthinobacterium lividum*, *Lactobacillus paracasei*, *Propionibacterium acnes*, *Pseudomonas Antarctica* / *meridiana*, and *Brevundimonas bulata* exist in the oesophagus (Pei *et al.*, 2004). Other selected members of the bacterial genera found in human distal oesophagus are given in Figure 1.

1.3. The stomach

In general, the human stomach has a remarkably low pH. The normal resting gastric juice's pH is below 3.0, which prevents virtually all bacterial growth, and which is bactericidal for most transient species, especially the LABs. During and shortly after a meal, the pH may increase to values around 6.0. This will allow passing bifidobacteria to survive the gastric juice prior to proceeding onto the small intestine (to battle the bile salts). The resident flora of the gut lumen is highly acidic tolerant and consists mainly of lactobacilli and streptococci.

In the stomach mucosa, the pH is much higher, and bacterial populations may be higher, as well. In addition to lactobacilli and streptococci, some other bacterial species and yeasts may be present (Hartemink, 1999). The gastric juice plays a significant role in digestion of proteins, by activating digestive enzymes, making ingested proteins unravel so that

digestive enzymes can alter protein down to individual amino acids. Fermentation of ingested carbohydrates in the stomach hardly occurs.

1.4. The small intestine

When the partially digested food enters the small intestine, it is mixed with intestinal secretions, such as bile, pancreatic enzymes and bicarbonates. The bile in particular has a strong bactericidal effect. Together with a strong-fluid secretion by the intestinal mucosa, this also prevents extensive colonization of the small intestine. Colonization usually takes place in crypts and blind loops. In this lower part of the small intestine, the movement is slightly reduced, the bile is diluted, the pH becomes more neutral, and the oxygen tension drops rapidly. This favours the growth and/or transit of different bacteria, initially mainly aerotolerant species, and in the ileum also strict anaerobes as revealed in Figure 3 (Hartemink, 1999). There is not much carbohydrate fermentation in the small intestine in healthy humans, due to the flow rate and the little bacterial mass.

In studies undertaken in pigs, it has been reported that the conditions in the small intestine differed widely. The pH is much higher, and the bile secretion is less abundant, which results in an extensive bacterial growth in the small intestine. This also results in substantial fermentation of ingested carbohydrates. The human body is projected to produce between 20 to 30 g of bile salts per day to replace the loss occurring in the excreta (250 to 500 mg), and these are typically stored in the gall bladder (Glickman, 1980).
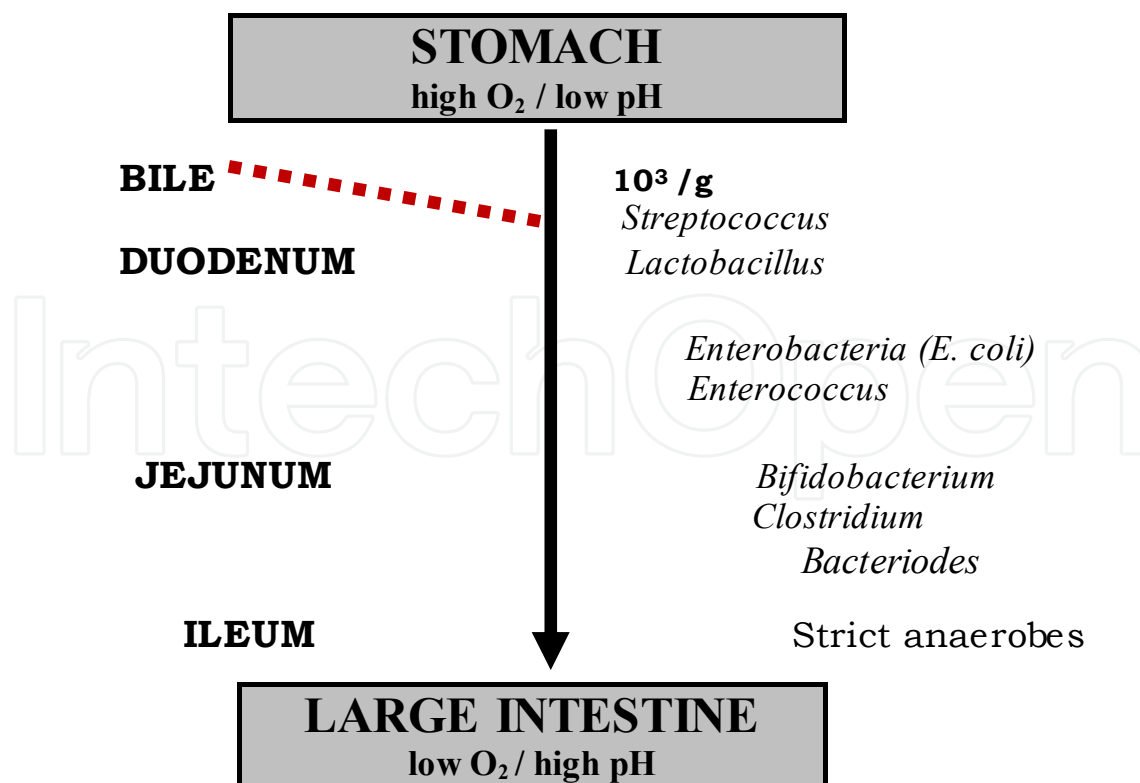

Figure 3. Appearance of bacterial species, oxygen tension and habitat in the small intestine.

1.5. The large intestine

In the large intestine, the flow rate of the digesta decreases considerably. In addition, the bile is even more diluted, and the pH is close to neutral. Total logarithmic counts may reach up to 10^{11} bacteria/gram contents. Higher numbers have been reported, but it is physically impossible to achieve a number over 10^{12} bacteria/gram faecal dry weight, taking into account the average balance of faeces and the dimensions of an average bacterium. It is estimated that over 400 different bacterial species reside in the human large intestine. Of these, about 200 have been validly described, but often non-identifiable strains are reported.

In addition to the resident bacteria, transient bacteria are often isolated. The dominant floras in the large intestine are relatively stable, and they include *Bifidobacterium*, *Bacteriodes* and anaerobic cocci. Large variations also exist in the less dominant species, especially among the facultative or aerotolerant species like *E. coli* and lactobacilli. The numbers of the dominant species are also comparable in different population. Differences in counts are more often due to the methodology used, rather than actual differences. As in individuals, the counts of less dominant species differ widely between different populations. Among the dominant bacterial groups are members of the genera *Bacteriodes*, *Bifidobacterium*, *Coprococcus*, *Peptostreptococcus*, *Eubacterium* and *Ruminococcus*. Members of the following genera are often isolated and are available in lower numbers: *Fusobacterium*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Veillonella*, *Megasphaera*, *Propionibacterium* and *Enterobacteriaceae*.

It is indispensable to emphasize here that the principal function of the GI tract includes breakdown and absorption of food components and water. In general, degradation takes place in the upper part of the GI tract, whereas the major sites of absorption are in the lower part of small intestines and the large intestines. Degradation and absorption are enhanced by the excretion of the large number of digestive enzymes, such as glycosidases, lipases, peptidases and proteinases. The colon receives digesta from the intestinal ileum approximately 5 h after food ingestion. Thereafter, rate of motion slows progressively from the caecum towards the distal colon. Concurrent with this is an increase in water absorption; thus gut contents in the proximal colon are more or less liquid in nature but have a faecal like appearance distally (Hartemink, 1999).

For most of the world's population, the standard gut transit time is 60 h, with a variety of 23 – 168 h. The colon itself has a capacity of approximately 500 ml with about 220 g contents. In general, stools weight correlates inversely with transit time. Studies with healthy volunteers have indicated that speeding up colonic transit times from 67 to 25 h resulted in an increase in stool weight from 148 to 285 g/day. Conversely, when transit time increased, stool weight decreases from 182 to 119 g/day (Hartemink, 1999). The differences are mainly due to changes in the water content of the faecal mass.

The structure of faeces is highly variable. Bacteria may constitute up to 55% of the total solids, whilst fibre and other non-digested, non-fermented compounds represent less than 17% of the weight of which about 24% is soluble material. Faecal water content may be as high as 70% of the total weight. Stool size is influenced by both dietary and endogenous

factors. Endogenous factors mainly operate through hormones on the intestinal motility. The well-known endogenous factors include: decreased peristaltic movements during exercises and menstrual cycle. Dietary factors, like non-digestible fibres and polyalcohols (sorbitol), may retain water and thus increases stool bulk. High amounts of these factors may cause diarrhoea, due to the increased osmotic pressure.

2. Screening and isolation procedures of bifidobacteria strains

Molecular methods have shown that the average percentage of bifidobacteria in the GI tract of humans is approximately 3% of total microbiota, or they occur at a concentration of $10^9 - 10^{10}$ CFU/g of faeces (Jia *et al.*, 2010). As to achieve intestinal colonization in humans or animals, bifidobacteria have to endure inhibitory substances secreted by the host, such as gastric acid in the stomach and bile salts (in the small intestine). Although, both the gastric pH (pH < 3) and bile salts are strongly bacteriocidal, some resistant bifidobacteria can handle the low pH's ranges of the stomach and also survive the effects of bile salts in the small intestine of humans. These can be isolated and screened for their leading roles as probiotics.

2.1. Isolation and cultivation of bifidobacteria resistant to acidic pH and bile salts

2.1.1. Isolation via stress-shock procedure

Selection of acid and bile resistant bifidobacteria has been based on the stressing isolation method developed by Chung *et al.*, (1999). Faecal samples are collected from infants and/or adults. The tube containing the faecal sample is promptly screened for the isolation of resistant strains, as follows: Faecal samples (0.8 g each) are inoculated into 8 ml of Transga-lactooligosaccharide-propionate (TP) medium as an enrichment medium for the bifidobacteria. After an anaerobic incubation for 12 h at 37 °C, 0.8 ml of the incubated cultures is transferred into fresh TP medium with pH adjusted to 2.0 and incubated anaerobically for another 12 h at 37 °C. After the acid exposure, an aliquot (0.8 ml) of the incubation medium is transferred into fresh TP medium supplemented with 1.5% ox-gall, and the incubation continued for another 2 h at 37 °C. The resulting incubation medium is serially diluted and plated on TP agar, to select colonies of the resistant bifidobacteria strains. To isolate reference strains, serially diluted *Bifidobacterium* cells grown in the regular TP medium are plated on TP agar medium. In most of the isolation studies, *B. adolescentis, B. longum, B. infantis, B. bifidum* and owner identified *Bifidobacterium* strains (commonly called "own isolates" in microbiology) are used as reference strains. The reference strains are utilized for the convenience of comparison to the resistant strains. Microscopic analysis (1000 × with immersion oil) is routinely performed to confirm *Bifidobacterium* morphology.

In addition, *Bifidobacterium* cells are examined for their biochemical and morphological characteristics according to the Bergey's Manual of Determinative Bacteriology. The cultures

are grown in Man, Rogosa, and Sharpe (MRS) medium under anaerobic conditions, in a microprocessor-controlled anaerobic chamber. Cultures are incubated for 18 h at 37 °C and stored at 3 – 5 °C between transfers. For the fermentation test, 0.5 ml of 10% substrate solutions (which were membrane filtered through 0.45 μM filter), are added to 9.5 ml of Peptone Yeast-extract Fildes (PYF) basal medium (Mitsuoka, 1990). After 2.5 days of strictly anaerobic incubation, the pH of the growth medium is measured. Tubes showing pH values below 5.5 are considered to be positive for fermentation. The presence of acetate and lactate in the fermented PYF containing glucose medium is assayed by using gas chromatography (GC) or high performance liquid chromatography (HPLC).

2.1.2. Isolation and screening via stress-shock

Briefly, faecal samples of 3 to 5 days old new-born babies are collected and taken to the laboratory for immediate analysis and isolation of bifidobacteria. About 2 g of each faeces sample is placed in a sterile test-tube (30 ml) and closed tightly with a rubber-stopper. For optimal survival of these highly sensitive anaerobic bacteria, the samples are treated within 15 min after faeces emission, or else the samples are kept in an anaerobic environment until analysis (maximum of 10 h). Screening for the isolation of resistant strains is as follows: faecal samples (2 g each) are inoculated into 10 ml test-tubes of Raffinose-Bifidobacterium (RB) broth (pH 6.8). After an anaerobic incubation for 12 h at 38.5 ºC, 1 ml of the incubated culture is transferred into 10 ml of fresh RB medium with pH adjusted to 3.0 and incubated anaerobically for 2 h at 38.5 ºC. After the acid exposure, an aliquot (1 ml) of the incubation medium is transferred into 10 ml of fresh RB medium supplemented with 1% ox-gall, and the incubation continues for another 2 h at 38.5 ºC. The resulting incubation medium is serially diluted (10-folds) in a pre-reduced Ringer solution with 5 – 10% glycerol for the inhibition of the cellulolytic activity of the fungus. An aliquot of 100 μl from each dilution is plated directly on RB and MRS agars using the surface streak method and incubated anaerobically at 38.5 °C for 3 – 4 days to determine colonies of the resistant *Bifidobacterium* strains.

Likewise, the isolate designated *B. longum* GB-03 was isolated from a pharmaceutical product called Golden Bifid (containing a combination of unspecified *Bifidobacterium* spp., *Streptococcus thermophilus* and *Lactobacillus bulgaricus*) using a similar approach. The first step is crucial to reveal that a single piece (0.5 g) has to be dissolved in 0.2 ml test-tube of sterilized distilled water before being inoculated into 10 ml test tube of fresh RB-medium.

3. Morphological identification of bifidobacteria by phase contrast microscopy (PCM)

In the morphological analysis of bifidobacteria population, *in situ*, in human faeces and/or other foods products, microscopes have been used to determine the degree of heterogeneity of these probiotic's populations. The morphology of bifidobacteria determined microscopically has been used as an aid to phenotypic differentiation within the group, while the effect of medium type, low pH and high bile salt concentrations on the bifidobacterial cell morphology has also been studied by this method. Individual

Bifidobacterium strains are characterized phenotypically, including morphology identification by phase contrast microscopy (PCM).

Bifidobacteria are gram-positive, anaerobic, rods of various shapes (short, regular, thin cells with pointed ends, coccoidal regular cells, long cells with slight bends or protuberances) or a variety of branching (pointed, slightly bifurcated, club-shaped or spatulated extremities), single or chains of various arrangements (in star-like aggregates or disposed in "V" or "Y" or else "palisade" arrangements) (Scardovi, 1986).

As a pattern to characterize the heterogeneous population of bifidobacteria associated with human origin and other sources, the PCM examinations and two different media (RB & modified MRS) were used to demonstrate a better phenotypical correlation of the natural isolates to the reference strains on RB, MRS and modified MRS media as shown in Figures 4.1 – 4.12). These media are unique and appear to be still the most predominant in culturing the bifidobacteria strains.

Isolates of bifidobacteria are normally cultured anaerobically on appropriate agars at 38 ºC for 3 – 4 days. For gram-staining, a loopful of the culture is streaked on microscope slides (46 × 25 mm) and the staining technique followed thoroughly. Subsequently, the slide is observed under phase contrast microscopy, preferably at 1000 × magnification by oil immersion and can be photographed as well, using the images advanced software package if available.

3.1. Morphological characterization of *Bifidobacterium* reference strains

The basic morphologies, namely short, regular, thin cells with pointed ends, coccoidal regular cells, and long cells with slight bends or protuberances are discernible among the 2 *Bifidobacterium* reference strains (*B. adolescentis* and *B. infantis)* shown in Figures 4.1, 4.2, 4.3, 4.4 and 4.11) on modified MRS and RB media. From these micrographs alone, it is obvious to validate that individual variations of the average phenotypic morphologies of bifidobacteria are present as described earlier. The PCM also provided a rapid and clear visualization of the basic bifidobacteria cell morphology, while at the same time, allowed only broad comparisons amongst the bifid structures within a mixture of 2 other LABs (*Streptococcus thermophilus* & *Lactobacillus bulgaricus*) (see Figure 4.12).

The typical colonies of bifidobacteria are altogether round and white on RB and modified MRS media. Colonies are usually picked off of a suitable plate and may be kept sub-cultured 2 – 3 times on a freshly prepared agar as to obtain pure culture without contamination. The morphologies of the 2 reference strains and their relationship to each will now be discussed separately. When the strain of *B. adolescentis* is resuscitated and cultured on modified MRS medium (Figure 4.1) or RB medium (Figure 4.2), it may be differentiated clearly from the *B. infantis* (Figures 4.3 & 4.4) on the basis of morphology. The *B. infantis* was also resuscitated and cultured under the similar conditions. As it can be observed from Figure 4.2, *B. adolescentis* on RB displayed long and thick rod-shaped and regular coccoidal cells. The cells of *B. adolescentis* strain on RB were almost paired and assembled, a feature which was highlighted by PCM. The existence of distinct "V"- and/or "Y"-shapes and some long cells with protuberances or slight

curvature of this isolates when grown on RB agar, is a powerful diagnostic feature, particularly when distinguishing this specie from closely related *B. minimum* when grown on Trypticase-Phytone-Yeast extract (TPY) agar stabs (Biavati *et al.*, 1982). In addition, curved cells with smooth and rounded ends are the most one dominating in the micrograph. These features were not compatible with descriptions of this particular species' morphology as described by Reuter (1963), but were common to other species of the genus.

The *B. infantis* strain displayed slender, often short rod-shaped and of the typical club-shaped extremities, which cells of these species are reported to exhibit (see Figures 4.3 & 4.4). The morphology of this strain is almost the same when grown on both the MRS and RB solid growth media. Furthermore, *B. infantis* showed a distinct tendency for chain formation on RB medium. These cells often occurred in "V" and "Y"-shapes and were similar to that of many other species of the genus. Nevertheless, it was also possible to differentiate between this strain and the closely related *B. longum* GB-03 (own isolate, Fig. 4.6) on the basis of small variety of club-shaped extreme morphology.
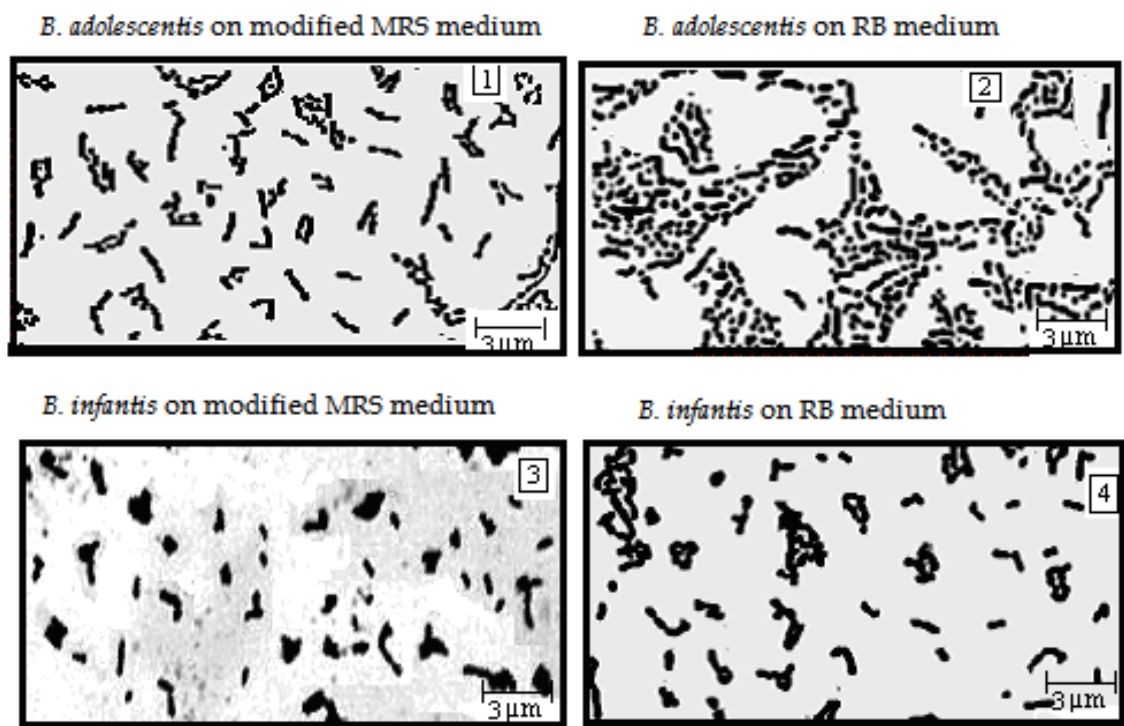
3.2. Morphological differentiation of isolates of bifidobacteria

Morphological consistency is greater among the *Bifidobacterium* isolate (*B. longum* GB-03 and *B. bifidum* WN-04) as shown in Figures 4.5 to 4.11) than the *Bifidobacterium* reference strains. Cell shapes ranged from long and thick–rods with protuberances to long and thin–rods with blunted ends and slightly bifurcated club-shaped extremities, with a number of variations on these basic shapes. Two morphological groups and their potential significance are discussed separately below.
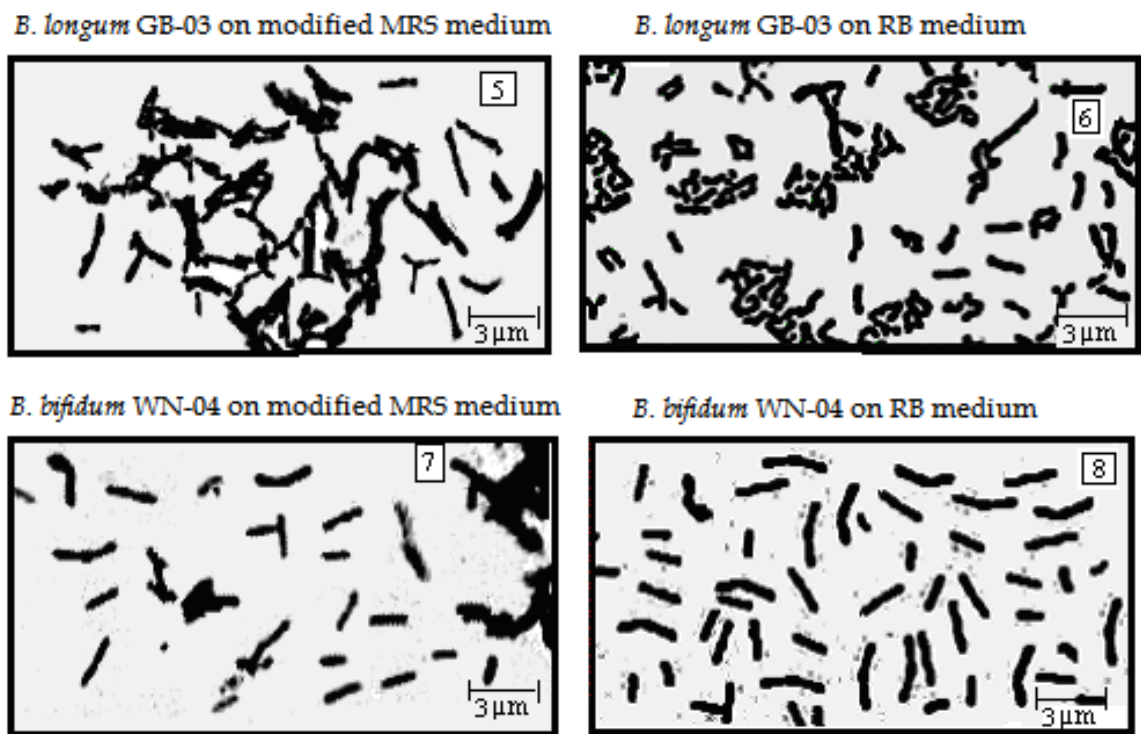
3.2.1. Long and thick–rods with protuberances cell morphology

Figures 4.6 and 4.8 display both isolates of *B. longum* GB-03 and *B. bifidum* WN-04 on RB medium, which consisted of long and thick cells with slight bends. The regular morphology of these cells and the star-like aggregates arrangement (Figure 4.6) was evident under the PCM when grown on RB agar. Also, the presences of sparsely distributed single cells were also evident under the PCM (Figure 4.8). The morphology of these cells was consistent with any of the *Bifidobacterium* reference strains discussed previously. The isolates' morphologies resembled the reference strain of *B. infantis* which are never elongated but have a penchant for group formation (Figure 4.4).

Although no conclusions could be drawn on the basis of morphology alone, the presence of "V"-shaped rods, protuberances with a large variety of bending in *B. bifidum* WN-04 isolate appeared to resemble the reference strains of *B. bifidum*, especially the "amphora-like" cells that are characteristic (Sundman & Bjorksten, 1959). On the RB media, PCM analysis allowed a better correlation of the natural isolates to the reference strains. Speciation of *B. longum* GB-03 (in Figure 4.6) conversely appeared to favour the reference strain of *B. longum*, especially the ultra-elongated and relatively thin cellular elements with slightly irregular contours (Reuter, 1963).

B. *adolescentis* on modified MRS medium   B. *adolescentis* on RB medium

B. *infantis* on modified MRS medium   B. *infantis* on RB medium

Phase Contrast Micrographs of *Bifidobacterium* reference strains: **Fig. 4.1**, *B. adolescentis* on modified MRS; **Fig. 4.2**, *B. adolescentis* on RB; **Fig. 4.3**, *B. infantis* on modified MRS and **Fig. 4.4**, *B. infantis* on RB, taken at 1000 × magnifications.

B. *longum* GB-03 on modified MRS medium   B. *longum* GB-03 on RB medium

B. *bifidum* WN-04 on modified MRS medium   B. *bifidum* WN-04 on RB medium

Phase Contrast Micrographs of the isolate strains: **Fig. 4.5**, *B. longum* GB-03 on modified MRS; **Fig. 4.6**, *B. longum* GB-03 on RB; **Fig. 4.7**, *B. bifidum* WN-04 on modified MRS and **Fig. 4.8**, *B. bifidum* WN-04 on RB, taken at 1000 × magnifications.
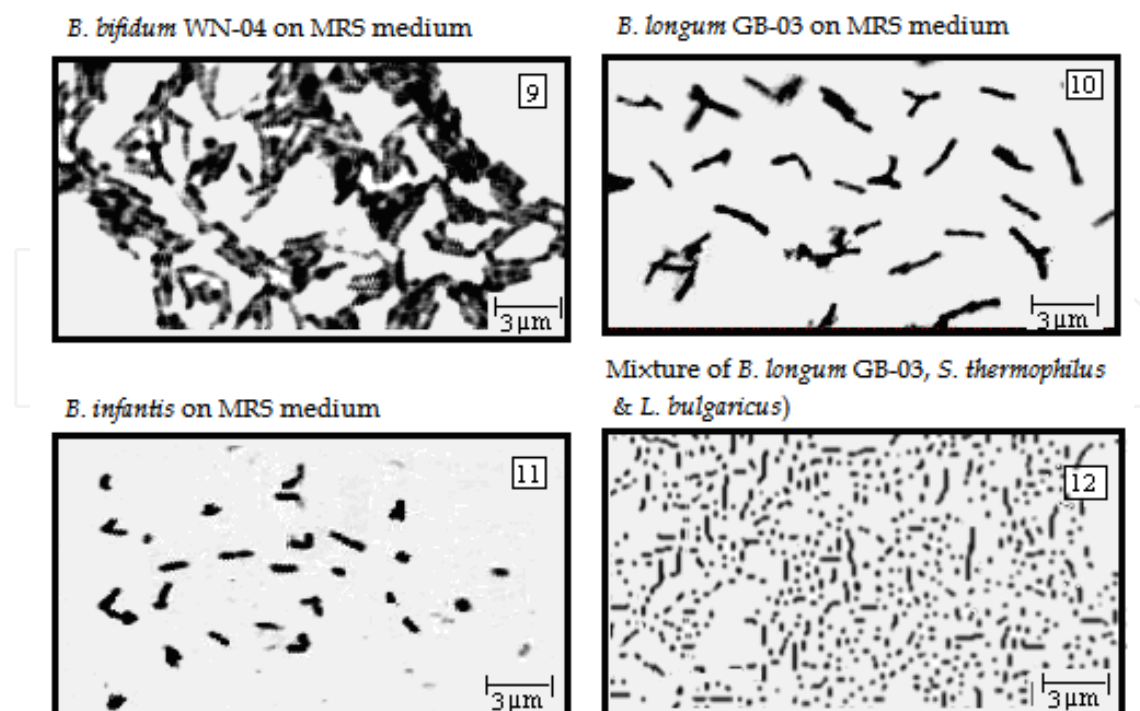
Figure 4. Phase Contrast Micrographs of *Bifidobacterium* strains: **Fig. 4.9**, *B. bifidum* WN-04 on unmodified MRS; **Fig. 4.10**, *B. longum* GB-03 on unmodified MRS; **Fig. 4.11**, *B. infantis* on unmodified MRS and **Fig. 4.12**, *B. longum* GB-03 and an assortment of other 2 Lactic Acid Bacteria (*Streptococcus thermophilus* & *Lactobacillus bulgaricus*), taken at 1000 × magnification.

3.2.2. Long and thin–rods with blunted ends cell morphology

This was the most common type of morphology encountered among the *Bifidobacterium* isolates of *B. longum* GB-03 and *B. bifidum* WN-04 on the unmodified MRS agar (Figures 4.5, 4.7, 4.9 & 4.10). Since only the general cell structure was used to differentiate this species from the other bacteria, PCM proved sufficient for this purpose. Variations of morphology within these small groups were visible under PCM as indicated by the following examples. The isolate of *B. longum* GB-03 in Figure 4.12 exemplified the diversity of rods and coccus cells morphology including bifid structures also; with the absence of any coccus build cells when grown on RB agar in Figure 4.6. By comparison with the reference strains, the cells morphology of *B. longum* GB-03 isolate is more peculiar to that displayed by *B. infantis* (Figure 4.4) and the isolate of *B. bifidum* WN-04 (Figures 4.7 & 4.8). All the *Bifidobacterium* isolates displayed long and short club-shaped rods, most of which were long and thin with blunted ends and of conventional "V" and/or "Y"-shaped cells.

3.3. Confirmation of identity of *Bifidobacterium* strains

3.3.1. Fructose-6-Phosphate Phosphoketolase (F6PPK) verification test

F6PPK is certainly a key enzyme in the "bifidus pathway" and it allows the discrimination of the specific feature on expression of fructose-6 phosphate in cellular extracts that assigned the bifidobacteria to the genus level (Sgorbati, 1979).

The procedure to test for the F6PPK activity in the *Bifidobacterium* strains is still practised as described by Scardovi (1986). In brief, cells harvested from 10 ml RB or MRS broth are washed twice with 50 mM phosphate buffer (pH 6.5). The cells are disrupted by sonication in the cold, and 0.25 ml of each of NaF and Na iodoacetate solution and fructose-6-phosphate (Na Salt: 70% purity) are added to the sonicate. The reaction is stopped by the addition of 1.5 ml of hydroxylamine HCl, and 1 ml each of trichloroacetic acid and 4 M HCl. Finally, 1.0 ml of a colour-developing agent ($FeCl_3.6H_2O$ 5% (w/v) in 0.1 M HCl) is added. A tube without fructose-6-phosphate serves as a blank, to facilitate the visual comparison. The formation of acetyl phosphate from fructose-6-phosphate, shown by the reddish violet colour formed by the ferric chelate of its hydroxamate is an indicator for F6PPK. This is the distinctive and key enzyme of the "bifid shunt" that characterizes the genus. There are three subtypes of F6PPK in bifidobacteria as shown in Figure 5.

3.3.2. Determination of acetic and lactic acids

One possible method of validating the presence of acetic and lactic acids in the fermented milk by bifidobacteria can be assayed by using High Performance Liquid Chromatography (HPLC). Samples for this analysis are prepared by using a modified method described by Dubey & Mistry, (1996).
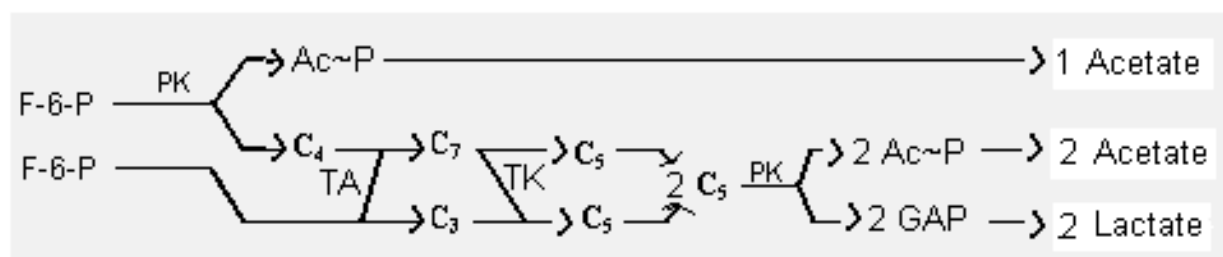

Figure 5. Fermentation of hexose for carbohydrate metabolism (the "bifid shunt"), based on Schlegel (1993), where PK, phosphoketolase; TA, transaldolase; TK, transketolase, Ac~P, acetyl phosphate; GAP, glyceraldehydes-3-phosphate.

The strains were maintained anaerobically by propagation in MRS broth (peptone: 10 g/l; meat extract: 8 g/l; yeast extract 5 g/l; D(+)glucose: 20 g/l; di-potassium hydrogen phosphate: 2 g/l; di-ammonium hydrogen citrate: 2 g/l; Tween-80: 1 ml/l; sodium acetate: 5 g/l; magnesium sulfate: 0.2 g/l; manganese sulfate: 0.04 g/l, supplemented with 0.05% (w/v) cysteine-hydrochloride).

The production of acetic and lactic acids, spore formation, aerobic and anaerobic growth, gram reactions, motility, gas production from lactose and carbohydrates fermentation tests are some of the confirmation tests that proves highly diagnostic personality characteristics of different *Bifidobacterium* spp as summarized in Table 1. Furthermore, the taxonomy of bifidobacteria has changed ever since they were first isolated. They had been assigned to the genera *Bacillus*, *Bacteroides*, *Nocardia*, *Lactobacillus* and *Corynebacterium* among others, before being recognized as a separate genus in 1974.

Many of the *Bifidobacterium* species groupings are heterogeneous and the entire genera have been re-examined using DNA-DNA hybridization. A point is made here that, instant phenotypic characterization of most bacteria within their respective genera relies on biochemical tests such as the proportion of acetic and lactic acid relative to the end product of metabolism; the ratio of acetic and lactic acid produced; some key carbohydrate fermentations; colonies and phenotypic morphologies; and the presence of fructose-6-phosphate phosphoketolase (F6PPK), a key enzyme in the bifidus pathway.

Characteristics	*Bifidobacterium* Strains			
	B. bifidum[1]	B. longum[1]	B. infantis[1]	B. adolescentis[2]
Spore forming	−	−	−	−
Motility	−	−	−	−
Gram reaction	+	+	+	+
Morphology: rods, pleiomorphic	+	+	+	+
Anaerobic growth	+	+	+	+
Aerobic growth	−	−	−	−
Gas from lactose	−	−	−	−
Catalase	−	−	−	−
F6PPK	+	+	+	+
Acetic and lactic production (ratio 3:2)	+	+	+	+
Carbohydrates Fermentation Test				
Cellobiose	−	+	−	+
Fructose	+	+	+	+
Fructooligosacharides	−	+	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Isomaltose	−	+	+	+
Lactose	+	+	+	+
Maltitol[3]	−	−	−	−
Mannose	−	−	−	−
Melezitose	−	−	−	−
Raffinose	+	+	+	+
Stachyose	+	+	+	+
Trehalose	−	−	−	−
Xylose	−	+	−	+

Legends on Table 1: [1] Obtained from American Type Culture Collection, Rockville, USA. [2] Obtained from China General Microorganisms Culture Collection Center, Beijing, China. [3] Maltitol is still widely used as a non-cariogenic sweetener and sugar substitute but is as yet not used as a possible prebiotic. + positive results or fermentation; − negative results or no fermentation observed. F6PPK (fructose-6-phosphate phosphoketolase).

Table 1. Phenotypic characteristics of some of the pH- and bile salts-resistant bifidobacteria tested.

4. Common media used in isolation and detection of bifidobacteria

Many different media for bifidobacteria are outlined in Table 2.

Medium	Selectivity based on*	Used for
Acetylglucosamine-Lactose (AL) agar	lactose, acetylglucosamine	faeces
AMC-agar	nal, polymyxin B, kan, iac, TTC, LiCl, prop	*B. longum*
Bifidobacterium selective (BS) agar	LiCl, neo, paro, prop	faeces
Bifidobacterium selective medium (BBM-agar)	nal, rifampicine, raffinose	faeces
Bifidus Blood Agar	aniline blue, blood	faeces
Bif-medium	human whey, nal, paro, aztreonam, netilmycin	dairy products
Bifidobacterium Iodoacetate Medium (BIM-25 agar)	kan, nal, iac, neo, polymyxin B	sewage
BS-agar	LiCl, neo, paro, prop	faeces
China Blue (CB) agar	specific impact of china blue	faeces
GL-agar	galactose, LiCl	dairy products
Liver Cysteine Lactose (LCL) agar	lactose, liver infusion	faeces
LP agar	lactose, LiCl, prop	dairy products
Modified Rogosa agar	neo, paro, prop, LiCl	dairy products
MPN-agar	lactose, nal	faeces
MRS-LP-agar	prop, LiCl	dairy products
Neomycin Paromomycin Lithium Nalidixic acid (NPLN) agar	LiCl, nal, neo, paro, prop	faeces, dairy products
Propionate or Beerens agar	propionic acid, pH 5.0	faeces
Raffinose-Bifidobacterium (RB) Agar	raffinose, LiCl, propionate	faeces, dairy products
RCM (modified)	low pH	dairy products
RCM + stain	Loeffler's methylene blue stain	dairy products
Rogosa agar	low pH	faeces, dairy products
Rogosa (modified)	neo, paro, prop, LiCl	dairy products
Rogosa-N	low pH, nal	faeces
Tomato Casein Peptone Yeast Agar (TCPY)	tomato juice	faeces
Transgalactosyloligosaccharide (TOS-Agar)	TOS	faeces, dairy products
TOS-Agar (modified)	TOS, nal, neo, paro	dairy products
TPYd-agar	dicloxacillin	dairy products
TTC-agar	TTC	faecal contamination
VF-agar (modified)	LiCl, prop, neo, sodium lauryl sulfate	dairy products
YN-6 agar	lactose, nal, neo, bromocresol green	faeces, sewage

Legends on Table 2: *iac = iodoacetic acid, kan = kanamycin, LiCl = lithiumchloride, nal = nalidixic acid, neo = neomycin, paro = paromomycin, prop = propionate, TOS = transgalactosyl oligosaccharides, TTC = 2,3,5-triphenyl-tetrazoliumchloride

Table 2. Popular media used for the enumeration of bifidobacteria from faeces, dairy- and pharmaceutical products, (Adapted from prebiotic effect on non-digestible oligo- and polysaccharides by Hartemink, 1999).

Media used for the detection of bifidobacteria can be classified in 5 different groups. These are non-selective medium (such as MRS and Rogosa), medium without antibiotics but with elective carbohydrate, medium with antibiotics, medium with propionate, and medium with elective substance and/or low pH (Table 3).

Medium	Group[*]
Acetylglucosamine - Lactose (AL) agar	2
Bifidobacterium selective (BS) agar	3, 5
Bifidobacterium selective medium (BBM) agar	2, 3
Bifidus Blood agar	5
Bifidobacterium Iodoacetate Medium (BIM-25) agar	3
China Blue agar	5
Liver Cysteine Lactose (LCL) agar	2
Rogosa agar	1
Modified Rogosa agar	3, 5
MPN-agar	2, 3
MRS	1
MRS agar with LiCl and antibiotics (MRS-NN)	3, 5
Neomycin Paromomycin Lithium Nalidixic acid (NPLN) agar	3, 4
Propionate agar or Beerens agar	4
Raffinose-Bifidobacterium (RB) agar	2, 4
Reinforced Clostridial agar with Cephalothin and blood (RCB)	3
Tomato Casein Peptone Yeast agar (TCPY)	5
Tomato Casein Peptone Yeast agar (TPCY) with azide	5
Tomato Casein Peptone Yeast agar (TPCY with sorbic acid	5
Tomato Casein Peptone Yeast agar (TPCY with antibiotics	3
Transgalactosyloligosaccharide (TOS - agar)	2
TTC-agar	5
x-Gal medium	5
YN-6 agar	2, 3, 5

Legends on Table 3: [*] group: 1 = non selective medium, 2 = medium without antibiotics but with elective carbohydrate, 3 = medium with antibiotics, 4 = medium with propionate, 5 = medium with elective substance and/or low pH

Table 3. Media used for the detection of bifidobacteria from faeces (Source: Hartemink, 1999).

Combinations and media belonging to more than one group are also used. From the large number of media used, it can be concluded that there is no standard medium for the detection of bifidobacteria. *Bifidobacterium* spp. in the GI tract of humans are normally present in an adequate amounts and estimated to be between 10^9 and 10^{10} colony forming units (CFU) per gram wet weight or around 3% of total microbiota (Jia *et al.*, 2010). However, the selectivity of independent media for the quantification of bifidobacteria is thoroughly examined and tested with different baby faeces.

The experimental results of 3 media (PROP, RB and NPLN) tested on bifidobacteria show a wide variation in counts for the different samples (see Figure 6). Absolute counts are highest for the faecal samples on NPLN, followed by RB in 8 of 9 samples. PROP showed the lowest counts. However, as it can be observed from the same Figure 6, the principal difference between these 3 media is exceedingly little, actually less than one log unit.
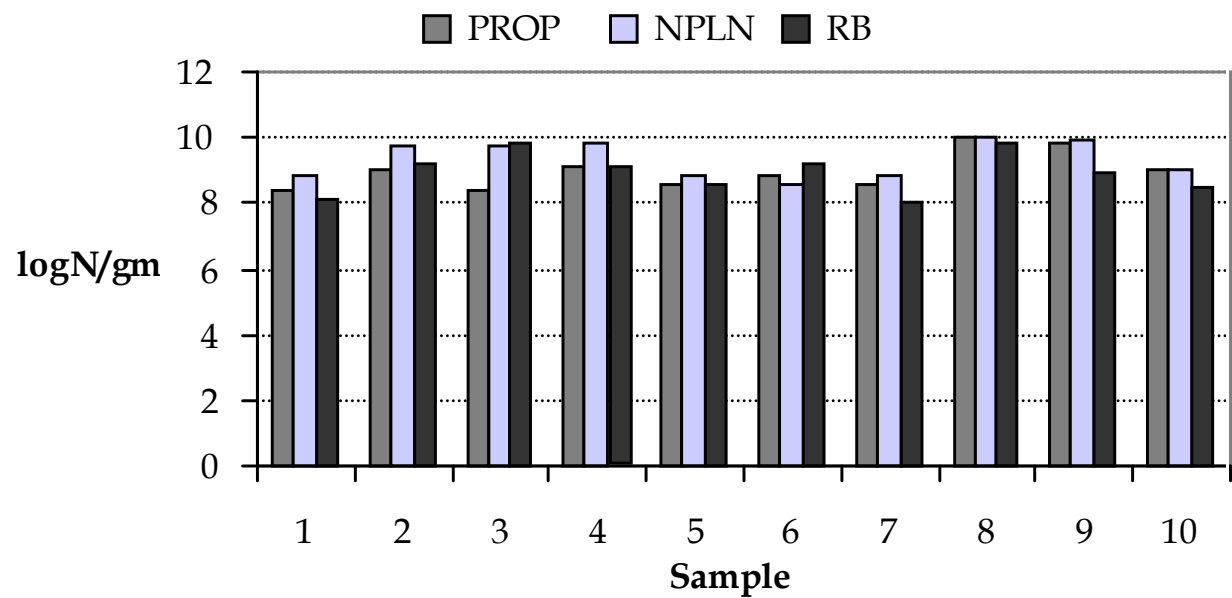
Figure 6. Counts (log CFU/gm wet weight) on PROP, NPLN and RB media in babies' faeces.

Selectivity is also determined by microscopic observations of all different colony morphologies on all countable (between 10 and 150 colonies/plate) plates (see Table 4). Based on morphologies, selectivity is highest for babies' faeces with NPLN with 29% false positive colonies (growth, but no bifidobacterial morphology). PROP showed 39% false positive and RB with 50% false positives. False negatives (non-typical colonies, but bifid morphology) can be determined on RB, as this is the only medium for which typical colonies are described. However, no false negatives were observed in this work.

Medium	Babies' faeces		
	morphology		
	n	typical	non-typ. [b]
RB pos [c]	24	12	12 (50)
RB neg	4	0	4 (0)
PROP	18	11	7 (39)
NPLN	28	20	8 (29)

Legends on Table 4: [b] number in brackets is the percentage of false positive (typical colony, non-typical morphology) or false negatives (non-typical colony, typical morphology) of the colonies tested. [c] pos = colonies showing characteristics for bifidobacteria, neg = colonies not showing characteristics for bifidobacteria. Bifidobacteria characteristics were defined as yellow-green colonies with a yellow halo. This attribute could only be determined on RB, as no characteristics were defined for other media.

Table 4. Selectivity of media for bifidobacteria.

Most false positive colonies are reported to be different cocci (mono-, diplo- or streptococci), spore-forming rods and short rods. No yeast is observed on any of the media tested. Based on the actual counts, selectivity can only be determined for RB, as the colonies of bifidobacteria and non-bifidobacteria cannot be determined for the other media and not all colonies are tested for their morphology. Selectivity as percentages of non-typical colonies ranges from around 5 – 7%.

Colonies of different shapes can be tested microscopically. Bacterial morphology is determined, and typical and non-typical morphology is also determined. Typical morphology of bifidobacteria is branched or bifid-shaped rods. For the determination of bifidobacteria, none of the 3 media tested was decidedly selective. In this study, the occurrence of false positive or false negative colonies was determined. The lowest incidence of potential false positive colonies was observed on NPLN, but in all 3 media, the number of non-bifidobacteria capable of growing on the selective media was remarkably high. When many different species are capable of growing on the medium, an increase of one of these species may result in serious mistakes in calculating bifidobacteria. NPLN and RB gave slight higher counts than PROP. The incidence of false positive, based on morphologies on RB was comparable with that on the PROP and slightly higher than that on NPLN. The incidence of competitive flora was relatively low (less than 10% of the total colonies on the plates), as bifidobacteria are one of the main groups of intestinal bacteria in humans.

PROP medium has been described as the best medium for the determination of bifidobacteria by Silvi *et al.*, (1996), but they also concluded that the total bifidobacterial counts were significantly lower on PROP than on the other media tested. Similarly, Favier *et al.*, (1997) concluded that PROP underestimated bifidobacteria in some of their samples. Both studies used human faeces as the test substrate. Several other studies, in which PROP agar is used, also show significantly lower bifidobacterial counts than most other studies (Favier *et al.*, 1997).

NPLN, which has been described as the medium of choice to choose bifidobacteria in dairy products, showed many cocci. This was in accordance with results observed by Silvi *et al.*, (1996). In the same study, BIM-25 was tested, and this medium was found to be non-specific. All these 3 media performed reasonably well for human faeces and bifidobacteria can reliably be counted. The typical colonies morphological trait and the basic cellular-morphology of bifidobacteria were demonstrated well by RB media, with reference to NPNL and PROP medium. The RB medium presented strains with double thickness diameter and more bifurcated cellular morphology under phase contrast microscopy.

5. Experimental procedures for the enumeration of bifidobacteria and determining microbial inactivation by low acidic pH or bile salts

LAB or bifidobacteria strains can be selected or isolated from commercial or alleged "own isolates" strains, from freeze-dried cultures which are resuscitated to stationary phase in MRS broth at a ratio of 2% of the volume of the fresh broth. Decimal dilutions are put onto Raffinose–Bifidobacterium (RB) agar plates whose pH had to be adjusted to 6.8 – 7.0 with 2

N NaOH. The agar plates are then incubated anaerobically at 38.5 ºC for 3 – 4 d and number of colony forming units (CFU)/mL are determined. Two hundred microliters of each strain containing about 10^8 CFU/mL is aseptically transferred into test tubes containing 9 mL of diluted MRS medium with pH adjustments of 3.0, 3.5, 4.0, or 4.5, using 2 N HCl. These suspensions are incubated anaerobically at 38.5 ºC and numbers of survivors are determined after various times as shown in Figure 7 (A). Cells were harvested by centrifugation at 5 ºC, were washed with phosphate-buffered saline (PBS) and were re-suspended in diluted MRS medium without pH adjustment. After thorough mixing on a vortex mixer, the concentration of surviving cells is determined by anaerobic pour plate counts, using 2 plates of RB agar per dilution, and incubated at 38.5 ºC for 3 – 4 days.

Similarly, treatments for the bile salts are carried out at the final concentrations of 0.15%, 0.30%, 0.45%, and 0.60% ox-gall in diluted MRS medium (pH 6.8), exposed to appropriate times as to low pH and incubated anaerobically at 38.5 ºC (see Figure 7 (B). The cells are harvested by centrifugation, washed with PBS, re-suspended in diluted MRS medium without pH adjustment, and mixed using a vortex mixer as described for acidic pH conditions before. Numbers of CFU of bifidobacteria surviving the lytic effect of bile salts are also determined by anaerobic pour plate counts on RB agar after anaerobic incubation for 3 – 4 d at 38.5 ºC.

5.1. Characterizations for $D_{(acid)}$-, $D_{(bile)}$-, $z_{(acid)}$, and $z_{(bile)}$-values

$D_{(acid)}$-value is defined as the time (in min) required at a specified acidic pH to reduce the number of cells by 90%, while $D_{(bile)}$-value is defined as the time (in min) required at a specified concentration of bile salts to reduce the number of cells by 90%. In fact, the $D_{(bile)}$-value of any LAB or bifidobacterial strain is directly proportional to the bile salt concentrations, while the $D_{(acid)}$-value is inversely proportional to the acidic pH.

The $z_{(acid)}$ and/or $z_{(bile)}$-values, on the other hand, is defined as a decrease in pH value (pH < 4.5) or an increase in bile salt concentration (% ox-bile) required to reduce the D-values by 1 log cycle, however, respectively. The $D_{(acid)}$- and $D_{(bile)}$-values can be directly calculated from the absolute values of the reciprocal of the slopes of the linear-regression equations, using a Microsoft Office–Excel software. It is essential to emphasize that, the regression lines must be applied to all the treatments, for which restriction of the R-squared (R^2) value is pragmatic above 0.8920. Moreover, the $D_{(acid)}$- and/or $D_{(bile)}$-values can also be calculated algebraically from the regression equation derived using the method of least-squares to be able to produce the $z_{(acid)}$- and/or $z_{(bile)}$-values for the probiotic strains.

In order to determine the $z_{(acid)}$- and/or $z_{(bile)}$-values, the formula is exactly the same as that for heat resistance, replacing T (temperature) with pH values or bile salts (BS) concentrations as described by Equations (1) and (2), respectively. In both of these cases, the effect of acidic conditions and bile salts is determined from the reduction in concentration of colony-forming units. One has to pay attention that the dynamic $z_{(acid)}$- and/or $z_{(bile)}$-values are calculated for a period of exponential destruction of microbial cells (following the logarithmic order of death), using both Equations (1) and (2).
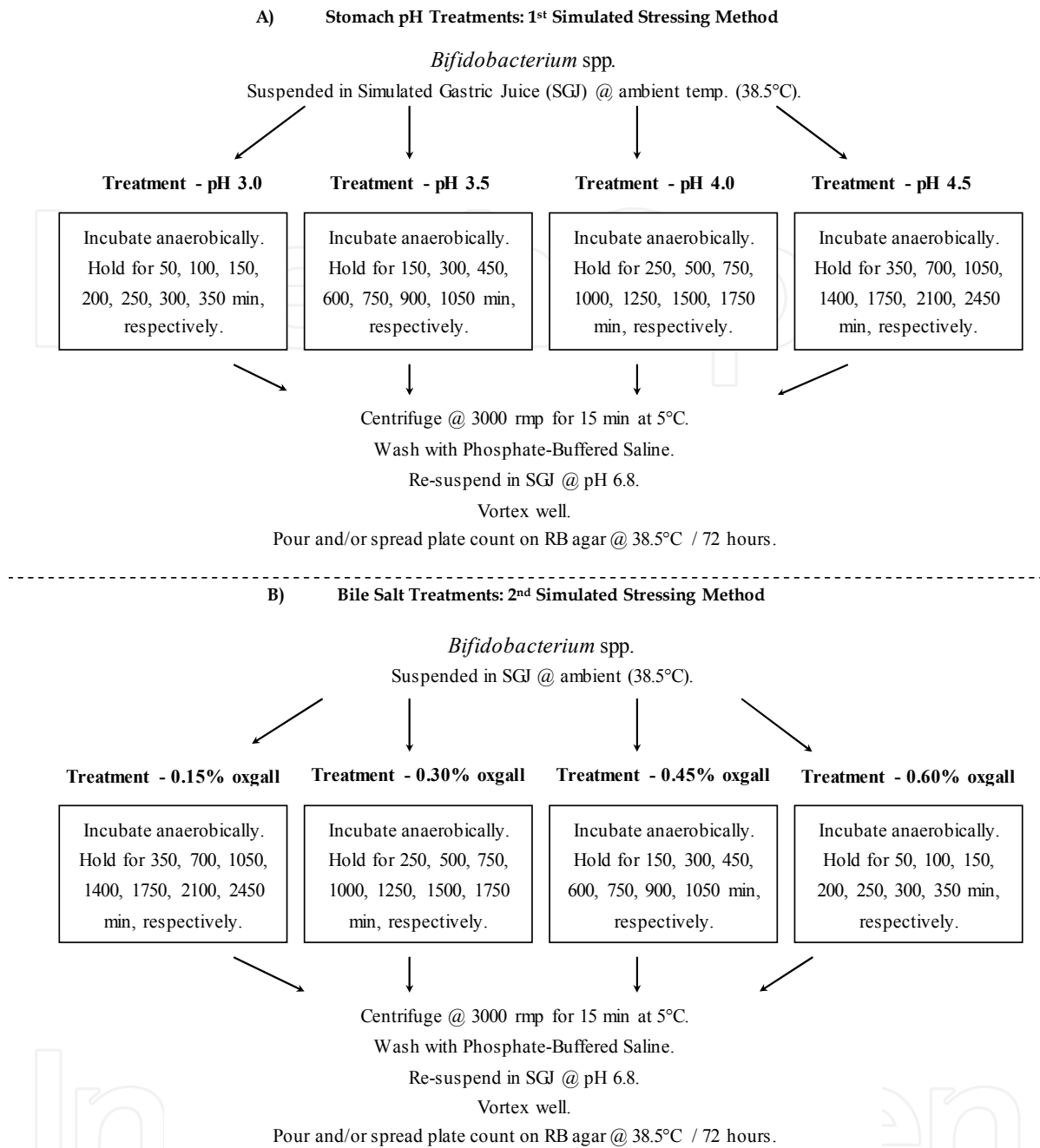
A)   Stomach pH Treatments: 1st Simulated Stressing Method

Bifidobacterium spp.

Suspended in Simulated Gastric Juice (SGJ) @ ambient temp. (38.5°C).

Treatment - pH 3.0

Incubate anaerobically. Hold for 50, 100, 150, 200, 250, 300, 350 min, respectively.

Treatment - pH 3.5

Incubate anaerobically. Hold for 150, 300, 450, 600, 750, 900, 1050 min, respectively.

Treatment - pH 4.0

Incubate anaerobically. Hold for 250, 500, 750, 1000, 1250, 1500, 1750 min, respectively.

Treatment - pH 4.5

Incubate anaerobically. Hold for 350, 700, 1050, 1400, 1750, 2100, 2450 min, respectively.

Centrifuge @ 3000 rmp for 15 min at 5°C.

Wash with Phosphate-Buffered Saline.

Re-suspend in SGJ @ pH 6.8.

Vortex well.

Pour and/or spread plate count on RB agar @ 38.5°C / 72 hours.

- -

B)   Bile Salt Treatments: 2nd Simulated Stressing Method

Bifidobacterium spp.

Suspended in SGJ @ ambient (38.5°C).

Treatment - 0.15% oxgall

Incubate anaerobically. Hold for 350, 700, 1050, 1400, 1750, 2100, 2450 min, respectively.

Treatment - 0.30% oxgall

Incubate anaerobically. Hold for 250, 500, 750, 1000, 1250, 1500, 1750 min, respectively.

Treatment - 0.45% oxgall

Incubate anaerobically. Hold for 150, 300, 450, 600, 750, 900, 1050 min, respectively.

Treatment - 0.60% oxgall

Incubate anaerobically. Hold for 50, 100, 150, 200, 250, 300, 350 min, respectively.

Centrifuge @ 3000 rmp for 15 min at 5°C.

Wash with Phosphate-Buffered Saline.

Re-suspend in SGJ @ pH 6.8.

Vortex well.

Pour and/or spread plate count on RB agar @ 38.5°C / 72 hours.

Figure 7. Schematic diagram of treatment groups for the selected bifidobacteria. A): Influence of gastric acidity and its residence time. B): Influence of bile salt(s) and its residence time.

$$z_{(acid)} = \frac{-(acid\ pH_2 - acid\ pH_1)}{Log_{10}\left(\dfrac{D_{(acid\,1)}}{D_{(acid\,2)}}\right)} \tag{1}$$

$$z_{(bile)} = \frac{BS_2 - BS_1}{Log_{10}\left(\dfrac{D_{(bile\,1)}}{D_{(bile\,2)}}\right)} \tag{2}$$

where z is the acid pH value (in Equation 1) or bile salts value (in Equation 2) required for a ten-fold reduction in D-values; pH_1 is the acidic value of pH 1; pH_2 is the acidic value of pH 2; BS_1 is the concentration of bile salts 1 (%); BS_2 is the concentration of bile salts 2 (%); $D_{(acid1)}$ or $D_{(bile1)}$ is a D-value obtained at either pH_1 or BS_1; and $D_{(acid2)}$ or $D_{(bile2)}$ is a D-value obtained at either pH_2 or BS_2.

5.2. Survival of bifidobacteria in simulated acidic pH of human stomach

Figure 8 shows the survival of selected *Bifidobacterium* strains exposed to various acidic pH levels. The bifidobacterial counts (range: 5.5 – 6.7 log CFU/ml) of all four strains at pH 3.5 after 5 h are an indication of resistance that may perhaps simulate gastric conditions. In fact, with *B. bifidum*, *B. infantis*, and *B. longum* the counts were > 2.0 log CFU/ml after exposure for 5 h, which indicates that these strains are relatively resistant at pH 3.0. However, as observed from all the experimental results in Figure 8, the *B. adolescentis* strain is more sensitive than the other three strains to all the acid treatments. For instance, numbers of *B. adolescentis* are 3.4 log CFU/ml after 10 h at pH 3.5, but below the level of exposure thereafter, while a similar count (~3.4 log CFU/ml) is observed with the other three strains after 12.5 h. This 2.5 h difference in survival at pH 3.5 is approximately the time chosen by Olejnik *et al.*, (2005) to control acid resistance, as these times simulate residence time in the stomach. On this basis, this specific strain of *B. adolescentis* is considered a less-resistant strain with respect to gastric acidity.

Many other researchers have found pH 2.0 and pH 3.0 to be lethal and sublethal pH values respectively for lactic acid bacteria (LAB), including bifidobacteria (Khalil *et al.*, 2007). It is vital to stress that probiotics are able to confer health benefits despite the brief exposure to exact acidic conditions following ingestion. Although a log-scale reduction of viability may occur, it may still mean that a sufficient number of bifidobacteria survive the gut, depending on the dose. Moreover, the exposure to acid does not mean that the potential health benefits are lost. Some cells may die, and some may be injured. However, these cells may recover later, and they may also have beneficial effects on health. The mechanism may be mediated, for example, through the components of the cell wall of the probiotics which will then be available in both dead and living cells. It should be also borne in mind that, the food matrix in which the probiotics are consumed is likely to have a strong effect on the survival of the bacteria in the gut.

In Figure 8, for example, it is possible to say that the high survival counts of *B. bifidum*, *B. infantis*, and *B. longum* exposed to pH 4.5 after 41 h is a representation of culture stability curves of the acid resistant strains (log CFU/ml) in a food matrix. While the pH of 4.5 does not represent gastric acid conditions, it is a typical representation of fermented products, and in such products, the survival counts are expected to last for much longer periods during shelf life, especially at the refrigeration temperature of 4 °C.

5.3. Survival of bifidobacteria in simulated bile salts nature

Resistance to bile salts is considered an intrinsic property for probiotic strains to survive the conditions in the small intestine. The physiological bile salt concentration in the GI tract of

humans is estimated to be 0.3 – 0.4% w/v (Jia *et al.*, 2010). As shown in Figure 9, the linear regressions of the loss of CFU did express satisfactorily that *B. bifidum*, *B. infantis*, and *B. longum* are more-resistant strains to the bile salts. These 3 strains survived well in 0.45% (w/v) bile salts, with more than 4.5 log CFU/ml present after 10 h. Their capacity to survive high bile salt concentrations suggests the existence of defence mechanisms and confirms that strains showing antagonistic effect against enteric pathogens should be able to compete successfully with the pathogens in the GI tract. It is clear that *B. adolescentis* is again the less-resistant strain encountered with only 2.8 log CFU/ml surviving after 10 h in 0.45% bile salts (see Figure 9). Therefore, *B. adolescentis* is considered the less-resistant strain, while *B. bifidum*, *B. infantis*, and *B. longum*, in that order, are considered the more-resistant. It is well known that, the bile salt hydrolytic (BSH) activity may be the contributing factor towards the resistance of the LABs and to the toxicity of conjugated bile salts in the duodenum, and therefore, is an essential colonization factor.

5.4. The feasibility of $D_{(acid)}$-, $D_{(bile)}$-, $z_{(acid)}$- and $z_{(bile)}$-values for selection of probiotic strains and for determining the mechanisms of resistance to acid and bile salts stress

Table 5, shows that accurate tabulation of the $D_{(acid)}$- or $D_{(bile)}$-values and their respective $z_{(acid)}$- or $z_{(bile)}$-values is tremendously helpful in evaluating the resistance and susceptibility of probiotics to acidic pH and high bile salt concentrations, respectively. Both the estimated $D_{(acid)}$- and $D_{(bile)}$-values validated that the most acid- and bile-resistant strain is *B. bifidum* followed by *B. infantis*, *B. longum*, and final *B. adolescentis.* It is also possible to observe in Table 5, that, increasing the bile salt concentration from 0.15 to 0.60% had a greater impact on survival than decreasing the pH values from 4.5 to 3.0, with the $D_{(bile)}$-values of *B. bifidum* decreasing from 17.40 to 1.40 min and the $D_{(acid)}$-values decreasing from 23.80 to 1.10 min. Similar trends are observed with all other *Bifidobacterium* strains. However, decreases of depicted $z_{(acid)}$-value in the pH value (pH<4.5) or increases of depicted $z_{(bile)}$-value in the bile salt concentrations (% ox-bile) are expected to cause a 1-log reduction in their respective *D*-values. In practice, $z_{(acid)}$- or $z_{(bile)}$-value measures how the sensitivity of probiotic strains is to small changes in [H+] and/or [OH-] or bile salts. As for probiotics to gain intestinal colonization in humans or animals for their proclaimed therapeutic health benefits, obviously, they have to tolerate inhibitory substances secreted by the host, such as gastric acids (in the stomach) and bile salts (in the small intestine).

Of all ions, H+ and OH− are the most mobile, and minor changes in their concentrations show significant effects on microorganisms. Most organisms survive better when these ions are present in approximately equal concentrations, that is, pH 7.0. Although many bacteria tolerate higher pH values, only a few are acid tolerant or acidophilic. In addition, many other bacteria are tolerant of small pH variations, especially in the pH range of 6.0 to 9.0. For instance, if the pH of the medium changes rapidly, there may be a transient change in the intracellular pH, and this is usually readjusted to the original pH within 30 min. Consequently, any damage produced by adverse pH is not actually due to the H+ and/or

OH⁻, but to the effect of these ions on the proportion of undissociated weak acids or bases, which penetrate more readily into the bacterial cell than the ionized forms. In contrast, bile salts are biological detergents synthesized in the liver from cholesterol, conjugated to either glycine or taurine, and are then secreted into the intestine where they facilitate fat absorption. Bile salts are well known to be toxic for many cells as they disrupt the lipid bilayer structure of the cellular membranes. Many earlier studies revealed that the autochthonous gastrointestinal microbiota must develop strategies to protect themselves against bile salts.

Figure 8. Linear regressions of the loss of CFU for the selected bifidobacteria strains when exposed to simulated gastric acidity of pH 3.0, pH 3.5, pH 4.0 and pH 4.5, respectively: (a) *B. bifidum*, (b) *B. longum*, (c) *B. infantis*, (d) *B. adolescentis*.
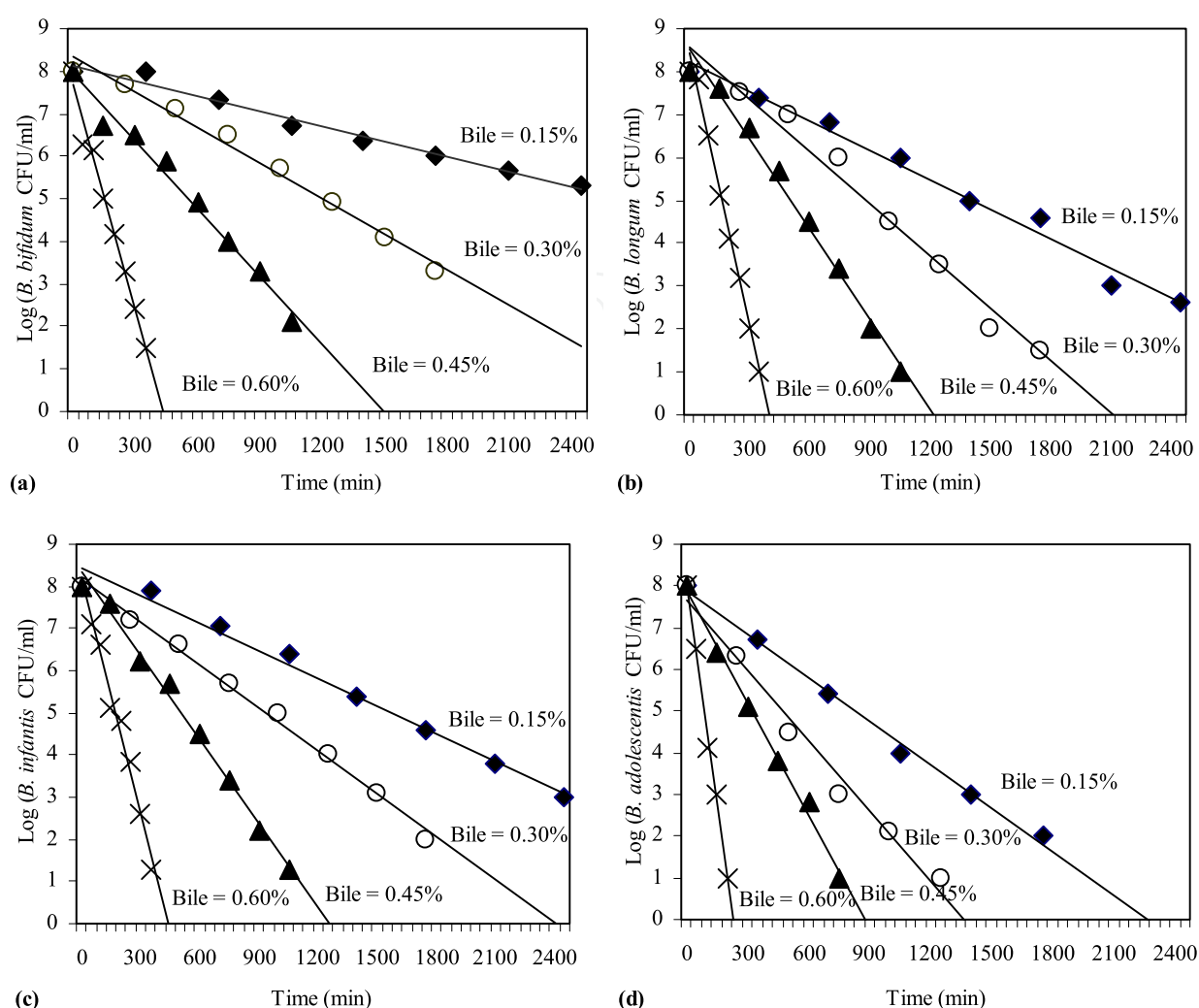
Figure 9. Linear regressions of the loss of CFU for the selected bifidobacteria strains when exposed to high bile salt (oxgall) concentrations of 0.60%, 0.45%, 0.30% and 0.15%, respectively: (a) *B. bifidum*, (b) *B. longum*, (c) *B. infantis*, (d) *B. adolescentis*.

The individual $z_{(acid)}$- or $z_{(bile)}$-values calculated from their $D_{(acid)}$- and $D_{(bile)}$-values ranged from 1.11 – 1.55 pH units and 0.40 – 0.49%, respectively (Table 5). Although the combination of both the low acidic pH and bile salts is not assessed, it is assumed that at pH < 3.0, and 0.60% of ox-bile, the combined effects could be more synergistic and even greater in magnitude for probiotic bacteria to survive. Additionally, the $D_{(acid)}$- and $D_{(bile)}$-values reveal a modern and efficient sorting order of the more-resistant probiotic strains to these two distinct hostile GI tract conditions in humans. Many authors have investigated the effect of bile on survival of LAB. For example, Kim *et al.*, (1999) examined the effect of bile concentration in the range of 0 – 0.4% on survival of *Lb. lactis* and found bile to be toxic at concentrations over 0.04%. Shimakawa *et al.*, (2003) reported that 0.2% oxgall in the growth medium inhibited growth of *B. breve* strain Yakult. Others detected that all bacterial cells were killed by 0.2% bile and higher (Olejnik *et al.*, 2005). However, Khalil *et al.*, (2007) reported higher resistance to bile salts, with viability of strains apparently increasing when exposed to high levels of oxgall (0.4%).

pH	$D_{(acid)}$-value (min)			
	B. bifidum	*B. infantis*	*B. longum*	*B. adolescentis*
4.5	23.80	14.10	12.00	7.60
4.0	8.40	6.00	5.70	3.98
3.5	3.00	2.70	2.60	2.05
3.0	1.10	1.10	1.20	1.10
	$z_{(acid)}$-value (in pH units)			
	1.11	1.55	1.35	1.55
Bile Salts	$D_{(bile)}$-value (min)			
0.15%	17.40	10.50	9.60	6.80
0.30%	7.40	5.20	4.70	3.20
0.45%	3.20	2.55	2.30	1.58
0.60%	1.40	1.30	1.10	0.75
	$z_{(bile)}$-value (% ox-bile concentration)			
	0.40	0.48	0.49	0.46

Table 5. Selected *Bifidobacterium* strains and their calculated $D_{(acid)}$-, $D_{(bile)}$-, $z_{(acid)}$- and $z_{(bile)}$-values.

As compared to previous studies, the practicality of $D_{(acid)}$-, $D_{(bile)}$-, $z_{(acid)}$- and $z_{(bile)}$-values as new kinetic-measurements applied in this study, are indeed, quick to identify comparably higher survival of bifidobacteria cells (> 4.1 log CFU/ml after 2.5 h) at elevated bile salt concentrations of 0.6% (w/v), thereby confirm also that the individual *Bifidobacterium* strains are resistant to harsh intestinal conditions in the following order: *B. bifidum > B. infantis > B. longum > B. adolescentis*. A number of researchers reported that *B. infantis* had the highest survival rates followed by *B. bifidum, B. breve and B. longum*, when exposed to bile salt at concentrations ranging from 0 to 3 g/l. In contrast, the literature contains also one preliminary report that *B. longum* exhibited the highest tolerance to bile salts followed by *B. bifidum* and *B. infantis*, which was almost the exact opposite in order of their tolerance to acidic pH. These contrasting observations may reflect the strain-specific resistance to acid or bile salts stress. It also indicates that tolerance is strain- rather than species-specific. Likewise, the source of isolation of the probiotic strains is particularly influential too.

6. Conclusion

Apart from the isolation, enumeration, unequivocal taxonomical characterization, screening and selection of tolerant strains of bifidobacteria to gastric acid and bile salts studies, the assessment of the tolerant bifidobacteria to bile salts and low pH has been made possible by use of D- and z-value concept. After log-conversion, inactivation followed first-order kinetic law whereby validating the kinetic assumptions of the latter concept. The projected $z_{(acid)}$- and $z_{(bile)}$-values were all fairly similar for the bifidobacteria strains and suggested the effect of increasing the bile salt concentration or decreasing the pH on the $D_{(acid)}$- and $D_{(bile)}$-values. This approach is useful for measuring the resistance and sensitivity of lactic acid bacteria or bifidobacteria to these two hostile gastrointestinal conditions. The approach pursued in this chapter would be extremely useful for predicting the suitability of bifidobacteria and/or other LAB as probiotics for use in real life situations. While the mechanisms of probiotic survival in the GI tract could be more complex, the practical utility of the $D_{(acid)}$- and/or $D_{(bile)}$- and their $z_{(acid)}$- and $z_{(bile)}$-values is significant.

Author details

Nditange Shigwedha[*] and Li Jia
University of Namibia & Meat Corporation of Namibia, Namibia

Acknowledgement

---

[*] Corresponding Author

7. References

Biavati, B.; Scardovi, V. & Moore, W.E.C. (1982). Electrophoresis Patterns of Protein in the Genus *Bifidobacterium* and Proposal of Four New Species. *International Journal of Systematic Bacteriology*, Vol.32, No.3, (July 1982), pp. 358-373

Chung, H.S.; Kim, Y.B.; Chun, S.L. & Ji, G.E. (1999). Screening and Selection of Acid and Bile Resistant Bifidobacteria. *International Journal of Food Microbiology*, Vol.49, No.1-2, (March 1999), pp. 25-32

Dubey, U.K. & Mistry, V.V. (1996). Effect of Bifidogenic Factors on Growth Characteristics of Bifidobacteria in Infant Formulas. *Journal of Dairy Science.* Vol.79, No.7, (July 1996), pp. 1156-1163

Favier, C.; Neut, C.; Mizon, C.; Cortot, A.; Colombel, J.F. & Mizon, J. (1997). Fecal β-D-Galactosidase Production and Bifidobacteria are Decreased in Crohn's Disease. *Digestive Diseases and Sciences*, Vol.42, No.4, (April 1997), pp. 817-822

Glickman, R.M. (1980). Intestinal Fat Absorption, In: *Nutrition and Gastroenterology*, M. Winick, (Ed.), 29-41, Wiley, New York, USA

Hartemink, R. (1999). *Prebiotic Effects of Non-Digestible Oligo- and Polysaccharides*, Ponsen & Looijen, ISBN 90-5808-051-X, Wageningen, The Netherlands

Jia, L.; Shigwedha, N. & Mwandemele, O.D. (2010). Use of D_{acid}-, D_{bile}-, z_{acid}- and z_{bile}-Values in Evaluating Bifidobacteria with Regard to Stomach pH and Bile Salt Sensitivity. *Journal of Food Science*, Vol.75, No.1, (January/February 2010), pp. M14-M18

Joint FAO/WHO. (2002). Guidelines for the Evaluation of Probiotics in Food. Ontario, Canada: 30 April - 1 May 2002, Available from
http://www.who.int/foodsafety/publications

Kazor, C.E.; Mitchell, P.M.; Lee, A.M.; Stokes, L.N.; Loesche, W.J.; Dewhirst, F.E. & Paster, B.J. (2003). Diversity of Bacterial Populations on the Tongue Dorsa of Patients with Halitosis and Healthy Patients. *Journal of Clinical Microbiology*, Vol.41, No.2, (February 2003), pp. 558-563

Khalil, R.; Mahrous, H.; El-Halafawy, K.; Kamaly, K.; Frank, J. & El Soda, M. (2007). Evaluation of the Probiotic Potential of Lactic Acid Bacteria Isolated from Faeces of Breast-Fed Infants in Egypt. *African Journal of Biotechnology*, Vol.6, No.7, (April 2007), pp. 939-949

Kim, W.S.; Ren, J. & Dunn, N.W. (1999). Differentiation of *Lactococcus lactis* Subspecies *lactis* and Subspecies *cremoris* Strains by their Adaptive Response to Stresses. *FEMS Microbiology Letters*, Vol.171, No.1, (February 1999), pp. 57-65

Liepke, C.; Adermann, K.; Raida, M.; Magert, H-J.; Forssmann, W-G. & Zucht, H-D. (2002). Human Milk Provides Peptides Highly Stimulating the Growth of Bifidobacteria. *European Journal of Biochemistry*, Vol.269, No.2, (January 2002), pp. 712-718

Lourens-Hattingh, A. & Viljoen, B.C. (2001). Growth and Survival of a Probiotic Yeast in Dairy Products. *Food Research International*, Vol.34, No.9, (November 2001), pp. 791-796

Macfarlane, S. & Dillon, J.F. (2007). Microbial Biofilms in the Human Gastrointestinal Tract. *Journal of Applied Microbiology*, Vol.102, No.5, (May 2007), pp. 1187-1196

Mitsuoka, T. (1990). Bifidobacteria and their Role in Human Health. *Journal of Industrial Microbiology & Biotechnology*, Vol.6, No.4, (April 1990), pp. 263-267

Olejnik, A.; Lewandowska, M.; Obarska, M. & Grajek, W. (2005). Tolerance of *Lactobacillus* and *Bifidobacterium* Strains to Low pH, Bile Salts and Digestive Enzymes. *Electronic Journal of Polish Agricultural Universities*, Vol.8, No.1, Available from http://www.ejpau.media.pl/volume8/issue1/art-05.html

Palacios, M.C.; Haros, M.; Rosell, C.M. & Sanz, Y. (2008). Selection of Phytate-Degrading Human Bifidobacteria and Application in Whole Wheat Dough Fermentation. *Food Microbiology*, Vol.25, No.1, (February 2008), pp. 169-176

Patrignani, F.; Lanciotti, R.; Mathara, J.M.; Guerzoni, M.E. & Holzapfel, W.H. (2006). Potential of Functional Strains, Isolated from Traditional Maasai Milk, as Starters of the Production of Fermented Milks. *International Journal of Food Microbiology*, Vol.107, No.1, (March 2006), pp. 1-11

Pei, Z.; Bini, E.J.; Yang, L.; Zhou, M.; Francois, F. & Blaser, M.J. (2004). Bacterial Biota in the Human Distal Esophagus. *Proceedings of the National Academy of Sciences (PNAS)*, Vol.101, No.12, (March 2004), pp. 4250-4255.

Reuter G. 1963. Vergleichede Untersuchungen über die Bifidus-Flora im Säuglings- und Erwachsenenstuhl. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt I Orig*, Vol.191, (1963), pp. 486-507

Scardovi, V. (1986). Genus *Bifidobacterium*, In: *Bergey's Manual of Systematic Bacteriology*, P.H.A. Sneath, N.S. Mair, M.E. Sharpe, & J.G. Holt (Eds.), 1418-1434, Williams & Wilkins, Baltimore, USA

Schlegel, H.G. (1993). *General Microbiology*, Cambridge University Press, ISBN 0-521-43372-X, Melbourne, Australia

Sgorbati, B. (1979). Preliminary Quantification of Immunological Relationships among the Transaldolases of the Genus *Bifidobacterium*. *Antonie van Leeuwenhoek*, Vol.45, No.4, (April 1979), pp. 557-564

Shimakawa, Y.; Matsubara, S.; Yuki, N.; Ikeda, M. & Ishikawa, F. (2003). Evaluation of *Bifidobacterium breve* Strain Yakult-Fermented Soymilk as a Probiotic Food. *International Journal of Food Microbiology*, Vol.81, No.2, (March 2003), pp. 131-136

Silvi, S.; Rumney, C.J. & Rowland, I.R. (1996). An Assessment of the Three Selective Media for Bifidobacteria in Faeces. *Journal of Applied Microbiology*, Vol.81, No.5, (November 1996), pp. 561-564

Sundman, V.; Björksten, K.A.F. & Gyllenberg, H.G. (1959). Morphology of the Bifid Bacteria (Organisms Previously Incorrectly Designated *Lactobacillus bifidus*) and Some Related Genera. *Journal of General Microbiology*, Vol.21, No.2, (October 1959), pp. 371-384

Yildirim, Z. & Johnson, M.G. (1998). Characterization and Antimicrobial Spectrum of Bifidocin B, a Bacteriocin Produced by *Bifidobacterium bifidum* NCFB 1454. *Journal of Food Protection*, Vol.61, No.1, (January 1998), pp. 47-51