CHARACTERIZATION OF BIFIDOBACTERIAL STRAINS OBTAINED FROM UKRAINE

A Thesis in
Food Science
by
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ABSTRACT

Speciation of bifidobacterial isolates using traditional biochemical and phenotypic methods is tedious and often provides inconclusive results. Characterization using DNA-based methods may provide less ambiguous results. Ten bifidobacterial strains, identified by the Ukrainian Technological Institute of Milk and Meat (TIMM) as Bifidobacterium adolescentis (2), Bifidobacterium bifidum (2), Bifidobacterium longum ssp. longum (4), Bifidobacterium animalis ssp. animalis (1), Bifidobacterium longum ssp. infantis (1) using biochemical and phenotypic methods, were characterized to the species level using Polymerase Chain Reaction (PCR) and also evaluated by Pulsed Field Gel Electrophoresis (PFGE). Bifidobacterial strains were grown on Liver Lactose Agar (LLA) and on MRS-cysteine (MRS) at 37°C for 72 hours under anaerobic conditions. Single isolated colonies of each strains were picked from LLA or MRS plates and evaluated by PCR using primers specific for Bifidobacterium genus and then with primers specific for B. animalis ssp. lactis, B. longum ssp. longum, B. bifidum, B. adolescentis, B. animalis ssp. animalis, and B. longum ssp. infantis, respectively. Although all ten TIMM isolates were identified as members of the genus Bifidobacterium, results obtained using species-specific primers revealed all ten isolates obtained from TIMM were misidentified and were actually isolates of B. animalis ssp. lactis. Further evaluation using PFGE to assess strain relatedness showed all ten isolates gave PFGE patterns identical to the type strain DSMZ 10140T when digested with SpeI. When digested with XbaI, 9 of the isolates gave patterns identical to DSMZ 10140T. One strain, RT09, had one extra band when digested with XbaI. Allelic profiling of the
Ukrainian bifidobacterial strains, revealed four distinct groups. Interestingly, 6 (60%) of the isolates fell into the same cluster as that containing the common commercial probiotic strain BB-12. Our results demonstrate the conventional phenotypic methods used to characterize these isolates were sufficient to assign the correct genus, but not the correct species. These findings highlight the importance of employing molecular methods when typing bifidobacterial isolates.
# TABLE OF CONTENTS

LIST OF FIGURES ..................................................................................................... vii  
LIST OF TABLES ....................................................................................................... ix  
LIST OF ABBREVIATIONS ...................................................................................... x  
ACKNOWLEDGEMENTS ......................................................................................... xii  

Chapter 1  Literature Review ......................................................................................... 1  
1.1. Definition of probiotics .................................................................................. 1  
1.2. Evidence for putative health effect ................................................................. 2  
1.3. Bifidobacteria as probiotics microorganisms (groups of probiotic bacteria) .......................................................................................................... 4  
2. Genus *Bifidobacterium* ..................................................................................... 4  
2.1. Growth conditions (pH, optimum temperature, sensitivity to oxygen) ........ 5  
2.2. Nutrients requirement and metabolism .......................................................... 5  
2.3. Species of *Bifidobacterium* commonly used as Probiotics .................... 7  
2.4. *Bifidobacterium animalis* ssp. *lactis* .............................................................. 7  
3. Importance of identification and characterization of bifidobacteria ................. 8  
3.1. Consumer safety and confidence ................................................................. 10  
4. Identification and characterization methods used for bifidobacteria ............... 12  
4.1. Phenotypic methods used to characterize bifidobacteria ............................... 13  
4.1.1. Bacteriological culture methods .......................................................... 14  
4.1.1a. Microscopy ................................................................................. 16  
4.1.2. Biochemical methods .......................................................................... 17  
4.2. Molecular methods ........................................................................................ 18  
4.2.1. DNA based methods ............................................................................ 18  
4.2.1. 1. DNA hybridization ................................................................... 19  
4.2.1. 2. Polymerase Chain Reaction (PCR) .......................................... 20  
4.2.1. 2a. Random Amplification of Polymorphic DNA PCR (RAPD-PCR) .......................................................... 22  
4.2.1. 3. Pulsed Field Gel Electrophoresis (PFGE) ................................ 23  
4.2.1. 4. DNA sequence-based methods ................................................. 24  
4.2.1. 4a. Multi Locus Sequence Typing (MLST) .................................... 24  
4.2.1. 4b. Single Nucleotide Polymorphism Typing (SNP) ..................... 25  
5. Probiotics in Ukraine ........................................................................................ 26  
5. 1. Bacterial starter culture for dairy products in Ukraine ................................. 29  
5. 2. Technological Institute of Milk and Meat (TIMM) ................................... 30  
5.2.1. History and function of TIMM ............................................................ 30  
2.2.2. Bifidobacteria identification at TIMM ............................................... 31  
References ............................................................................................................ 33
LIST OF FIGURES

Figure 2-1: Evaluation of *RT strains* using *Bifidobacterium* genus specific primers. .................................................................75

Figure 2-2: Detection of *Bifidobacterium* species using *B. animalis* ssp. *lactis* DSMZ 10104<sup>T</sup> specific primer pair .................................................................76

Figure 2-3: Characterization of RT bifidobacteria by PFGE using *XbaI* ............77

Figure 2-4: Characterization of RT bifidobacteria by PFGE using *SpeI* ..............78

Figure 2-5: Phylogenetic tree of placing RT bifidobacterial isolates into the scheme of Briczinski et al. (2009) and *B. animalis* ssp. *lactis* DSMZ 10104<sup>T</sup> .....79

Figure B1-1: Detection of *Bifidobacterium* genus in isolates obtained from commercial supplier using the Lm3 and Lm26 primer pair. .......................................85

Figure B2-2: Evaluation of bifidobacterial strains obtained from commercial supplier using *B. animalis* ssp. *lactis* specific primers (Bflact 1, 2) in 1% agarose gel. .........................................................86

Figure C1-1: Genus and species-specific PCR in control stains (ATCC and DSMZ). ......................................................................................................................................88

Figure C1-2: Detection of RT bifidobacteria (set II) using *Bifidobacterium* species-specific primers ....................................................................................................................89

Figure C1-3: Detection of *B. animalis* ssp. *lactis* from RT bifidobacteria (set II) using *B. animalis* ssp. *lactis* specific PCR ..................................................................................................................90

Figure C1-4: Detection of *B. bifidum* in lyophilized culture samples of *B. bifidum* RT04 using BiBif1, 2; and Bflact-2, 5 primer pairs for detection of *B. animalis* ssp. *lactis* ...........................................................................................................91

Figure C1-5: Detection of *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* in lyophilized culture sample of RT03 using Ban2, 23 Si; and Bflact-2, 5 primer pairs, respectively ..................................................................................92

Figure C1-6: Detection of *Bifidobacterium* in RT lyophilized bifidobacteria culture using species-specific PCR ..................................................................................................................93

Figure C1-7: Detection of *Bifidobacterium animalis* ssp. *lactis* in RT bifidobacterial isolates using Bflact-2, 5 *B. animalis* ssp. *lactis* - specific primer pair ...........................................................................................................94
Figure **D1-1**: Pulsed Field Gel Electrophoresis (PFGE) of chromosomal DNA digested with *XbaI* ................................................................. 96

Figure **D2-2**: PFGE in RT bifidobacteria. *XbaI* (set II) ............................................. 97

Figure **E1-1**: Growth of *B. bifidum* ATCC 29521 on LLA and MRS- cysteine media..................................................................................................................... 99

Figure **F1-1**: Anaerobic incubator .............................................................................. 101

Figure **F1-2**: Anaerobic chamber............................................................................... 102
LIST OF TABLES

Table 2-1: Microorganisms used in this study............................................................. 72

Table 2-2: Sequences of PCR primers used in genus and species verification of
          *Bifidobacterium*............................................................................................. 73

Table 2-3: Identification of RT bifidobacateria by SNP analysis............................ 74
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>DSMZ</td>
<td>Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>ERIC</td>
<td>Enterobacterial Repetitive Intergenic Consensus</td>
</tr>
<tr>
<td>F6PPK</td>
<td>Fructose-6-phosphate phosphoketolase</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FOS</td>
<td>Fructooligosaccharides</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal Transcribed Spacer</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic Acid Bacteria</td>
</tr>
<tr>
<td>LLA</td>
<td>Liver Lactose Agar</td>
</tr>
<tr>
<td>LLB</td>
<td>Liver Lactose Broth</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MRS-agar</td>
<td>MRS- agar deMan Rogosa and Sharpe agar</td>
</tr>
<tr>
<td>MRS-broth</td>
<td>deMan Rogosa and Sharpe broth</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
</tbody>
</table>
PFGE  Pulsed Field Gel Electrophoresis  
RAPD- PCR  Randomly Amplified Polymorphic DNA-PCR  
RT  Roberts-Tmanova culture collection  
rDNA  Ribosomal DNA  
RFLP  Restriction Fragment Length Polymorphism  
RNA  Ribonucleic acid  
SDS  Sodium dodecyl sulfate  
TBE  Tris-boric acid-EDTA  
™  Trademark  
TIMM  Ukrainian Technological Institute of Milk and Meat  
Tris  Tris(hydroxymethyl)-aminomethane  
WHO  World Health Organization of the United Nations
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Chapter 1

Literature Review

1. Definition of probiotics

Although the probiotic concept is new for many consumers it can be traced as far back as 2500 B.C., which dates back to the emergence of fermented milk products (Kroger et al. 1989). The definition of the term “probiotic” has varied throughout history, for example: Lilly and Stillwell described probiotics as a “substance secreted by one microorganism, which stimulates the growth of other microorganisms” (Lilly and Stillwell 1965). For Parker probiotics were “every substance or microorganism that contributes to gastrointestinal balance” (Parker 1974). In attempt to narrow the definition Fuller defined probiotics as “live microbial feed supplement, which beneficially affects the host animal by improving its intestinal balance” (Fuller 1989). In 2001 a consensus definition was developed by a group of experts from the FAO/WHO which emphasized the importance of the viability of microorganisms in order to exert the health benefits in the host. This consensus definition “Live microorganisms which when administered in adequate amount confer a health benefit on the host” (FAO/WHO 2001) has been reasonably well accepted by the “probiotic community”.
1.2. Evidence for putative health effects

The positive role of Lactic Acid Bacteria (LAB) on human health was first noted by Eli Metchnikoff and Henry Tissier. Metchnikoff suggested that a high intake of fermented milk products enhanced longevity in Bulgarian peasants (Metchnikoff 1907). In his book “The prolongation of life: Optimistic studies” he proposed that LAB decrease putrefaction and toxic effects of gut microbiota (Metchnikoff 1907). The other researcher, Henry Tissier (1900), observed bacteria with a Y-shaped morphology, called bifid-bacteria, predominate in the stools of healthy breast-fed infants. He believed these bacteria were important in maintaining a healthy human gut. Tissier suggested bifidobacteria could be beneficial in the treatment of diarrhea in children (Tissier 1905).

Metchnikoff’s and Tissier’s observations were accepted within commercial circles resulting in production of fermented milk products that claimed beneficial health effects despite the absence of scientific evidence supporting such claims (Tannock 2003).

Lack of supporting evidence regarding the positive effect of probiotics on host health, inaccurate identification of microorganisms, poor knowledge of the intestinal microbiota, and skepticism of Metchnikoff’s theory slowed the process of scientific and medical validation of probiotics (Podolsky 1998).

Interest in investigation of the beneficial effect of LAB reappeared in USA in 1920 (Podolsky 1998). The first clinical trials to validate effect of probiotics on constipation were conducted in 1930 (Kopp-Hoolihan 2001). Then, a commercial product “Acidophilus milk” containing Lactobacillus acidophilus was noted useful in treatment of constipation, colitis, and chronic diarrhea (Rettger et al. 1935; Podolsky 1998).
Interest in probiotics and their beneficial effect on host health has reemerged during the last 20 years (Fuller 1989; Fuller 1992; Goldin and Gorbach 1992; Gibson et al. 1997; Sanders 1998; Sanders 1999; Gronlund et al. 2000; Ouwehand et al. 2002; Guarner and Malagelada 2003; Adolfsson et al. 2004; Saran 2004; Sheil et al. 2007). Probiotics have been reported to aid in the prevention of diarrheal disorders and other gastrointestinal disturbances, have anti-colon cancer and antimitagenic effects, antihypertensive effect and modulate the immune system (Tissier 1905; Goldin and Gorbach 1992; Duffy et al. 1994; Saavedra et al. 1994; Singh et al. 1997; Salminen et al. 1998; Lee et al. 1999; Sanders 1999; Saarela et al. 2000). New fermented dairy products, containing probiotics, can alleviate lactose maldigestion (Gallagher et al. 1974). Martini et al. (1991) studied lactose digestibility comparing low-fat milk, yogurt, and milks fermented by single strains of Streptococcus salivarius ssp. thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus and Bifidobacterium bifidum. Lactose digestion was higher when yogurt was consumed followed by milks fermented with single strains and was lowest in milk (Martini et al. 1991). Although, the mechanism for increased lactose tolerance was clearly related to the presence of the bacterial β-galactosidase (Vesa et al. 2000), the mechanism by which other probiotic health benefits occur remains to be elucidated.

The use of properly defined strains in probiotic-containing products is important since it is believed that the health effects are strain specific (FAO/WHO, 2001). Therefore, a systemic component of such investigations should be accurate identification of the organism to the genus, species, and strain level (Salminen et al. 2004).
1.3. Bifidobacteria as probiotic microorganisms (groups of probiotic bacteria)

Microorganisms considered to be human probiotic are found in the genera *Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Streptococcus, Bifidobacterium*, including other non-lactic acid bacteria such as *Bacillus subtilis, Escherichia coli* strain Nissle and the yeast *Saccharomyces* (Gibson et al. 1997). Species in the genera *Lactobacillus* and *Bifidobacterium* are the most common microorganisms used as human probiotics in food and dietary supplements (Sanders 1998; Salminen et al. 2004). Since bifidobacteria are the focus of this work, they are discussed below.

2. Genus *Bifidobacterium*

The genus *Bifidobacterium* were first described in the eighth edition of *Bergey’s Manual of Determinative Bacteriology* (Rogosa 1974). Bifidobacteria are nonmotile, anaerobic, Gram-positive, nonsporeforming rods that sometime assume Y and V shape forms (Holt et al. 1994). At the present time the genus *Bifidobacterium* contains 38 species (Euzéby 1997).

The first reported observation of bifidobacteria was in 1905 by French Pediatrician Henry Tissier who observed bacteria of Y shaped morphology in the stools of healthy infants and named them bifid – bacteria. To date, bifidobacteria have been isolated from human intestine, oral cavity and animal intestine, honeybees (Scardovi and Trovatelli 1969), sewage (Biavati et al. 1982), and fermented milks (Meile et al. 1997). Yoshioka et al. (1991) reported bifidobacteria constitute 95% of the total microflora in healthy breast-fed infants. In humans the number of bifidobacteria present in the colon
declines with age to 3-6% of the fecal flora (Hopkins et al. 2001; Sotokari et al. 2003). The species of bifidobacteria present in humans changes with age with *Bifidobacterium adolescentis* more predominant in adults and *B. longum* ssp. *infantis* and *B. breve* in children (Biavati et al. 2000; Ward and Roy 2005). Bifidobacteria are often added to commercial fermented dairy products for their putative probiotic properties.

### 2.1. Growth conditions

Most bifidobacterial strains exhibit optimum growth at temperatures between 37-41°C (Holt et al. 1994), although *B. thermacidophilum* isolated from waste water in Beijing (Dong et al. 2000) was reported to grow at a temperature of up to 49.5°C (Dong et al. 2000). Members of the genus *Bifidobacterium* are acid-tolerant bacteria and exhibit optimum growth at pH 6.5-7.0. No growth has been observed below pH 4.5 (Biavati et al. 2000), although *B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis* can survive in environments as low as pH 3.5 (Matsumoto et al. 2004). Although bifidobacteria are anaerobic microorganisms (Scardovi 1984) some species are oxygen tolerant. *Bifidobacterium animalis* ssp. *lactis* is able to grow under conditions of oxidative stress (Meile et al. 1997).

### 2.2. Nutrient requirement and metabolism

Bifidobacteria are fastidious organisms and require a number of specific nutrients to support growth. Bifidobacteria have been reported to ferment a range of sugars
including glucose, galactose, fructose, raffinose, and sucrose, whereas mannitol and sorbitol are utilized by only few species (Holt et al. 1994). Other essential components required for bifidobacterial growth include cysteine, biotin, and calcium pantothenate (Parker 1974).

The enzyme Fructose-6-phosphate Phosphoketolase (F6PPK) has been reported as a key characteristic in hexose fermentation by *Bifidobacterium* (Scardovi and Trovatelli 1965). Lactic and acetic acids are produced during the growth of bifidobacteria in theoretical ratio of 3:2 (Tamura 1983; Araya-Kojima et al. 1995; Biavati et al. 2000). The ability to utilize various carbohydrates differs among the species of *Bifidobacterium* genus (Biavati et al. 2000; Ventura et al. 2004). Kaplan and Hutkins (2000) reported that *B. breve*, *B. adolescentis*, *B. bifidum*, *B. longum ssp. infantis*, and *B. longum ssp. longum* strains utilize fructooligosaccharides (FOS).

The so called bifidogenic factors (fructooligosaccharides, lactoferrin, lactulose, xylooligosaccharides (D-xylan), and others) have been reported important in the growth of bifidobacteria (Modler 1994). The influence of these compounds on growth of bifidobacteria in human and bovine milk has been studied. Bifidobacterial species exhibited differences in their growth pattern in human and bovine milk (Duber and Mistre 1996). Addition of lactulose and FOS to infant milk formula did not enhance the growth of bifidobacteria in vitro for *Bifidobacterium bifidum* ATCC 15696, *Bifidobacterium breve* ATCC 15700, and *Bifidobacterium longum ssp. longum* ATCC 15708 (Duber and Mistre 1996). Enhanced growth of bifidobacteria in human milk was reported to be due to the N-acetyl-D-glucose-amine-containing saccharides, which are absent in bovine milk (Poupard et al. 1973).
2.3. Species of *Bifidobacterium* commonly used as probiotics

In order to use bifidobacteria for manufacture of fermented dairy products, the microorganism must be safe, as well as exhibit probiotic and appropriate technological properties (Saarela et al. 2000; FAO/WHO 2002). Species of bifidobacteria commonly used as probiotics include *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* ssp. *longum*, *Bifidobacterium longum* ssp. *infantis* and *Bifidobacterium animalis* ssp. *lactis* (Ziemer and Gibson 1998; Mercenier et al. 2002). Among all bifidobacteria *Bifidobacterium animalis* ssp. *lactis* has been very commonly added to fermented dairy products due to their ability to survive exposure to oxygen and to survive during processing and storage as well as because of the putative health effect of this subspecies (Masco et al. 2005; Salminen et al. 2005).

2.4. *Bifidobacterium animalis* ssp. *lactis*

*Bifidobacterium animalis* ssp. *lactis* was first isolated from a commercial French yogurt (Meile et al. 1997) during a search for oxygen tolerant bifidobacteria. This isolate appeared to be distinctive among the other bacterial strains used in the study. The strain was named *Bifidobacterium lactis* due to the fact it was isolated from a cultured milk products. The authors described this strain, *Bifidobacterium lactis* UR1 (DSM 10140) (Meile et al. 1997), as a Gram-positive, nonmotile, non-sporeforming rod, which grew on agar in the presence of 10% oxygen at temperatures ranging from 39°C to 42°C. This strain fermented L-arabinose, D-ribose, D-glucose, amygdalin, esculin, maltose, lactose, melibiose, saccharose, raffinose, gentiobiose, and 5-ketogluconate (Meile et al. 1997).
The taxonomic position of *B. lactis* and *B. animalis* was questioned soon after its first use. Cai et al. (2000) noted the *B. lactis* described by Meile et al. (1997) and *B. animalis* described by Scardovi and Trovatelli (1974) were closely related species and proposed to consider *B. lactis* as junior synonym of *B. animalis*. Analysis of the 16S-23S ITS region of *B. lactis* DSM 10140\(^T\), *B. animalis* 25527 and six additional *B. lactis* strains revealed the presence of two distinct clusters (Ventura and Zink 2002). Ventura and Zink (2002, 2003) and Zhu et al. (2003) proposed these two species be separated at the subspecies level. In order to differentiate *B. animalis* and *B. lactis* Masko et al. (2004) used a polyphasic approach, which demonstrated that these two species were closely related. Thus, *B. animalis* and *B. lactis* have been reclassified to *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*, respectively (Masco et al. 2004).

*Bifidobacterium animalis* ssp. *lactis* grows in milk and milk-based media. This microorganism is oxygen tolerant and is able to grow under the conditions of oxygen stress (Masco et al. 2004). The ability to tolerate oxygen exhibited by *B. animalis* ssp. *lactis* is important for the dairy industry since most other bifidobacterial strains require more anaerobic conditions for growth. The oxygen tolerance of *B. animalis* ssp. *lactis* helps to facilitate the propagation of this species and their further use in production of probiotic-containing products.

3. Importance of identification and characterization of bifidobacteria

Introduction of probiotics strains into functional foods has increased worldwide in recent years. Probiotic products are usually considered as Generally Recognized as Safe
(GRAS) (Sanders and Veld 1999). The beneficial effects of probiotic bacteria on human health have been widely publicized and promoted by manufacturing companies and health practitioners. New probiotic products and supplements containing probiotics strains constantly appear on the market. These products usually include one or a few known commercial probiotic bacterial species and strains. Recently, novel probiotic strains of various microorganisms (Propionibacterium spp., Bacillus spp. and yeast Saccharomyces boulardii) have been added to probiotic containing foods (Vankerckhoven et al. 2008). Since the organisms do not have a long history of use in foods, there are some questions about the safety of these novel “probiotic” strains.

Discrepancies between label information and actual organisms found in probiotic containing products have been reported (Klein et al. 1998; Mercenier et al. 2002). These discrepancies have often included the identity of the probiotic species as well as the viable population present in the product. Consequently, the public perception of the functionality of probiotic products might be undermined thereby leading to loss of consumer confidence and decreased consumption of these products.

The use of properly defined strains in probiotic products is important (FAO/WHO 2001). Selection and identification of probiotic microorganisms are two critical steps in the complex process of production of probiotic containing products. The identity of any novel strain, aimed to be introduced into the probiotic product, should be defined, characterized, and differentiated among other probiotic microorganisms using reliable methods and techniques. Huys et al. (2006) reported 28% of commercial strains were misidentified at the genus or species levels, suggesting, accurate identification,
differentiation, and characterization of bifidobacteria at the species and strains level could improve quality and credibility of the probiotic containing products on the market.

3.1. Consumer safety and confidence

The US market for probiotics reached $4.1 billion in 2009 and is expected to rise to $5 billion in 2014 (Mintel Report 2009). However, despite growth in the functional foods market in US and worldwide, many people continue to doubt the reported health effects of probiotics on the host (Saarela et al. 2000; Lahteenmaki and Ledeboer 2007). Andersson et al. (2001) and Saarela et al. (2000) noted that increased awareness among consumers about the putative health benefits of probiotics may lead to questions of credibility for probiotic foods and pharmaceutical preparations. An adequate system of information and scientific confirmation of the probiotics health benefits are required in order to maintain consumer safety and confidence (Reid et al. 2003; FSTB 2004; Sanders and Heimbach 2005). Education of customers and providing them sufficient information regarding the probiotic containing products and their health benefits remain necessary in order to change existing perception about probiotics, erase consumer doubts, and boost their interest in probiotic- containing foods (Bruhn 2002; Gorska-Warsewicz 2003; Viana et al. 2008).

Containers of probiotic-containing products should provide information to consumers regarding the type (genus, species, and strain) of the specific microorganism, its quantity, and health benefits (FAO/WHO 2002). However despite the guidelines of FAO/WHO published in 2002, the label on containers of probiotic-containing products,
often includes only the generic term “bifidobacteria” (Tannock 2003), the identity of the probiotic microorganisms is not always properly depicted on the label (Walker and Buckley 2006), the list of microorganisms used in the product often does not match the label (Huff 2004), and information regarding the level of probiotic microorganism, species or strain is scarce (Tannock 2003).

Another important aspect to consider during production of probiotic-containing products are the measures used to assure the purity of the specific probiotic species and strains used to prepare probiotic containing foods (FAO/WHO 2002). The European Food Safety Authority (EFSA) published a document (Frequently Asked Questions (FAQ) related to the EFSA assessment of Article 14 and 13.5 health claims applications 1), describing important aspects of probiotic strain characterization. The characterization of microorganisms and species identification should include “genetic typing at the strain level by internationally accepted molecular methods and strains should be named according to the International Code of Nomencalature” (EFSA 2009).

Good Manufacturing Practices (GMP) and standards are fundamental components in production of probiotic-containing products (Sanders and Veld 1999). However, lapses in these practices have a negative effect and influence the perception of probiotics by consumers. Temmerman et al. (2003) noted mislabelling of strains contained in some preparations and use of old or non-existing nomenclature (Temmerman et al. 2003; ISAPP 2004). Presence of contaminants like Enterococcus faecium in probiotic preparations has been reported (Hamilton-Miller et al. 1999).

In order to sustain consumer confidence in commercial probiotic products, manufactures need to produce probiotic products with acceptable sensory properties,
convenient packaging, suitable price, provide accurate information about the identity and level of probiotic bacteria in the product, and comply with regulatory guidelines. Moreover, the beneficial health effect of probiotics microorganisms needs to be placed on the label and must be the scientifically proven and supported by clinical trials.

In summary, the quality of probiotics products along with scientifically supported evidence of their health benefits and use of reliable methods for identification of the probiotics microorganisms are an important stepping stones for establishing reputable probiotic containing products.

4. Identification and characterization methods used for bifidobacteria

For many years identification and characterization of bifidobacteria was based on phenotypic methods. The taxonomic designation of bifidobacteria based on classical phenotypic and cultural methods has been controversial among microbiologists since its discovery by Tissier (Poupard et al. 1973). The phenotypic and biochemical methods, and morphological resemblance of bifidobacteria to lactobacilli resulted in classification of this group into the genus *Lactobacillus* for many years (Leahy et al. 2005).

In the end, a polyphasic approach has been reported to aid in bifidobacterial identification, characterization, and differentiation (Ventura et al. 2004). Combination and integration of information (phenotypic, genotypic, and phylogenetic) received from various techniques and methods helps to insure correct identification and classification of bifidobacteria.
4.1. Phenotypic methods used to characterize bifidobacteria

Until recently phenotypic methods were used in most laboratories worldwide for identification of bifidobacteria. Basic phenotypic criteria used to identify bifidobacteria included location of isolation from the host, growth on various media, cell morphology, and utilization of carbohydrates. Pleomorphic characteristics, exhibited by bifidobacteria during culturing on certain media can aid in distinguishing these bacteria from others organisms (Poupard et al. 1973). In addition, utilization of various carbohydrates has been used for identification and differentiation of bifidobacteria in the past (Scardovi 1984). However these assays are difficult to interpret and lack accuracy in differentiation of bifidobacteria (Briczinski and Roberts 2006; Briczinski and Roberts 2007). Masco et al. (2004), Roy and Sirois (2000), and Roy et al. (1996) reported difficulties in differentiation of B. longum ssp. infantis and B. longum ssp. longum or B. animalis ssp. animalis and B. animalis ssp. lactis utilizing phenotypic assay.

Phenotypic tools, used for identification of bifidobacteria are time consuming and do not always provide clear results (Herreman et al. 1994; Gomez Zavaglia et al. 2000; Matsuki et al. 2003; Ventura et al. 2004). In addition, conventional methods often lead to ambiguous or inaccurate identification at the species level (Roy et al. 1996; Masco et al. 2004). Phenotypic methods used for differentiation of bifidobacteria, particularly B. longum ssp. longum, B. longum ssp. infantis, B. animalis ssp. animalis and B. animalis ssp. lactis, are not reliable (Ward and Roy 2005).
4.1.1. Bacteriological culture methods

The growing interest in probiotics requires reliable and inexpensive methods for detection of bifidobacteria in food products. Bacteriological analysis has been often used for quality assurance of probiotic products (Vikova et al. 2004, Vinderola and Reinheimer 2000). A viable plate count method is utilized by many laboratories for screening and identification of probiotic microorganisms (Vinderola et al. 2008). Identification of bifidobacteria using culture method can be complicated by a presence of a mixed bacterial culture in probiotic-containing product. Selective media that support the growth of bifidobacteria while suppressing growth of other bacteria are usually utilized in such cases (Shah 2000). Selective agents known for bifidobacteria include antibiotics (kanamycin, naladixic acid, paramycin and polymixin B) and propionic acids (Hartemink and Rombouts 1999). Several media have been evaluated for detection of bifidobacteria in food products and feces, however, none were found suitable for all cases (Hartemink et al. 1996; Hartemink and Rombouts 1999; Rada and Petr 2000; Simpson et al. 2004). Some selective media reported for isolation of bifidobacteria do not support growth of all species of *Bifidobacterium* or are not completely selective. For example, media containing Neomycin-Paromomycin Nalidixic acid-Lithium chloride agar (NPNL) and BIM-25 (antibiotics) supported not only growth of bifidobacteria but some other non-bifidobacteria (Hartemink et al. 1996). Hartemink et al. (1996) reported use Raffinose-*Bifidobacterium* (RB) agar as an antibiotic free media that is selective for bifidobacteria.

At the same time, some selective media were observed to be satisfactory for identification of bifidobacteria (Boylston et al. 2004). Trypticase-phytone-yeast extract
(MTPY) agar supplemented with glacial acetic acid and mupirocin was reported to be selective for glucose fermenting and glucose non-fermenting bifidobacteria (Rada and Petr 2000). Liver Infusion Broth can also be used for cultivation of anaerobes (Difco 1984) and Liver Lactose Agar is used by some commercial suppliers for cultivation of bifidobacteria (Lyoferm Inc 2002). Growth of bifidobacteria was reported on De Man, Rogosa and Sharpe media (MRS) (De Man et al. 1960), which was used for enrichment, cultivation and isolation of *Lactobacillus* and other anaerobic microorganisms. MRS media contains ingredients (polysorbate, acetate, magnesium, manganese), which favor growth of lactobacilli (De Man et al. 1960). Cysteine can be added to MRS medium as a reducing agent, which provides reducing conditions that allow growth of bifidobacteria (Lyoferm Inc 2002). *Bifidobacterium* selective media (BSM) prepared from MRS media supplemented with cysteine hydrochloride and mupirocin was reported to be selective media for bifidobacteria and suggested to use for enumeration of bifidobacteria from animal feeds (Simpson et al. 2004). MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paramomycine sulfate) agar was reported to be useful in enumeration of bifidobacteria in mixed populations containing bifidobacteria, *Lactobacillus dulbrueskii* and *St. thermophilus* (Tharmaraj and Shah 2003).

In summary, bacteriological analysis of bifidobacteria comprise a variety of media, reagents, supplementary tests, and time. Identification and differentiation of bifidobacteria from the other Lactic Acid Bacteria (LAB) based on cultivation using various media is confusing, inaccurate, and time consuming. A representative number of viable cells, which is required for the satisfaction of quality standards necessary for production of fermented dairy products, might be obscured due to presence of bile or
antibiotics in growth media that possibly have influence on growth of starter culture microorganisms (Dave and Shah 1996). In addition to that, the detection of bifidobacteria can be complicated by the presence of other closely related species as well as to differentiate among them and other starter culture bacteria such as lactobacilli, streptococci, and bifidobacteria (Ghoddusi 2008, Dave and Shah 1996). Thus, microbiological analysis of bifidobacteria is a complex process and identification of microorganisms to the species and strain level are often ambiguous.

4.1.1a. Microscopy

Morphological properties obtained with the aid of microscopy are sometime useful in identification of bifidobacteria (Poupard et al. 1973). Tissier described bifidobacteria as curved rod or rods with Y – shaped ends characterized as “bifid”. Branching and swelling of the bacteria was noted. This morphology often disappears after adaptation to laboratory culture media and the rod form of the bacteria becomes predominant (Sundman et al. 1959). Glick et al. (1960) speculated that inadequate supply of N-acetyl-amine sugar results in pleomorphism of these bacteria (Glick et al. 1960). It was noted that a high degree of a branching occurred on some media (tomato agar), whilst the bacteria exhibit rod or curved-rod forms when cultivated on nutritionally rich complex media. Pleomorphism has been also observed in lactobacilli and other microorganisms (Sundman et al. 1959). Thus, morphology is not always a reliable method for identification and classification of bifidobacteria.
4.1.2. Biochemical methods

Carbohydrate metabolism, cell wall composition, and membrane composition (lipid, and phospholipids) are sometimes important in identification of bifidobacteria. Kuhn and Tiedemann (1953) described carbohydrate metabolism in bifidobacteria as fermentation of glucose via glycolytic system resulting in the formation of lactate and acetate via phosphoenol pyruvate. An alternative pathway for fermentation of hexoses in bifidobacteria, the F6PPK pathway, was reported by Scardovi and Trovatelli (Scardovi and Trovatelli 1965). The presence of the key enzyme fructose-6-phosphate phosphoketolase (F6PPK) has been used for identification of *Bifidobacterium* to the genus level (Masco et al. 2004).

The composition and structure of cell wall peptidoglycan and cell wall fatty acid analysis has also been used to differentiate bifidobacteria from lactobacilli and other bacteria (Kandler 1970; Scardovi et al. 1979). The composition of bifidobacterial cell wall differs from that of lactobacilli. Veerkamp reported the *B. bifidum* differ from other bifidobacteria and lactobacilli by its specific cross-linking depiptide (Veerkamp 1971). Differentiation of bifidobacteria and lactobacilli can also be achieved by evaluating differences in phospholipid composition (Exterkate et al. 1971). However, variation of the fatty acid composition with regards to composition of growth media and temperature can obstruct correct identification. Polyacrylamide gel electrophoresis of soluble cellular proteins from bifidobacteria was reported useful in taxonomy of bifidobacteria (Biavati et al. 1982), although this method is not commonly employed.
4.2. Molecular methods

Developments in molecular techniques have brought new insight into the identification and characterization of bifidobacteria. These methods allowed verification of previous classification system for bifidobacteria. Analysis of the 16S rRNA gene is a key in understanding modern taxonomy of bacterial species (Ward and Roy 2005).

Molecular (DNA-based) methods are useful for detection and characterization of bifidobacteria to the genus species and subspecies level. Polymerase Chain Reaction (PCR) has been reported useful for detection of bifidobacteria and Pulse Field Gel Electrophoresis (PFGE) and Random Amplification of Polymorphic DNA (RAPD) have been used for typing of bifidobacteria. Real time PCR (RT-PCR), microarrays, multilocus sequence typing (MLST), and single nucleotides polymorphism (SNP) are also powerful technique for subtyping bifidobacteria (Salminen et al. 2005a; Ward and Roy 2005; Ventura et al. 2006; Barrangou et al. 2009; Brickzinski et al. 2009).

4.2.1. DNA-based methods

DNA-based methods such as DNA-DNA hybridization, ribotyping, hybridization with specific probe, PCR, and PFGE are regarded as valuable tools for characterization of bifidobacterial species.
4.2.1.1. DNA hybridization

DNA-DNA studies were introduced as a tool in bacterial classification when phenotypic and biochemical methods became insufficient for identification of bifidobacteria. Nucleic acid hybridization is a common technique that can be used to assess similarities between two species (Farber 1996; Holzapfel et al. 2001). A degree of homology higher than 70% between the tested and control isolates along with phenotypic identity, group these isolates into the same species (Herreman et al. 1994). The reported homology between \textit{B. longum} ssp. \textit{longum} and \textit{B. longum} ssp. \textit{infantis} is near 70\% (Herreman et al. 1994). Results of DNA hybridization tests indicated that \textit{B. animalis} ssp. \textit{lactis} and \textit{B. animalis} ssp. \textit{animalis} are closely related (85.5\% - 92.3\%) (Cai et al. 2000).

The sequence of the highly conserved 16S rRNA gene is often used for identification of \textit{Bifidobacterium}. Yamamoto et al. (1992) developed species-specific oligonucleotide probes specific for \textit{B. adolescentis}, \textit{B. bifidum}, \textit{B. breve}, \textit{B. longum} ssp. \textit{infantis}, and \textit{B. longum} ssp. \textit{longum} by sequencing 16S rRNA gene from these closely related species of \textit{Bifidobacterium}.

The DNA-DNA hybridization method is not widely used in laboratories anymore. This method has been noted as laborious and time consuming (Biavati et al. 2000). In addition, rapid development in molecular biology has resulted in development of new techniques useful in bifidobacterial taxonomy. These methods might provide more accurate identification and characterization of bifidobacteria.
4.2.1.2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) – amplification of DNA molecules in an exponential manner driven by the enzyme DNA polymerase was developed by K. Mullis (1983) and patented by Cetus Corporation (Lerner and Lerner, 2003). DNA polymerase, naturally present in living organisms, was used to duplicate DNA. The specificity of the reaction is based on primer selection and annealing temperature. This method is widely used for medical and biological research, detection of microorganisms, diagnosis of disease, bacterial subtyping, and gene cloning (McPherson et al. 1995).

PCR is a sensitive, specific, rapid, and effective method for characterization of bifidobacteria. Primers are selected that amplify regions of the 16S rDNA or 16S to 23S internal transcribed spacer sequences (Bourget et al. 1993; Matsuki et al. 2003). As a general rule, primer specificity can be achieved by designing primers of 16-30 nucleotides long, avoiding repetitive sequences and mispriming at GC-rich regions, absence of internal complimentarity and formation of primer-dimers (McPherson and Moller, 2006). Amplicons generated by PCR are visualized by agarose gel electrophoresis and staining with EtBr (ethidium bromide) after separation.

Genus and species-specific primers have been employed for identification of bifidobacteria by PCR (Matsuki et al. 2003, Yeung et al. 2002). Primer sequences derived from 16S rRNA gene sequences have been regarded as genus and species-specific sequences (Kok et al. 1996; Matsuki et al. 1998). Bifidobacterial microorganisms isolated from food were classified to the genus Bifidobacterium by PCR using the genus-specific primer pair lm3, lm26, which target the 16S rRNA region (Kaufmann et al. 1997).
Primers, based on 16S rDNA and 16S-23S ITS sequences, were reported to be species-specific sequences for identification of *Bifidobacterium* species (Ward and Roy 2005). Matsuki et al. (1998) and Matsuki et al. (1999) developed species-specific primers that allowed rapid and accurate detection of *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, *B. adolescentis*, and *B. bifidum* from the human intestinal tract. Primer sequences derived from 16S rDNA regions were limited to differentiation of commercial *Bifidobacterium* species (Yeung et al. 2002) due to the high level of similarity in the 16S rRNA genes of bifidobacterial organisms (Youn et al. 2008). The Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) technique, based on use of primers targeting short repetitive sequences in bacterial genome, was utilized to identify and trace eighty-nine bifidobacterial strains from 26 species. This method appeared to be rapid, specific, and sensitive for bifidobacterial identification due to its ability to target complete genome and process many isolates in one time (Ventura and Zink, 2002).

*Bifidobacterium animalis* ssp. *lactis* has been identified among different environmental isolates. The primer pair Bflact-2 targeted 16S rRNA gene and Bflact-5 targeted 16S-23S interspacer region resulted in specific amplification of *B. animalis* ssp. *lactis* in the presence of other bifidobacteria and lactobacilli species (Ventura et al. 2001a). ERIC-PCR differentiated *B. lactis* DSMZ 10140^T^ and *B. animalis* ATCC 25527, but showed distinct profiles for these two closely related species (Ventura et al. 2003). Analysis of reference and commercial strains of *B. animalis* spp. *lactis* by PCR using subspecies-specific primer sequences suggested assign commercial *B. lactis* Bb12 and reference strain *B. animalis* ssp. *animalis* ATCC 27536 were both *B. animalis* spp. *lactis* (Mayer et al. 2007).
Thus, based on published records of a successful identification of bifidobacteria using PCR we consider this technique suitable for detection and identification of bifidobacteria to the genus and species level.

4.2.1.2a. Random Amplification of Polymorphic DNA PCR (RAPD-PCR)

RAPD technique is used for identification of bifidobacteria. This method allows separation of closely related strains within a species (Vincent et al. 1998). Several arbitrary short primers (about 10 nucleotides) are created to amplify random segments of DNA within the bacterial genome by PCR (Ward and Roy 2005). This method does not require previous knowledge regarding DNA sequence of the microorganism being analyzed. The RAPD profile is obtained after the resolution of amplicons via agarose gel electrophoresis and staining with ethidium bromide. The disadvantage of this method is low reproducibility, alteration of RAPD profile by presence of transposons, plasmids, and mutations (Vincent et al. 1998). The outcome of the RAPD test depends strongly on the concentration of DNA template and PCR conditions. In additions to that, low stringency conditions during the intial PCR cycles and low annealing temperature could affect the result of RAPD. Occurrence of mismatches between the template and primers can obscure RAPD results (McPherson and Moller, 2006). *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, and *B. suis* were characterized by RAPD method (Sakata et al. 2002; Satokari et al. 2003). Multiple RAPD-PCR primer sets, employed to differentiate among the commercial strains of *B. animalis* ssp. *lactis*, failed to separate these strains
(Briczinski et al. 2009), suggesting additional techniques must be used for differentiation of these genomicsly monomorphic subspecies.

4.2.1.3. Pulse Field Gel Electrophoresis (PFGE)

In order to type strains below the strain level Pulsed Field Gel Electrophoresis (PFGE) has been applied (Bourget et al. 1993). The DNA fragment pattern of restricted bacterial genomic DNA is used to differentiate among closely related species. This method involves harvesting bacterial cells, embedding in agarose plugs, lysing of cells followed by digesting of intact chromosomal DNA with restriction enzymes that recognize specific rarely occurring sequences within the bacterial genome and cut the bacterial DNA into fragments of different size. The restriction enzymes most often used for bifidobacteria are SpeI recognition site (ACTAGT) or XbaI recognition site (TCTAGA) (Roy et al. 1996). The fragments are then separated on an agarose gel using a pulsed electric field, yielding a unique pattern or “fingerprint” for each microorganism that visualized after staining the gel with ethidium bromide.

PFGE has been reported useful in determination of relatedness among different commercial strains (Yeung et al. 2004). This method is considered to be the “gold standard” in bacterial typing (Olive and Bean 1999) and has proved to be a valuable method for the probiotic industry for bifidobacterial strain identification and for determining relatedness among strains (Roy et al. 1996; Yeung et al. 2002). Roy et al. (1996) reported genomic diversity existed for *B. breve, B. longum ssp. infantis, B.*
bifidum, and B. longum. However, Masco et al. (2005) observed identical PFGE profiles for multiple B. animalis ssp. lactis or B. longum biotype longum isolates examined.

The amount of time required to perform the assay (Tenover et al. 1997), lengthy specimen preparation, inadequate cell lysis (Farber 1996), and inability to separate strains of B. animalis ssp. lactis are disadvantages of PFGE in bifidobacteria typing. However, a less time consuming protocol was reported (Briczinski and Roberts 2006), which reduced the time required for analysis of bifidobacteria to less than 24 h. The technique still suffers from a lack of discriminatory ability for subtyping genomically monomorphic species like B. animalis ssp. lactis (Barrangou et al. 2009).

4.2.1.4. DNA sequence-based method

Accurate identification of bifidobacterial species to the genus, species, and strain level can be achieved by sequence analysis. Typing methods such as Multi Locus Sequence Typing (MLST) and Single Nucleotide Polymorphism (SNP) can be employed for discrimination analysis of bifidobacteria.

4.2.1.4a. Multi Locus Sequence Typing (MLST)

MLST, developed by Martin Maiden, Dominique Caugant, Ian Feavers, Mark Achtman, and Brain Spatt, is a sequence based approach used in typing bacteria, based on DNA sequence variation in housekeeping genes and strains separation due to their unique allelic profile (Urwin and Maiden 2003; Sullivan et al. 2005). This technique is an
extension of the principles of multilocus enzyme electrophoresis (MLEE) (Selander et al. 1986). In MLST variations in alleles are detected by nucleotide sequencing rather than from electrophoretic mobility of their gene products. A sequence type (ST) is assigned based on differences in house-keeping genes sequences described as distinct alleles of each seven loci, which may differ at a single or many nucleotide sites (Briczinski and Roberts 2007; Briczinski et al. 2009).

MLST is a highly discriminatory, accurate, and portable typing method. The MLST method has been reported useful in tracking of bacterial pathogens (Chan et al. 2001; Urwin and Maiden 2003; Sullivan et al. 2005; Platt et al. 2006). This method might also be useful for probiotic strain characterization and differentiation (Ventura et al. 2006).

4.2.1.4b. SNP typing

Single nucleotide polymorphism (SNP) analysis is relatively new approach to strain taxonomy (Filliol et al. 2006). This method is based on identification of single nucleotide changes in the genome of the organisms being studied. These mutations can occur within the coding or in intergenic regions. Variations in the SNP content of the genomes of strains of specific probiotic microorganisms may be useful in differentiation of specific strains within a species (Achtman 2008). Analysis of the complete genome sequences of \textit{B. animalis} ssp. \textit{lactis} DSM 10140\textsuperscript{T} and BI-04 showed remarkable genome conservation and identified 47 single nucleotide polymorphisms (SNPs) between these two strains (Barrangou et al. 2009). Subsequent sequencing of these SNPs in a larger
collection of *B. animalis* ssp. *lactis* strains, previously analyzed by PFGE and carbohydrate analysis, allowed differentiation of the strains into fourteen distinct groups (Briczinski et al. 2009).

In summary, new DNA sequence-based typing methods may allow development of a deeper understanding of the taxonomy between closely related bifidobacterial organisms (Achtman 2008; Barrangou et al. 2009).

5. Probiotics in Ukraine

The milk processing industry (about 400 dairy companies) is a large sector of the economy in Ukraine (Melnyk et al. 2006). There are three main sectors in the Ukrainian dairy industry: whole milk, cheese, and butter production (Melnyk et al. 2006). Cheese and some types of whole milk followed by soured milk are considered profitable in the Ukrainian dairy market. Ukraine also exports dairy products to Germany, Poland, Japan, Mexico, Netherlands, and Kazakhstan with the main export market for milk products being Russia.

Healthy and nutritious food is always important for Ukrainians. The importance and value of nutritional foods in Ukraine is based on the theories of balanced and adequate nutrition developed by Pokrovskii and Ugolev (Chakhovskii 1991). Pokrovskii’s theory is based on balanced food (Покровский 1979). Ugolev’s nutrition theory is based on Pavlov’s principles, which includes physiological processes of food digestion, food intake, endocrine system (hormones, which are produced by endocrine cell of gastro-intestinal tract) and bacterial metabolites of the intestine (Уголев 1991).
This approach accounts for the influence of the gastro-intestinal tract on the health of humans. The theory of E. Metchnikoff, where lactic-acid bacteria present in the digestive tract could prolong life by preventing putrefaction, has been acknowledged not only in scientific circles, but also by the public (Karpa 2006).

Shenderov (Шендеров) (1988) noted relationships between the microflora of the colon and human health. Many Ukrainians, who believe in the beneficial aspects of probiotics health effects, historically consumed fermented dairy products such as kefir, kusle moloko (sour milk), etc. Moreover, the recent concept of “functional foods”, as a diet based means of assuring a healthy GI microbiota and improving the health of the host (human and animal) health, has changed the food choices of consumption of products by Ukrainians (Шендеров 2001).

“Probiotic therapy” is relatively new concept in Ukrainian medicine, which serves as a preventative and curative remedy in treatment of diseases. Species of the genus *Bifidobacterium* (*B. bifidum, B. longum ssp. longum, B.longum ssp. infantis, B. breve, B. adolescentis* and *B. animalis ssp. lactis* and *Lactobacillus* (*L. casei, L. acidophilus, L. cellobiosus, L. buchneri, L. plantarum, L. fermentum*), *Lactococcus*, *Streptococcus*, and *Enterococcus* are used in production of pharmaceutical and fermented products used with the aim to cure various disease or syndromes (Квасников 1992; Tamime et al. 1995; Shah 1997; Коваленко 2002).

Many milk products produced in Ukraine (liquid and dry) are supplemented with specific combinations of probiotic strains (Бондаренко et al. 1998; Сорокулова 1998). One of the most popular fermented milk products in Ukraine is kefir (Hallé et al. 1994).
In addition to the starter culture kefir grains are used to manufacture the product, “bio-kefir” is supplement with species of *Bifidobacterium* or *Lactobacillus* species.

Fermented dairy products containing specific probiotics in Ukraine include bio-yogurt, bio-kefir, ryazhenka, and other products, which may also be enriched with the medicinal herbs. The bioactive ingredients of these products are thought to be health beneficial (Ouwehand and Salminen, 1998). The health effects of “Bio-kefir”, and “Bio-yogurt” have been clinically tested. Improvement in liver function (Григоров et al. 2002; Отт and Чагаровский 2002), improvement and normalization of GI tract function, blood pressure, and decrease of cholesterol level were observed.

A combination of the functional properties of probiotics and remedial properties of medicinal herbs is realized in Fito-ryazhenka (Lupinskaia and Nikolkina 2001).

Another combination of medicinal herbs and probiotics microorganisms (*L. acidophilus*, *B. longum* ssp. *longum*, and *B. bifidum*) is in a product called “Бифілакт-А” (Bifilakt-A). Species of the genus *Bifidobacterium* are also supplemented into dry products (dry milk).

The beneficial health properties of probiotics and validation of observations of Metchnikoff have been explored continuously in Ukraine. A new wave of research aiming to understand the postulated protective properties of probiotics against cancer started in 1960 in Russia. Bogdanov hypothesized that certain strains of probiotics have anti-cancer properties. The hypothesis was proven to be credible and was used in treatment of patients after the Chernobyl catastrophe (PR Newswire 2008). Del-Immune V preparation is currently used as a therapeutic product in treatment of flu, hepatitis C, bronchitis, allergies, and other health conditions (Tymoshok et al. 2007).
Consumer interest in functional foods in Ukraine has resulted in the emergence of a strong market for probiotic-containing products and concomitant manufacture of various fermented products for sale in the market place. Quality control of cultured products enriched with probiotics and an assessment of their putative health claims are important to assure food safety and consumer confidence.

5.1. Bacterial starter culture for dairy products in Ukraine

Bacterial starter cultures are used during manufacture of fermented dairy products. LAB and species of *Bifidobacterium* are the most commonly used microorganisms in fermented dairy products in Ukraine. The quality of raw product and starter culture is an important element in manufacturing of fermented dairy products.

One of the main problems associated with milk products in Ukraine is the quality of raw milk. Despite recent increases in milk production in Ukraine, the availability and quality of raw milk remain critical (low technological level of dairies, low control of quality of raw and finished product, lack of European standards on finished products) affecting manufacture of dairy products and consumers’ safety (Melnyk et al. 2006).

Regionally produced fermented milk products are known for their probiotic functional properties by consumer in that particular region and might represent a set of diverse strains with unique properties for further study and application. Probiotic bacteria constitute a significant portion of the starter culture used in the dairy industry in Ukraine. In Ukraine, the Technological Institute of Milk and Meat (TIMM) produces approximately 1/3 of the starter culture used to manufacture fermented products in
Ukraine. The remaining starter cultures and probiotics are supplied by multinational manufacturers.

5.2. Technological Institute of Milk and Meat (TIMM)

The Ukrainian Technological Institute of Milk and Meat (TIMM) is a branch of the institute of the Ukrainian Academy of Agrarian Sciences. TIMM is conducting scientific investigations in the areas of chemical composition of dairy and meat, the technology for manufacturing meat and dairy products, mechanization and automation of food product production and technological aspects of preparation of bacterial starters for food products and forage production (TIMM 2007).

5.2.1. History and function of TIMM

TIMM was founded as the Ukrainian Research Institute of Meat and Dairy Industry by decree of the Ukrainian Government №1731 on 10.10.1959. In 1992, the Institute was renamed the Technological Institute of Milk and Meat. The institute is comprised of seven departments: Whole-milk products, Technology and butter production, Technology and cheese production, Technology and meat processing, Biotechnology, Standardization, Marketing, and Pharmaceutical preparations. The TIMM provides scientific information regarding the processing of raw material, new technologies and equipment for manufacture of dairy and meat products in order to
implement state sponsored scientific programs, investigate problems in the industrial and agrarian complex, educate manufactures, and collaborate with other research universities.

5.2.2. Bifidobacteria identification at TIMM

Detection of bifidobacterial microorganisms in TIMM is performed according to protocol MBK 10.10.2.2.-2004 “Enumeration of bifidobacteria in fermented milk products”. Samples are collected and handled according the standard protocol ГОСТ 9225 and ГОСТ 10444.11. Samples are spread plated on Bloyrok media (modified liver media) after dilution of the sample and then incubated at 37°C for 72 h under anaerobic conditions. After incubation on Bloyrok media under anaerobic conditions bifidobacteria form white, shiny and soft, convex colonies of entire margin. Identification and characterization of bifidobacteria in TIMM is performed according to the Bergey’s Manual of Systematic Bacteriology (Holt et al. 1994). Bifidobacterial microorganisms are assigned to species according to their carbohydrate utilization profile (the exact method is unknown and unavailable).

Introduction of probiotic strains into production of functional foods and starter cultures at TIMM requires rigorous screening and characterization of the bifidobacteria isolates to be used. Accurate identification and characterization of bifidobacteria used in starter culture preparation and manufacturing fermented dairy products and pharmaceutical preparations to the genus, species, and strain level is necessary.

The hypothesis of the present work is that the conventional carbohydrate-utilization based methods used by TIMM may misidentify strains of Bifidobacterium
used in Ukraine. Further that application of molecular methods would provide a more accurate and definitive identification.

The specific objectives of this work were to:

1. Obtain strains of multiple species of bifidobacteria from Ukrainian Academy of Agricultural Sciences Technological Institute of Milk and Meat
2. Isolate the bifidobacterial strains present in each culture.
3. Verify that the TIMM strains are members of the genus *Bifidobacterium* with bifidobacteria genus specific primers by PCR
4. Identification TIMM strains to the species level by PCR
5. Characterize Ukrainian strains of bifidobacteria by PFGE and compare these with the appropriate ATCC type strains used in the US.
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pitaniya na osnove molochnokislykh bakteriy i ikh prakticheskoe ispol'zovanie. 
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farmakologi i toksikologii pishchi. *Meditina*. 184 с.)


Chapter 2

Identification, characterization, and differentiation of bifidobacteria obtained from Ukraine

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KEY WORDS: PCR, PFGE, Bifidobacterium, identification, characterization, differentiation, Ukraine
Abstract

Speciation of bifidobacterial isolates using traditional biochemical and phenotypic methods is tedious and often provides inconclusive results. DNA-based methods often yield clearer results. Ten freeze-dried bifidobacterial strains used as probiotics in Ukraine and identified by the supplier as *Bifidobacterium adolescentis* (2), *Bifidobacterium bifidum* (2), *Bifidobacterium longum* (4), *Bifidobacterium animalis* (1), and *Bifidobacterium infantis* (1) were characterized using Polymerase Chain Reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE) and allelic profiling. After anaerobic growth on MRS-cysteine (MRS) at 37°C for 72 hours, single colony isolates were picked and evaluated using PCR primers specific for the genus, relevant species and for *B. animalis* ssp. *lactis*. All ten isolates were identified as members of the genus *Bifidobacterium*. However, species-specific PCR revealed all ten isolates were *B. animalis* ssp. *lactis*. Further evaluation using PFGE to assess strain relatedness showed all ten isolates gave PFGE patterns identical to the type strain DSMZ 10140^T^ when digested with *SpeI*. When digested with *XbaI*, 9 of the isolates gave patterns identical to DSMZ 10140^T^. One strain, RT09, had one extra band when digested with *XbaI*. Allelic profiling of the Ukrainian bifidobacterial strains, revealed four distinct groups. Interestingly, 6 (60%) of the isolates fell into the same cluster as that containing the common commercial probiotic strain BB-12. Our results demonstrate the conventional phenotypic methods used to characterize these isolates were sufficient to assign the correct genus, but not the correct species. These findings highlight the importance of employing molecular methods when typing bifidobacterial isolates.
Introduction

Probiotic bacteria are used in a large number of dairy products in Ukraine (Квасников 1992; Бондаренко et al. 1998; Коваленко 2002). These bifidobacteria are added to fermented milk products for their putative health-promoting properties such as modulation of host “immunity”, modification of the microbiota of human intestinal tract and prevention of the colonization by pathogenic bacteria (Григоров et al. 2002; Отт and Чагаровский 2002). Because the probiotic effects of bifidobacteria are considered to be strain specific, unequivocal identification to the genus, species, and strain level is important (FAO/WHO 2002). Conventional physiological and biochemical methods are not always effective, are time consuming, require expertise, and often lack precision (Briczinski and Roberts 2006).

The Ukrainian Technological Institute of Milk and Meat (TIMM) provides starter culture and probiotic culture for use by a portion of the Ukrainian dairy industry. A research partnership established between TIMM and PSU aimed to characterize Ukrainian bifidobacterial strains since information regarding the identity of bifidobacterial strains used in fermented dairy products in Ukraine is scarce.

Identification of bifidobacteria is significant in the process of starter culture development, production, and product quality control. Accurate identification of bifidobacteria to the species and strain level is a crucial element for the fermented dairy product industry (starter culture companies and dairy plants), pharmaceutical companies, and for the production of probiotic products. Huys et al. (2006) reported 28% of commercial strains were misidentified at the genus or species level. Temmerman et al.
(2003) noted mislabeling of strains contained in some preparations and use of old or non-existent nomenclature. Commercial starter culture companies can have difficulty differentiating of *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* (Mayer et al. 2007). Mayer et al. (2007) observed a high degree of homogeneity among the commercial bifidobacterial strains and some reference strains. The need for policies regulating microbiological quality and labeling of probiotic products and pharmaceutical preparations has been suggested by various authors (Sanders and Veld 1999; Szajewska et al. 2004; Harish and Varghese 2006).

Molecular methods, such as PCR (Polymerase Chain Reaction) and PFGE (Pulsed Field Gel Electrophoresis) can be effective for characterization of bifidobacteria to the genus and species level. PCR with species or subspecies specific oligonucleotide primers targeting the 16S rRNA gene can be useful method for identification of bifidobacteria. This method is often based on conserved regions of the 16S ribosomal gene (Woese 1987; Matsuki et al. 1998). Leblond – Bourget et al. (1996) reported use of 16S rRNA and 16S to 23S internally transcribed spacer (ITS) sequences in phylogenetic analysis of *Bifidobacterium*. Kaufmann et al. (1997) classified microorganisms to the genus *Bifidobacterium* using genus-specific primers lm3, lm26 targeting the 16S rRNA region. *Bifidobacterium animalis* ssp. *lactis* was identified among different environmental isolates by multiplex PCR using the primer pair Bflact-2, targeting the 16S rRNA gene, and Bflact-5 targeting the 16S-23S ITS region (Ventura et al. 2001a). Species specific primers have also been developed to characterize other bifidobacterial microorganisms including *B. longum* ssp. *longum*, *B. animalis* ssp. *animalis*, *B. longum* ssp. *infantis*, *B. adolescentis*, and *B. bifidum* (Matsuki et al. 1998; Matsuki et al. 1999).
Pulsed Field Gel Electrophoresis (PFGE) is considered to be the “gold standard” in bacterial typing and has proved to be a valuable method for the probiotic industry for bifidobacterial strain identification and determining relatedness among strains (Yeung et al. 2004; Mättö et al. 2006). Roy et al. (1996) reported genomic diversity existed for \textit{B. breve}, \textit{B. longum} ssp. \textit{infantis}, \textit{B. bifidum}, and \textit{B. longum} ssp. \textit{longum}. However, Masco et al. (2005) observed a high degree of relatedness among strains \textit{B. animalis} ssp. \textit{lactis} and also among \textit{B. longum} biotype \textit{longum}, which limit the utility of these test organisms. The amount of time required to perform the assay, (Tenover et al. 1997) lengthy specimen preparation, inadequate cell lysis (Farber 1996) and inability to separate strains of \textit{B. animalis} ssp. \textit{lactis} are disadvantages of PFGE in bifidobacteria typing. However, less time consuming protocols have been reported (Briczinski and Roberts, 2006).

Analysis of single nucleotide polymorphism has been reported to be useful in differentiation of strains of \textit{B. animalis} ssp. \textit{lactis} (Briczinski et al. 2009). Analysis of genome sequences of \textit{B. animalis} ssp. \textit{lactis} DSM 10140 and Bl-04 showed genome conservation and identified 47 single nucleotide polymorphisms (SNPs) between these two strains (Barrangou et al. 2009). Subsequent sequencing of these SNPs in a collection \textit{B. animalis} ssp. \textit{lactis} commercial and reference strains resulted in identification of 9 SNPs/INDELs, which could be used to differentiate a set of \textit{B. animalis} ssp. \textit{lactis} strains (Briczinski et al. 2009).

Since PCR and PFGE are effective, rapid, and often discriminatory methods for characterization of \textit{Bifidobacterium} and Single Nucleotide Polymorphism (SNP) analysis is helpful in differentiation of closely related bifidobacterial organisms (Achtman 2008;
Barrangou et al. 2009) implementation of these molecular methods for typing probiotic strains could improve identification, characterization, differentiation of TIMM bifidobacteria and provide assurance that the appropriate probiotic bifidobacterial strains are added to probiotic-containing products produced in Ukraine.

The objective of the present research was to characterize ten bifidobacterial cultures used in Ukraine as starter culture for fermented dairy products to the species and strain level using molecular methods.

**Material and Methods**

1. **Bifidobacterial strains (TIMM, ATCC, and DSMZ) and growth conditions**

   The bifidobacterial strains evaluated in this work were obtained from the commercial starter culture supplier in Ukraine as freeze-dried stock culture (Table 2-1). Each strain was assigned a unique RT number when it was received in the Roberts Research Laboratory. Reference strains used in this work were obtained from the ATCC (American Type Culture Collection, Manassas, VA), and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, The German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) (Table 2-1).

   All bifidobacterial strains were cultured in Liver Lactose Broth (LLB) (35 g/L Liver Infusion Broth, 10 g/L lactose, 10 g/L trypticase peptone (Becton Dickinson and Company, Sparks, MD), 2 g/L sodium chloride (EMD Chemicals, Gibbstown, NJ) (Lyoferm Inc 2002) or de Man Rogosa Sharpe –cysteine broth (MRS broth) (55 g/L MRS
broth (Becton Dickinson and Company, Sparks, MD), 0.5 g/L L-Cysteine-Hydrochloride (Sigma, St. Louis, MI). When solidified media were required 15 g/L agar was added to LLB and MRS broth.

Duplicate sets of the ten bifidobacterial isolates were obtained from the culture supplier as lyophilized preparations. The two preparations, designated as set I and set II, were stored at -70º C and analyzed separately. When a vial containing a RT isolate was aseptically opened, a sample of the dried powder was obtained by touching with a sterile inoculating loop moistened with LLB and transferring into an autoclaved PCR tube. These samples, for use in “direct PCR” were stored frozen at -70º C prior to analysis. The remaining portion of the lyophilized powder was rehydrated with MRS-cysteine broth, then a loopful of rehydrated culture was streaked for isolation onto fresh MRS-cysteine agar plates and incubated under anaerobic conditions at 37º C for 72 hours. A portion (100 µl) of the rehydrated culture was also inoculated into 9 ml of fresh MRS-cysteine broth and incubated at 37º C for 48 h for use in the event growth did not appear on the anaerobic plates.

After incubation tubes and plates were evaluated for growth. White, shiny, slightly raised colonies of similar size were considered pure culture. If colonies on the plate were morphologically similar and uniform, a stock culture was prepared. If different morphologies were observed, then each different colony type was picked from the plate and streaked on new agar plate for isolation. These manipulations continued until uniform and morphologically similar colonies were observed on the plate. Stock culture was prepared for each separate isolate. Briefly, one well isolated colony was picked from the plate and inoculated into 10 ml LLB or MRS and incubated at 37°C for 24 h or until
turbid, then mixed with 20% glycerol in ratio of 1:1 and distributed in cryovials (1ml/vial). Samples were stored frozen at – 70°C.

3. Polymerase Chain Reaction

Fresh RT, ATCC, and DSMZ bifidobacteria stock cultures were activated before each experiment by culturing on solid media as described above. A portion of a single well isolated colony of the bifidobacterial strain grown on LLA or MRS-cysteine Agar, was picked from the agar plate and transferred into PCR tubes. Cells were lysed by microwaving PCR tubes containing the bifidobacterial cell for 7 min at high power (Bollet et al. 1991).

3.1. Bifidobacterium genus-specific PCR

All strains were identified to the genus level by PCR using the Bifidobacterium specific primer set lm26 and lm3 described by Kaufmann et al. (1997) and to the species level as described by Matsuki et al. (1999) and Ventura et al. (2001a). PCR primers used for genus and species determination are in Table 2-2.

The PCR reaction mix contained 12.5 µl (Go Taq Green Master Mix, Promega), 1 µl of each primer pair (1µM, IDT), lysed bifidobacterial cells, and nuclease-free water in a total volume of 25 µl. The negative control for the PCR reaction contained 25µl of the amplification mixture without any sample. The positive control for all PCR reaction was B. animalis ssp. lactis DSMZ 10140T.
PCR was conducted using an Eppendorf thermocycler (Eppendorf, Hamburg, Germany). PCR conditions for detection of *Bifidobacterium* genus were as follows: 35-cycles of denaturation at 94°C for 1 min, annealing at 57°C – 3 min, and elongation at 72°C – 4 min. Upon completion PCR reaction mixtures were cooled to 4°C. Amplicons were separated by electrophoresis (80-100 volts) in 1% agarose gels stained with ethidium bromide, and visualized using AlphaImager 3300 Gel Documentation System, (Alpha Innotech Corporation, San Leandro, CA).

### 3.2. *Bifidobacterium* species-specific PCR

PCR was used to assess the species (subspecies) level identity of each of the RT bifidobacteria isolates using primers specific for *B. adolescentis*, *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis*, *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, and *B. bifidum* (Table 2-2). Positive controls for species-specific PCR was the appropriate ATCC or DSMZ type strains for the species being examined (Table 1). Amplification consisted of one initial cycle of denaturation at 94°C – 5 min, and 35 cycles of denaturation at 94°C – 20 sec, annealing at 55°C – 20 sec, elongation at 72°C – 30 sec, and a final extension cycle at 72°C – 5 min. PCR reaction mixtures were cooled to 4°C after amplification. Amplicons were detected as described previously.

All RT bifidobacterial isolates were also evaluated using the *B. animalis* ssp. *lactis* specific primer described by Ventura et al. (2001a) (Table 2-2). The positive control for this reaction was *B. animalis* ssp. *lactis* DSMZ 10140T. PCR parameters for detection of *B. animalis* ssp. *lactis* species were as follows: an initial denaturation at 95°C
– 5 min, and 30 cycles of denaturation 95°C – 30 sec, annealing 58°C – 1 min, elongation 72°C – 4 min, and final elongation at 72°C – 7 min. After all cycles were completed samples were cooled to 4°C and amplicons were separated and visualized as described above.

3.3. Direct PCR analysis of lyophilized TIMM culture

Lyophilized material collected from RT cultures was retrieved. Cells were lysed using the microwave method described above. Following lysis, the PCR analysis as described above was conducted to determine if the species identified by TIMM could be detected. In addition to that, TIMM bifidobacteria were tested using *B. animalis* ssp. *lactis* specific primer described by Ventura et al. (2001a). Appropriate reference strains were also analyzed at the same time.

4. Pulsed Field Gel Electrophoresis

All RT bifidobacteria isolates and *B. animalis* ssp. *lactis* DSMZ 10104T were streaked on LLA and incubated at 37°C under anaerobic conditions for 72 h. Cells were collected and re-suspended to an optical density (OD) of 0.9 – 1.1 in resuspension buffer (100mM Tris, 100 mM EDTA pH 7.6). One hundred and sixty microliters (160 μl) of cell suspension was transferred to a 1.5 ml microcentrifuge tube. Then 40 μl lysozyme (100 mg/ml), 10 μl proteininase K (20 mg/ml), and 1.6% Incert /SDS agarose were added to the cell suspension. Plugs were prepared by loading prepared mix in Bio-Rad disposable
plug molds (Bio-Rad). Two plugs were prepared for each sample.

Cells were lysed using 12 μl of mutanolysin (5000 U/ml), then 40 μl of proteinase K (20 mg/ml) in ES buffer (0.5 M EDTA, pH 9.0 and 1% Sarkosyl). Plugs treated with mutanolysin were incubated in a water bath shaker at 55°C for 4 hours. Then Proteinase K (20 mg/ml) was added and plugs were incubated at 55°C overnight. Following lysis plugs were washed with sterile distilled water for 15 min in a 50°C shaking water bath followed by four washing in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) in shaking water bath at 50°C for 15 min each time.

DNA contained in the plugs was digested using XbaI or SpeI. The restriction mixture contained 10 μl of 10 X restriction buffer (Sigma), 1 μl of BSA (0.1 mg/ml), and 3 μl of XbaI and SpeI (30 000 U/μl), respectively. Plugs were incubated for three hours in a 37°C water bath. B. animalis ssp. lactis DSMZ 10140T strain was used as a positive control. The experiment was performed in duplicate.

Electrophoresis was performed using Chef Mapper System (Bio-Rad Laboratories, Inc., Hercules, CA). Plugs were placed in a gel composed of 1% SeaKem Gold agarose. DNA fragments were separated using the following conditions: switch time from 0.19 to 35.38 s, run time for 13.56 h, angle 120°, gradient 6 V/cm, temperature 14 at linear ramping (Briczinski and Roberts 2006). Following electrophoresis, the gel was stained with ethidium bromide (0.4 mg/l, Promega) and visualized using the AlphaImager system 3300 Gel Documentation System (Alpha Innotech Corporation, San Leandro, CA).
5. Single nucleotide polymorphism (SNP) analysis

DNA was isolated from TIMM bifidobacterial strains as described by Vincent et al. (1998). Amplification and sequencing, and allelic profile constructs for the RT bifidobacterial isolates was performed as described by Briczinski et al. (2009).

Results

Growth and isolation of RT bifidobacteria on LLA and MRS-cysteine media

RT bifidobacteria, grown on LLA and MRS-cysteine agar for 72 h under anaerobic conditions (anaerobic incubator) at 37°C, formed white, well defined round shape, slightly raised, soft, shiny colonies. RT bifidobacteria strains grown in the anaerobic chamber on MRS-cysteine media formed larger colonies and were more uniform then cells grown in an anaerobic incubator on LLA.

PCR

Analysis of both sets of lyophilized RT bifidobacterial isolates reveled they were all members of the genus *Bifidobacterium* (Fig. 2-1). None of the RT bifidobacterial isolates showed a positive result with the corresponding species-specific primers (Appendix B-C). Further analysis of the RT isolates by PCR using the *B. animalis* ssp. *lactis* species-specific primer pair revealed all were isolates of *B. animalis* ssp. *lactis* (Fig. 2-2).
In an effort to determine if the results observed when RT bifidobacteria were cultured were due to selective growth of *B. animalis* ssp. *lactis*, PCR was performed using the lyophilized powder from TIMM bifidobacteria culture as DNA template. Results obtained from this analysis using species-specific primers pairs, were negative when tested for the species designated by the supplier (Table 2-1). However, when these same samples were examined using *B. animalis* ssp. *lactis* species-specifics primers (Table 2-1) an amplicon of 680 bp was produced, confirming that all the RT culture were *B. animalis* ssp. *lactis*.

**PFGE**

All TIMM bifidobacteria isolates were evaluated using Pulsed Field Gel Electrophoresis (PFGE). The data obtained (Fig. 2-3; 2-4) revealed no differences between bifidobacteria isolates obtained from TIMM and the type strain *B. animalis* ssp. *lactis* DSMZ 10104\(^T\) using *XbaI* and *SpeI* with the exception of isolate RT09. The PFGE patterns of all ten RT bifidobacteria isolates were similar to that observed in DSMZ reference strain. Only, one additional band was observed in sample RT09, when restricted using *XbaI* (Fig. 2-3).

**Single nucleotide polymorphism (SNP) analysis**

Allelic profiles were created after sequencing of SNP regions. The phylogenetic tree, created using these allelic profiles, allowed clustering of the RT strains into specific
groups (Table 2-3, Fig. 2-5). SNP analysis revealed the allelic profiles of the RT strains matched four distinct groups (Fig. 2-5) previously described by Briczinski et al. (2009). The majority of TIMM strains were clustered into a group that matched the commercial control strain *B. animalis* ssp. *lactis* BB-12. The other three strains (RT02, RT09, and RT04) can be classified into two separate groups; one being distinct from other by the inclusion of a SNP in the Balat_0051 and Igr9 genes. There is agreement between the PFGE pattern and SNP typing obtained for isolates RT 09 and ATCC 27536 strains. Another strain, RT08 exhibited the second SNP identified in the glcU gene, which allowed further differentiation of this strain from all other TIMM strains (Fig. 2-5).

**Discussion**

Commercial bifidobacterial isolates from Ukraine formed colonies typical of bifidobacteria when grown on LLA and MRS-cysteine agar. Growth was better on MRS-cysteine media in the anaerobic chamber where these organisms formed uniform and larger colonies than when grown on LLA in anaerobic incubator. It appears either anaerobic conditions or growth media influenced colony size. Culture maintenance and manipulations in the anaerobic chamber along with growth on MRS-cysteine agar resulted in better bifidobacterial growth.

This is not surprising since the bifidobacteria are sensitive to the oxygen (De Vries and Stouthamer 1969) and growth under oxidative stress differs among the bifidobacterial species (Shimamura et al. 1992). Meile et al. (1997) reported no growth of *Bifidobacterium longum* and *B. animalis* at elevated oxygen concentration (Meile et al.
Bifidobacterium animalis ssp. lactis tolerates oxygen (Meile et al. 1997). The formation of small colonies by TIMM bifidobacteria when grown on LLA incubated under anaerobic conditions in anaerobic incubators might have been due to the prolonged exposure to oxygen during culture manipulations. To remove this variable, the second set of isolation experiments were carried out using MRS-cysteine and all culture manipulations were performed inside in the anaerobic chamber. We hypothesized these conditions would allow better recovery of bifidobacteria designated by commercial supplier (Table 2-1). In addition, the MRS-medium was supplemented with cysteine, which acts as a reducing agent (Hartemink et al. 1997) favoring growth of anaerobic bacteria including bifidobacteria. Cultivation of strains and maintenance of RT isolates inside the anaerobic chamber still resulted in recovery of only B. animalis ssp. lactis, as verified by species-specific PCR (Ventura et al. 2001a). Thus, regardless of the culture method employed only B. animalis ssp. lactis was recovered from the RT culture samples.

In effort to determine if the reason only B. animalis ssp. lactis was recovered from TIMM cultures was due our inability to culture the target strains, PCR was conducted using samples of the lyophilized powder as the source of template DNA. Results obtained from this analysis were negative with the respect to the species designated by the commercial supplier using species-specific PCR. When these same samples were analyzed using B. animalis ssp. lactis species-specific primers (Table 2-2) all were positive (Fig. 2-2). These data suggest all ten bifidobacterial isolates obtained from Ukraine contained high level of B. animalis ssp. lactis and that if the designated strains were present, the number was too small to detect using the conditions employed. Based
on these results, it appears the typing methods used by the commercial supplier resulted in assignment of the correct genus, but incorrect species level.

To further characterize *B. animalis* ssp. *lactis* cultures obtained from Ukraine, all isolates were subjected to PFGE. No differences were detected between the RT bifidobacterial isolates and *B. animalis* ssp. *lactis* DSMZ 10104<sup>T</sup> digested with *XbaI* and *SpeI* (Fig. 2-3; 2-4) with the exception of RT09 restricted with *XbaI*. As has been reported previously the limited genome variability for *B. animalis* ssp. *lactis* limits the utility of PFGE for differentiation of strains of this organism.

SNP analysis has been shown to be suitable for differentiation of isolates of *B. animalis* ssp. *lactis* (Briczinski et al. 2009). In the present study, SNP analysis was able to separate RT strains into 4 distinct groups. Six strains had allelic profiles identical to the commonly used commercial strain *B. animalis* ssp. *lactis* BB-12. Four other RT strains (RT02, RT04, RT08, and RT09) were separated into separate groups distinct from other TIMM bifidobacterial strains. SNP analysis in RT02 and RT09-1 revealed an allelic profile identical to that of *B. animalis* ssp. *lactis* ATCC 27536 (Table 2-5). Strain RT08 had an allelic profile identical to that of the previously characterized strain RB 7239. Strain RT04 was found to have a unique profile and was placed in its own group. These results further support the notion that SNP analysis is a useful tool for differentiation of strains of *B. animalis* ssp. *lactis*. 
Summary and conclusions

Assuming the initial determination of the identity of bifidobacterial strains obtained from commercial supplier from Ukraine was correct, and knowing that bifidobacteria are anaerobic and that the sensitivity to the oxygen differs dramatically, it is hypothesized the RT strains were exposed to the oxygen during culturing and were contaminated with or overgrown by *B. animalis* ssp. *lactis*.

Results obtained in this study demonstrate that PCR and PFGE can be used as part of a rapid and discriminatory program for maintenance of bifidobacteria. PCR using *B. animalis* ssp. *lactis* and *B. longum* ssp. *longum*, *B. adolescentis*, *B. animalis* ssp. *animalis*, and *B. bifidum* species-specific primers is a reliable method in identification of bifidobacteria species. PFGE, regarded as useful tool in differentiation of closely related species, was only able to differentiate RT *B. animalis* ssp. *lactis* strains (in respect to RT09), and thus exhibited limited utility when applied to differentiation of *B. animalis* ssp. *lactis* due to low level of polymorphism in these subspecies. SNP analysis was able to separate the RT strains into four separate groups and was found to be more discriminative then PFGE.

Based on these results, it is clear that great attention must be given to assume the identity of the bifidobacteria used in probiotic-containing dairy products and recommended that molecular methods be used routinely to assure accurate and reliable culture identification.
References


Journal 17: 565-573.


Table 2-1: Microorganisms used in this study

<table>
<thead>
<tr>
<th>Lab ID</th>
<th>Species (designated by supplier)(^1)</th>
<th>Species (designated in this study)(^2)</th>
</tr>
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<tbody>
<tr>
<td>RT01</td>
<td>Bifidobacterium adolescentis</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT02</td>
<td>Bifidobacterium adolescentis</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT03</td>
<td>Bifidobacterium animalis</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT04</td>
<td>Bifidobacterium bifidum</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT05</td>
<td>Bifidobacterium bifidum</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT06</td>
<td>Bifidobacterium infantis</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT07</td>
<td>Bifidobacterium longum</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT08</td>
<td>Bifidobacterium longum</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT09</td>
<td>Bifidobacterium longum</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>RT10</td>
<td>Bifidobacterium longum</td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
</tbody>
</table>

Reference strains

1. Bifidobacterium adolescentis ATCC\(^3\) 15703
2. Bifidobacterium animalis ATCC 25527
3. Bifidobacterium animalis ssp. lactis DSMZ\(^4\) 10140\(^T\)
4. Bifidobacterium bifidum ATCC 29521
5. Bifidobacterium longum ATCC 15708
6. Bifidobacterium infantis ATCC 15697

Note: \(^1\) - The species identified by the supplier of the culture; \(^2\) – The species identified as a result of this study; \(^3\) – ATCC (American Type Culture Collection); \(^4\) – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (German Collection of Microorganisms and Cell Cultures).
Table 2-2: Sequences of PCR primers used in genus and species verification of Bifidobacterium

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ – 3’</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>Bifidobacterium genus</td>
<td></td>
<td>1350 bp</td>
<td>(Kaufmann et al. 1997)</td>
</tr>
<tr>
<td>Lm - 3</td>
<td>F&lt;sup&gt;1&lt;/sup&gt; CCGGTGCTICCCACTTTCATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lm - 26</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; GATTCTGGCTCAGGATGAACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bifidobacterium animalis ssp. lactis</td>
</tr>
<tr>
<td>Bflact-2</td>
<td>F&lt;sup&gt;1&lt;/sup&gt; GTGGAGACACGGTTTCCC</td>
<td>680 bp</td>
<td>(Ventura et al. 2001)</td>
</tr>
<tr>
<td>Bflact-3</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; CACACCACAACAATCCAATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium infantis</td>
<td></td>
<td>828 bp</td>
<td>(Matsuki et al. 1999)</td>
</tr>
<tr>
<td>BiINF-1</td>
<td>F&lt;sup&gt;1&lt;/sup&gt; TCCCAGTTGATCGCATGGTC</td>
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<td></td>
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<tr>
<td>BiINF-2</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; GGAAACCACATCTCTGGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium longum</td>
<td></td>
<td>831 bp</td>
<td>(Matsuki et al. 1999)</td>
</tr>
<tr>
<td>BiLON-1</td>
<td>F&lt;sup&gt;1&lt;/sup&gt; TTCAGTTGATCGCATGGTC</td>
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<tr>
<td>BiLON-2</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; GGAAGCCGATCTCTACGA</td>
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Note: F<sup>1</sup> - Forward, R<sup>1</sup> - Reverse
### Table 2-3: Identification of TIMM bifidobacteria by SNP analysis

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Figure 2-1: Evaluation of RT strains using *Bifidobacterium* genus specific PCR.

Lane: M – 100 bp DNA ladder. 1 - RT01; 2 - RT02; 3 - RT03; 4 - RT04; 5 - RT05; 6 - RT06; 7 - RT07; 8 - RT08; 9 - RT09; 10 - RT10; 11 - *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140^T^; 12 - Negative control.
Figure 2-2: Detection of *Bifidobacterium* species using *B. animalis* ssp. *lactis* DSMZ 10140\textsuperscript{T} specific primer pair.

Lane: M – 100 bp DNA ladder; 1 - RT01; 2 - RT02; 3 - RT03; 4 - RT04; 5 - RT05; 6 - RT06; 7 - RT07; 8 - RT08; 9 - RT09; 10 - RT10; 11 - *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140\textsuperscript{T}; 12 - Negative control.
Figure 2-3: Characterization of RT bifidobacteria by PFGE using XbaI

Lane: M - λ DNA marker; 1 - RT01; 2 - RT02; 3 - RT03; 4 - RT04; 5 - RT05; 6 - RT06; 7 - RT07; 8 - RT08; 9 - RT09; 10 - RT10; 11 – B. animalis ssp. lactis DSMZ 10140T; 12 – RT09 – 1; 13 - RT09 – 1* (restricted plug from previous experiment).
Figure 2-4: Characterization of RT bifidobacteria by PFGE using SpeI
Lane: M - λ DNA marker; 1 - RT01; 2 - RT02; 3 - RT03; 4 - RT04; 5 - RT05; 6 - RT06; 7 - RT07; 8 - RT08; 9 - RT09; 10 - RT10; 11 – B. animalis ssp. lactis DSMZ 10140T;
Figure 2-5: Phylogenic tree placing RT bifidobacterial isolates into the scheme of Briczinski et al. (2009) and *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140T
Appendices
Appendix - A

Visit of Natalya Fedorivna Kigel to The Pennsylvania State University
Natalya Kigel Visit

The research partnership between the Ukrainian Technological Institute of Meat and Milk (Kiev, Ukraine) and The Pennsylvania State University (University Park, USA) was established after Dr. Roberts visit to Ukraine in March 2005.

The TIMM is the leading institute in the Ukraine in dairy science and probiotics research, oriented towards development, production, and supply of starter culture to the Ukrainian milk and meat commercial companies. The Dairy Microbiology and Probiotics Laboratory in the Food Science Department directed by Dr. Roberts conducts research focused on the identification and characterization of probiotics microorganisms using molecular methods as well as application of probiotics in dairy products.

The TIMM developed an interest in a research collaboration with our laboratory in the area of development of effective methods, which could be used at TIMM for the identification and differentiate of current and novel TIMM bifidobacterial species and strains in their collection. As results of this mutual interest, the Head of the Biological Department of Technological Institute of Milk and Meat, Natalya Kigel visited The Pennsylvania State University.

Prior Dr. Kigel’s arrival, I was involved in business communication between the TIMM and Dr. Roberts, document preparation, translation of business and traveling documents and procedures, and served as interpreter during conferences.

Natalya Fedorivna Kigel worked in our laboratory as a visiting scholar from September 26, 2005 to November 1, 2005. Upon her arrival, I began my work with
Professor Kigel on the joint project aimed to characterize and differentiate TIMM bifidobacteria. During her visit, Natalya Fedorivna was involved in the initial stages of this project while learning the basics of PCR and PFGE. I worked with Natalya Fedorivna during the initial experiments to analyze TIMM bifidobacteria using PCR and PFGE.

In addition, I introduced Natalya Fedorivna to the laboratory practices employed in our laboratory, and the educational and graduate studies procedures used in the Food Science Department at The Pennsylvania State University. I served as Russian and English interpreter for Natalia Fedorivna during the professional meetings and conferences at PSU and at the Worldwide Food Expo 2005 held in Chicago. I also assisted Natalya Fedorivna with her adjustment to the cultural environment of State College.

Results of the preliminary work indicated it would be beneficial for both research institutions. After Kigel’s visit, I repeated the preliminary experiment and began analysis of the lyophilized culture of TIMM bifidobacteria. TIMM was informed of the results of the preliminary experiment. After completing analysis of lyophilized TIMM bifidobacteria, the results were reported again to the TIMM in February 2007. However, over that time the communication between the two institutions was limited. Nevertheless, despite the weakening of the communication between two institutions, there is optimism that such collaboration can be renewed since “science has not boundaries – knowledge is the sacred treasure of all mankind, the torch that illuminates the World. Science is indeed the supreme representation of the country, for in the society of nations the one that holds the leadership in the realm of intellect and ideas, will always rank first” L. Pasteur.
Appendix - B

Preliminary analysis of *Bifidobacterium*

Visit of N. Kigel
Figure B1-1: Detection of *Bifidobacterium* genus in isolates obtained from commercial supplier using the Lm3 and Lm26 primer pair. *Bifidobacterium* genus is indicated by an amplicon of 1350 bp.

TIMM strains: Lane: 1. 100 bp DNA Ladder. 2. RT10; 3. RT01; 4. RT04; 5. RT07; 6. RT03; 7. RT05; 8. Negative control
Figure B1-2: Evaluation of bifidobacterial isolates obtained from commercial supplier using *B. animalis* ssp. *lactis* specific primers (Bflact 2, 5) in 1 % agarose gel.

TIMM strains: Lane: 1. 100 bp DNA Ladder; 2. RT01; 3. RT04; 4. RT07; 5. RT03, 6. RT05; 7. *B. animalis* ssp. *lactis* DSMZ 10140^T^; 8. Negative control.
Appendix – C

Results of species specific PCR analysis of TIMM *Bifidobacterium* isolates
Figure C1-1:  Fig. 3. Genus and species specific PCR in control strains (ATCC and DSMZ)

Lane: M - 100 bp DNA ladder. Lane: *B. animalis* ssp. *lactis* DSMZ 10140T- 1, 2 ; *B. animalis* ATCC 25527 - 3, 4 ; *B. longum* ssp. *longum* ATCC 15708 – 7, 8 ; *B. longum* ssp. *infantis* ATCC 15697 – 11, 12 ; *B. adolescentis* ATCC 15703 - 15, 16 ; *B. bifidum* ATCC 29521 - 19, 20.
Species : Lane: *B. animalis* ssp. *lactis* DSMZ 10140T - 23, 24 ; *B. animalis* -5, 6 ; *B. longum* ssp. *longum* ATCC 15708 – 9, 10 ; *B. longum* ssp. *infantis* ATCC 15697 – 13, 14 ; *B. adolescentis* ATCC 15703 - 17, 18 ; *B. bifidum* ATCC 29521 - 23, 24.
Figure C1-2: Detection of RT bifidobacteria (set II) using Bifidobacterium species-specific primers.

Lane: M – 100 bp DNA ladder. 1 - RT01; 2 - RT02; 3- B. adolescentis ATCC 15703 (279 bp); 4 - Negative control; 5 - RT03; 6 – B. animalis ssp. animalis ATCC 25527 (467 bp); 7 - Negative control; 8 - RT04; 9 - RT05; 10 – B. bifidum ATCC 29521 (278 bp); 11- Negative control; 12 - RT06; 13- B. longum ssp. infantis ATCC 15697 (828 bp); 14 - Negative control; 15 - RT07; 16 - RT08; 17 - RT09; 18 - RT10; 19 – B. longum ssp. longum ATCC 15708 (831 bp); 20 – Negative control.
Figure C1-3: Detection of *B. animalis* ssp. *lactis* in RT bifidobacteria (set II) using *B. animalis* ssp. *lactis* specific PCR

Lane: M – 100 bp DNA ladder. 1 - RT01; 2 - RT02; 3 - RT03; 4 - RT04; 5 - RT05; 6 - RT06; 7 - RT07; 8 - RT08; 9 - RT09; 10 - RT10; 11 – *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140T; 12 - Negative control.
Figure C1-4: Detection of *B. bifidum* in lyophilized culture samples of *B. bifidum* RT04 using BiBif1, 2; and Bflact-2, 5 primer pair for detection of *B. animalis* ssp. *lactis*.

Lane: M – 100 bp DNA ladder; 1-2 - RT04 dry sample; 3- RT04 - wet sample; 4 – *B. animalis* ssp. *lactis* DSM 10140<sup>T</sup>; 5 - Negative control.
Figure C1-5: Detection of *B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis* in lyophilized culture samples of RT03 using Bflact-2, 5 and Ban2, 23 Si primer pairs, respectively.

Lane: M – 100 bp DNA ladder. 1- Negative control; 2 - *B. animalis* ssp. *lactis* DSMZ 10140^T; 3- 5 - RT03 - dry samples; B. 6 - Negative control; 7 - *B. animalis* ssp. *animalis* ATCC 25527; 8-10 - RT03 dry samples.
Figure C1-6: Detection of *Bifidobacterium* species in RT lyophilized bifidobacteria culture using species-specific PCR.

A. Lane: M – 100 bp DNA ladder; 1 - RT01; 2 - RT02; 3 - *Bifidobacterium adolescentis* ATCC 15703 (279 bp); 4 - RT04; 5 - RT05; 6 - *Bifidobacterium bifidum* ATCC 29521 (278 bp); 7 - RT06; 8 – *Bifidobacterium longum* ssp. *infantis* ATCC 15697 (828 bp); 9 - RT07; 10 - RT08; 11 - RT09; 12 - RT10; 13 - *Bifidobacterium longum* ATCC 15708 (831 bp).
B. Negative control. 1 - *Bifidobacterium adolescentis* ATCC 15703 (279 bp); 2 - *Bifidobacterium bifidum* ATCC 29521 (278 bp); 3 - *Bifidobacterium longum* ssp. *infantis* ATCC 15697 (828 bp); 4 - *Bifidobacterium longum* ssp. *longum* ATCC 15708 (831 bp).
C. 1 - RT03; 2 - *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 (467 bp); 3. Negative control.
Figure C1-7: Detection of *Bifidobacterium animalis* ssp. *lactis* in RT bifidobacteria isolates using Bflact-2. *Bifidobacterium animalis* ssp. *lactis* - specific primer pair.

Lane: 1 - RT04; 2 - RT05; 3 - RT06; 4 - *Bifidobacterium animalis* ssp. *lactis* DSM 10140<sup>T</sup>; 5 - Negative control.
Appendix – D

PFGE analysis of RT *Bifidobacterium* isolates
Figure D1-1: Pulsed Field Gel Electrophoresis of chromosomal DNA digested with *XbaI*.  
Lane: M - λ DNA marker; Lane: 1- RT05; 2- RT01; 3 - , RT04; 4 - RT03; 5 - RT07; 6 - RT01; 7- RT04; 8 - RT05; 9 - RT07; 10 - RT03; 11- *B. animalis* ssp. *lactis* DSMZ 10140T
Figure D1-2: PFGE in RT bifidobacteria XbaI (set II).

Lane: M - λ DNA marker; 1 - RT01; 2 - RT02; 3 - RT03; 4 - RT04; 5 - RT05; 6 - RT06; 7 - RT07; 8 - RT08; 9 - RT09; 10 - RT10; 11 - *Bifidobacterium animalis ssp. lactis DSMZ 10140*. 

*Bifidobacterium animalis ssp. lactis DSMZ 10140* is a strain of bifidobacteria commonly used in probiotic supplements.
Appendix – E

Examples of growth of bifidobacteria on MRS or LLB media
B. bifidum ATCC 29521

MRS – cysteine Agar
(72 h)

LLA
(72 h)

B. bifidum ATCC 29521

MRS-cysteine Broth
(48 h)

LLB
(72 h)

Figure E1-1: Growth of B. bifidum ATCC 29521 on LLA and MRS-cysteine media.
Appendix – F

Anaerobic incubator setup
Figure F1-1: Anaerobic incubator
Figure F1-2: Anaerobic chamber