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IDENTIFICATION, ANTIMICROBIAL SUSCEPTIBILITY AND FUNCTIONALITY OF POTENTIALLY PROBIOTIC BIFIDOBACTERIA

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MERCIEKES...

Vier jaar en drie verhuizen, twee verbouwingen, een huwelijk, het verlies van een zeer gewaardeerde vriend, een zwangerschap en geboorte, en een doctoraat verder... Kortom, een bewogen periode waarin plaats was voor zeer veel leuke momenten en af en toe een traan. Zo ook is dit doctoraat een bron geweest van ervaring, gelach, spanning - die zich bij mij meestal heeft geuit in tranen-, kennis, en misschien het allerbelangrijkste, vriendschap. Heel wat mensen hebben hieraan op hun manier een steentje bijgedragen en mij een eeuwige indruk nagelaten...

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merciekes,
Liesbeth.

Table of content

>>> Part 3 - Experimental work <<<

Chapter 3: Classification and identification of *Bifidobacterium* species

Chapter 4: Culture-dependent and culture-independent microbial analysis of probiotic products claiming to contain bifidobacteria

List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
ARDRA	Amplified Ribosomal DNA Restriction Analysis
B.	*Bifidobacterium*
BCCM	Belgian Coordinated Collections of Microorganisms
CFU	Colony Forming Units
CFPL	Collection de la Faculté de Pharmacie de Lille
DBPC	Double Blind Placebo Controlled
DGGE	Denaturing Gradient Gel Electrophoresis
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked ImmunoSorbent Assay
ERIC	Enterobacterial Repetitive Intergenic Consensus
F6PPK	Fructose-6-Phosphate PhosphoKetolase
FAO	Food and Agriculture Organization
FISH	Fluorescent In Situ Hybridization
FOSHU	Foods for Specified Health Use
FT-IR	Fourier Transform-InfraRed
G.	*Gardnerella*
GI-tract	Gastrointestinal tract
GMO	Genetically Modified Organism
GRAS	Generally Recognized As Safe
ITS	Internally Transcribed Spacer
L.	*Lactobacillus*
LAB	Lactic Acid Bacteria
LD	Lethal Dose
LMG	Laboratory of Microbiology
MRS	de Man, Rogosa and Sharpe
QPS	Qualified Presumption of Safety
P.	*Propionibacterium*
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PI-PLC	Phosphatidylinositol-specific Phospholipase C
R-	Research collection, Laboratory of Microbiology, University of Ghent
RAPD	Randomly Amplified Polymorphic DNA
rRNA	ribosomal RNA
rep-PCR	repetitive DNA sequence-based polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
subsp.	subspecies
(T)-RFLP	(Terminal)-Restriction Fragment Length Polymorphism
WHO	World Health Organization

Part 1
Introduction and objectives

General introduction

Food products fermented by lactic acid bacteria (LAB) have long been used for their proposed health promoting properties. At the beginning of the 20th century, Elie Metchnikoff (1907) advocated the consumption of large quantities of cultured foods containing LAB, such as sour milk and yoghurt, for good health and long life and by doing so, the Russian scientist gave birth to the concept of 'probiotics' "avant la lettre". At present, the growing awareness that a well-balanced and healthy diet contributes to a good physical condition has marked an era of strong expansion of the functional food market, including probiotic products. As a result, the modern consumer has adopted a general desire of self-treatment and managing a specific health condition or illness using probiotic foods and dietary supplements. In the development of human probiotics, strains belonging to the bacterial genera *Lactobacillus* and *Bifidobacterium* are amongst the most commonly used primarily because these organisms have a long history of safe use commonly referred to as the GRAS (Generally Recognized As Safe) status. Despite their widespread use, however, not all of the available probiotic strains currently on the market have adequate scientific documentation. It is important that new and existing potentially probiotic strains are subjected to profound analyses addressing safety and functionality before conducting clinical trials and before entering a marketing strategy. In this way, successful probiotic products can be delivered with long-term marketing potential.

In general, it cannot be assumed that the properties concerning safety and functionality of any given strain will be shared by strains of the same genus or species. The strain-specificity of these properties thus justifies efforts to correctly identify a probiotic strain. Furthermore, a correct identification is crucial to link a strain to a specific health effect as well as to enable accurate surveillance and epidemiological studies (Reid *et al*., 2002). In addition, it is incumbent upon the producer to provide full information as to strain designation on the product label to enable the consumer to select a product whose component bacteria have been shown to possess useful properties.

There is a large collection of historical data indicating that lactobacilli and bifidobacteria are safe for human use (Adams and Marteau, 1995; Naidu *et al*., 1999). In addition, if a

probiotic strain is of human origin and thus a member of the normal commensal flora, the organism can generally be considered safe for use. However, due to the indiscriminate use of antibiotics in human and veterinary medicine and as animal growth promoters, acquired antibiotic resistance has become an increasingly common characteristic in microorganisms, sometimes leading to serious problems in the treatment of microbial infections. Expert panels have indicated that strains harbouring transferable antibiotic resistance genes are not suitable for use as probiotics (Salminen *et al*., 2001). In this context, the specific risks related to each probiotic strain must be carefully identified.

Regarding functionality aspects of a probiotic strain, the screening process of candidate strains should involve determination of their survival capacity during gastro-intestinal (GI) transit, which has been considered as an important prerequisite for probiotic action (Saarela *et al*., 2000). Many probiotic effects are mediated by immune regulation, particularly through a balanced control of pro-and anti-inflammatory cytokines (Isolauri *et al*., 2001). Consequently, probiotics may be used as innovative tools to alleviate intestinal inflammation, normalize gut mucosal dysfunction, and down-regulate hypersensitivity reactions. However, recent data evidence that differences exist in the immunomodulatory effects of candidate probiotic bacteria (Mercenier *et al*., 2004), hence necessitating the characterization of these properties before developing clinical applications for extended target populations.

Objectives of this work

Some general aspects related to probiotics were investigated during this Ph.D. study, which was focussed on the genus *Bifidobacterium*. In comparison to the large variety of probiotic products claiming to contain bifidobacteria, the diversity of marketed probiotic *Bifidobacterium* strains appears to be relatively low (Grand *et al*., 2003). In order to successfully commercialise probiotic products containing *Bifidobacterium* strains, research towards the correct **identification** as well as the **safety** and **functionality** properties of the included *Bifidobacterium* strains has to be conducted. These three aspects constituted the starting point of the research described in this Ph.D. thesis.

The first goal of this work was to establish a taxonomical framework of validly described *Bifidobacterium* species using a genotypic fingerprinting technique, which allows the **unambiguous identification** of unknown bifidobacteria. Consequently, using both culture-dependent and culture-independent methodologies including Denaturing Gradient Gel Electrophoresis (DGGE) and real-time PCR, the **qualitative as well as quantitative microbial aspects** of probiotic products claiming to contain bifidobacteria were investigated. Additionally, the work aimed to provide **scientific documentation related to safety and functionality** on commercially applied *Bifidobacterium* strains as well as on human reference strains.

- The use of the genomic fingerprinting technique repetitive DNA sequence-based polymerase chain reaction (rep-PCR), was evaluated for the taxonomic discrimination among the majority of validly described species within the genus *Bifidobacterium*.

- A set of commercially available, worldwide collected probiotic products claiming to contain bifidobacteria was subjected to culture-dependent microbial analysis using rep-PCR fingerprinting for identification of the bifidobacterial isolates.

- As an alternative/complement for culture-dependent qualitative analysis, Denaturing Gradient Gel Electrophoresis (DGGE) was used for the culture-independent detection of bifidobacteria present in probiotic products.

- To complete microbial analysis of probiotic products, real-time PCR was optimised for the culture-independent quantification of bifidobacteria in probiotic products.

- Finally, a selected subset of probiotic product isolates and human reference strains of *Bifidobacterium* were screened for the presence of antibiotic resistance, for GI transit survival capacity and for immunomodulatory properties in order to generate basic information documenting the safety and functionality of the strains.

Short overview of this thesis

Part 2 presents an **overview of the literature** relating to the content of this work. Firstly, a concise discussion on the probiotic concept is provided, including the technological, functional and safety aspects of probiotics. The second part presents an extensive description of the genus *Bifidobacterium*, including an overview of currently available culture-dependent and culture-independent methods for identification, typing, detection and quantification.

Part 3 presents the **experimental work** performed in the framework of this Ph.D. study.

- The first chapter describes the evaluation and use of rep-PCR fingerprinting as an identification tool for a broad range of *Bifidobacterium* species. Subsequently, the taxonomical standing of the closely related species *B. animalis* and *B. lactis* is further investigated using a polyphasic approach.

- The second chapter includes the culture-dependent and culture-independent microbial analysis of a range of worldwide collected probiotic products claiming to contain bifidobacteria. Culture-dependent analysis involved the isolation and subsequent identification of bifidobacteria using rep-PCR fingerprinting. Subsequently, the results are compared with Denaturing Gradient Gel Electrophoresis, which was chosen for the culture-independent qualitative detection of bifidobacteria. Finally, to complete the culture-independent microbial analysis, real-time PCR is evaluated for the quantification of bifidobacteria in probiotic products.

- In the third chapter, the antibiotic susceptibility of human reference strains and probiotic isolates of *Bifidobacterium* is investigated as part of the safety assessment of new potential probiotic *Bifidobacterium* strains.

- Finally, the last chapter includes trials addressing some aspects of functionality, such as the effect of bifidobacteria on the cytokine production by human peripheral blood mononuclear cells and the ability of bifidobacteria to survive transit through the GI-tract.

Part 4 comprises the **general conclusions**, future perspectives and a summary of this work.

Part 2

Overview of the literature

Calvin's thoughts on "Writing a doctoral dissertation"

SOURCE: http://www.iaim.ira.uka.de/

Chapter 1

Probiotics

Chapter 1 provides a concise overview of the definition and the selection criteria of probiotics. Several aspects will be discussed with specific reference to the target group of microorganisms investigated during this Ph.D., i.e. the bifidobacteria. Specific information on the genus *Bifidobacterium* will be presented in the following chapter.

1.1. Definition

Although the 'probiotic concept' was already established at the turn of the 19th century (Metchnikoff, 1907), it wasn't until the mid-1960s that the term 'probiotic' as such was defined (Lilley and Stillwell, 1965). Following its first citation, numerous definitions have been proposed in an attempt to address several key points of discussion regarding the site of activity, the need for viability of the strain, the concentration of cells required to obtain the claimed beneficial effect, etc... This resulted in the formulation of several excessively long definitions, of which none so far received universal acceptance. Recently, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have proposed the following concise definition of a probiotic: "*a live microorganism which when administered in adequate amounts confers a health benefit on the host*" (http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf). This definition is confined to effects exerted by viable microorganisms without explicit reference to the site of action (upper and lower gastro-intestinal (GI) tract, oral cavity, vagina,...), the route of administration or the clinical status of the host. On the other hand, it clearly indicates that the probiotic is a health-promoting microorganism and addresses the requirement of sufficient microbial numbers to exert those health effects. Although this definition has attempted to

harmonize the understanding of the term 'probiotic' with consideration of the current state of science, it clearly does not exclude further discussion.

Positive health effects have also been ascribed to "prebiotics", defined by Gibson and Roberfroid (1995) as "*non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon*". The combined use of pro- and prebiotics, commonly referred to as 'synbiotics', falls into the functional foods category, which is an expanding sector of the food industry (Stanton *et al*., 2001). Although no clear delineation of the definition for functional food has been developed so far (Menrad *et al*., 2002), these compounds can be described as "modified food or food-ingredients conferring a beneficial effect on health beyond or additional to the effects of the traditional nutrients present in the food" (ONFS Committee, 1994).

According to the current definition of a probiotic, a large variety of microbial species and genera are considered to have probiotic potential, most of which belong to the lactic acid bacteria (LAB). In the development of human probiotics, strains belonging to the genera *Lactobacillus* and *Bifidobacterium* have been most commonly used, even though some probiotic preparations are based on other LAB or even non-LAB species and yeasts (**Table 1**).

Table 1. List of species used in the development of probiotic products for human consumption (based on Holzapfel *et al* ., 1998 and Mercenier *et al* ., 2003)

Lactobacillus	***Bifidobacterium***	**Other LAB**	**Non-LAB**
L. acidophilus	*B. adolescentis*	*Enterococcus faecium*	*Escherichia coli*
L. casei	*B. animalis* subsp. *lactis*	*Lactococcus lactis*	*Saccharomyces cerevisiae('boulardii')*
L. crispatus	*B. bifidum*	*Leuconostoc mesenteroides*	
L. delbrueckii subsp. *bulgaricus*	*B. breve*	*Pediococcus acidilactici*	
L. fermentum	*B. longum* biotype infantis	*Streptococcus thermophilus*	
L. helveticus	*B. longum* biotype longum		
L. gasseri			
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			
L. salivarius			

1.2. Probiotic bifidobacteria

Essentially, fundamental and applied research on probiotic bifidobacteria started in the 1950s in Japan. In 1971, the Morinaga Milk Industry Company developed the first 'bifidus' product – a fermented milk containing *Bifidobacterium longum* and *Streptococcus thermophilus* (Ishibashi and Shimamura, 1993; Hughes and Hoover, 1991). Throughout the 1970s, advanced technologies triggered the delivery of products containing viable bifidobacteria on a commercial basis of which some were shown to have the potential to improve the health of the general public (Hughes and Hoover, 1991). Subsequently, the Morinaga Milk Industry Company launched a 'bifidus' milk (1977) as well as a 'bifidus' yoghurt (1979). At the same time (1978), Yakult introduced a fermented milk named MilMil™ in Japan that contained strains of *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (Ishibashi and Shimamura, 1993). Today, the market of probiotic preparations claimed to contain bifidobacteria has extended from milk-based products to powders and tablets and are partly responsible for a market value of approximately $1,3 billion in Europe alone (Leatherhead Food Research Association, Functional Food Markets, Innovation and Prospects, 2002). In present-day commercial probiotic products, bifidobacteria are used singly or in combination with other lactic acid bacteria such as lactobacilli and *Streptococcus thermophilus*.

Within the genus *Bifidobacterium*, the following (sub)species have been used as potential probiotics: *B. adolescentis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve* and *B. longum* biotypes infantis and longum (**Table 1**). However, since many probiotic characteristics are strain-specific, it is clear that these attributes have to be investigated on an individual strain basis rather than designating an entire species as probiotic. Potential probiotic strains need to be selected with respect to their safety, functionality, technological properties and health benefits, before they can be included into a commercial probiotic product. A list of well-documented probiotic *Bifidobacterium* strains is given in **Table 2**.

Table 2. List of well-documented probiotic *Bifidobacterium* strains (based on Mercenier *et al*., 2003)

Strain	Source
B. breve strain Yakult	Yakult (Japan)
B. lactis Bb-12	Chr. Hansen, Inc. (Denmark)
B. lactis FK120	Fukuchan milk (Japan)
B. lactis HN019 (DR10)	New Zealand Dairy Board (N.-Z.)
B. lactis LKM512	Fukuchan milk (Japan)
B. longum BB536	Morinaga Milk Industry Co., Ltd. (Japan)
B. longum SBT-2928	Snow Brand Milk Products Co., Ltd. (Japan)
Bifidobacterium species 420	Danlac (Canada)

Species names are those reported by the manufacturer, but do not necessarily reflect the true taxonomic position of these strains

1.3. Proposed guidelines for the selection of probiotics

The guidelines discussed below constitute a set of parameters that have been proposed for a strain, and hence the product containing the strain, to be considered and/or named 'probiotic'. Besides the availability of evidence supporting specific health promoting properties, these selection parameters include safety, basic functionality and technological properties (**Figure 1**).

1.3.1. Health benefit assessment

Essentially, two research tasks need to be performed in order to document the potential health promoting activity of a promising probiotic strain. First, clinical trials are needed to analyze the beneficial effects of administering a probiotic to a subject group. Secondly, if such an effect has been reported, the underlying microbial, biochemical and molecular mechanisms need to be unraveled. The limited correlation between *in vitro* observations and prediction of functionality of probiotic microorganisms in the human body has necessitated further substantiation of efficacy with human trials (Reid, 2005). It is generally recommended that such evidence result from randomised, double-blind, placebo-controlled (DBPC) or so-called phase II studies in which a test group, containing a sufficient number of subjects, is placed under complete dietary supervision (Marteau *et al*., 2002). The strength of these studies is

that results from the test group can be compared to those of a placebo-controlled group. When testing probiotic foods, the placebo should consist of the food carrier devoid of the probiotic compound. The principle outcome of these efficacy studies should be proven benefits such as (i) statistically and biologically significant improvement in condition, symptoms, signs, well-being or quality of life; (ii) prevention, reduced risk or longer period to next occurrence of disease and (iii) faster recovery from illness. Probiotics delivered in a food matrix are generally not tested in phase III studies, which concern parallel comparisons with a standard therapy. However, once reference is made to curing or treating a disease, most governmental regulatory agencies would regard this as drug therapy and will demand extensive testing including a phase III study. These randomised blinded designs are necessary to determine whether a probiotic therapy is as effective as or better than a standard treatment for a particular clinical condition. At present, no phase III studies have been reported on probiotics (Reid, 2005).

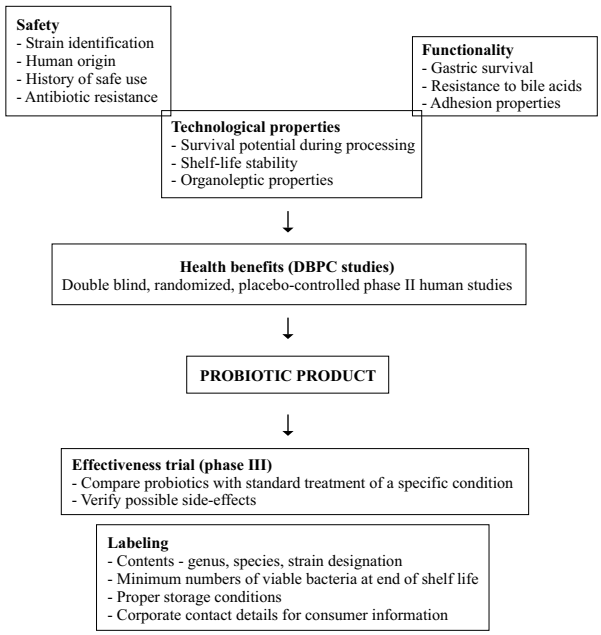

Figure 1. Proposed guidelines for the selection of potential probiotic strains for food use (based on Reid *et al*., 2002 and Reid, 2005)

Finally, no adverse effects should be observed when the probiotic is administered via a food product. Adverse effects should be monitored and incidents reported. Furthermore, it is generally recommended that human clinical trials are performed by independent institutions and that the results are published in peer-reviewed scientific or medical journals (Reid *et al*., 2002), thereby also including publications on negative results as these also contribute to the totality of the evidence supporting probiotic efficacy.

Although the list of potential probiotic health claims mainly attributed to lactic acid bacteria is still growing, most of these effects remain to be substantiated by human randomised DBPC clinical studies (for review see Ouwehand *et al*., 2002; Stanton *et al*., 2003; Mercenier *et al*., 2003). So far, the use of probiotics as biotherapeutics has been proven in cases of gastro-intestinal disturbances (Marteau *et al*., 2001), management of allergic diseases (Majamaa and Isolauri, 1997) and treatment and prevention of inflammatory bowel diseases (Marteau *et al*., 2001; Hanauer and Dassopoulos, 2001). A list of beneficial health effects evidenced by clinical studies or human intervention trials, resembling the traditional pharmacological DBPC approach is given in **Table 3**.

Table 3. Therapeutic use of probiotics (based on Mercenier *et al* ., 2003)

Therapeutic use considered successful:
Alleviation of lactose intolerance
Prevention of antibiotic associated diarrhoea
Prevention of gastro-enteritis caused by:
* Rotavirus in children
* *Clostridium difficile* after antibiotic treatment
Treatment of bacterial overgrowth
Effect on Inflammatory Bowel Diseases (IBD)
* Ulcerative colitis
* Crohn's disease
* Pouchitis
Reduction of allergy such as atopic dermatitis
Positive indications for therapeutic use:
Prevention and improvement of symptoms associated with Irritable Bowel Syndrome (IBS)
Suppression of colon cancer
Prevention or treatment of Traveler's diarrhoea
Inhibitory effects of *Helicobacter pylori*
Insufficient proof for therapeutic use:
Effect on viral infections
Cholesterol lowering effects

As mentioned earlier, it is important to bear in mind that not all strains within one species will be able to mediate comparable 'probiotic' effects. Conversely, it is also unlikely that a single strain will produce a multitude of potential benefits. Hence, each specific type of disorder requires careful selection of the most suitable probiotic strain in order to achieve the optimal health benefit. However, prior to submitting potential probiotic candidates to extensive human efficacy trials, more detailed knowledge is required regarding their safety, functionality and technological aspects.

1.3.2. Safety aspects

Despite the fact that most probiotics belong to bacterial taxa that have a very good safety record, it has been recommended that new and existing probiotic strains including genetically modified organisms (GMOs) need to be characterized with respect to the following safety aspects (for reviews, see Salminen *et al*., 1998; Reid *et al*., 2002 and Ouwehand *et al*., in press):

- As a first safety criterion, the **taxonomic identity** of the probiotic strain should be determined using validated and reproducible methodologies preferably by combining phenotypic and genotypic methods (see Chapter 2). This also implies the correct use of valid taxonomic names and the adequate designation of particular strains. The current state of evidence suggests that different strains can possess different features related to different safety risks, implying that it is not possible to identify the specific safety risks associated with a probiotic strain without proper identification. Proper identification is also necessary to avoid the inclusion of pathogenic microorganisms in probiotic products.

- Although a matter of debate, it has been suggested that probiotic strains should originate from the species of intended use. One can argue that a probiotic strain originating from a **healthy human GI-tract** can function better in a similar environment from where it was originally isolated. Although this point of view has been supported by the fact that most of the currently used strains are of human origin, some animal derived strains have also shown positive effects on humans. Perhaps the importance of this criterion is one of consumer perception, in that humans may not wish to consume strains that originated from pigs, rats or mice.

- In general, the microorganisms used in the production of fermented foods have a long **history of safe use**, and are often referred to as 'food grade' or GRAS organisms (Holzapfel *et al*., 1998). On the other hand, it should be kept in mind that any microorganism may cause unwanted side effects when administered in sufficiently high doses. The most immediate risk associated with the consumption of microorganisms is infection (e.g. meningitis, endocarditis), especially in immunocompromised individuals. In the particular case of bifidobacteria, reports on infections are very rare (Hata *et al*., 1988; Nakazawa *et al*., 1996; Ha *et al*., 1999) and such infections are most likely caused by bifidobacteria that are part of the patients' own microbiota (Wang *et al*., 1996; MacFie *et al*., 1999). Thus, despite the fact that bifidobacteria are present at high levels in the intestine, they appear to be among the least infectious organisms. However, also other risks such as administration toxicity, deleterious metabolic activities and excessive immune stimulation in susceptible individuals need to be taken into consideration (**Table 4**). The median lethal dose of orally administered *B. longum* BB536 and *B. lactis* HN019 in mice was shown to be > 50 g/kg/day (Momose *et al*., 1979; Zhou *et al*., 2000). No toxicity was reported after repeated oral administration of the former strain (Momose *et al*., 1979). Recently, Ouwehand and co-workers (2004) investigated the presence of known virulence factors in clinical blood isolates and dairy and faecal isolates of bifidobacteria. No significant differences with respect to these virulence factors could be observed between clinical and faecal isolates supporting the general opinion that bifidobacteria are safe for food and probiotic use.

- Due to the indiscriminate use of antibiotics in human and veterinary medicine and as animal growth promoters, **antibiotic resistance** has become an increasingly common characteristic in (food-borne) microorganisms (Threlfall *et al*., 2000) causing serious problems in treatment of microbial infections. Antibiotic resistance in bacteria may be intrinsic or acquired. Intrinsic resistance is a naturally occurring trait considered to be a species-specific characteristic, whereas acquired resistance stems from genetic mutations or from the acquisition of foreign DNA from other bacteria. Probiotic strains with non-transmissible antibiotic resistances do not usually confer a safety concern. To some extent, non-transmissible antibiotic resistance might even be a useful property if the probiotic strain is to be used as a prophylactic agent in the treatment of antibiotic associated diarrhoea (Charteris *et al*., 1998a). However, antibiotic

resistance linked to transferable mobile genetic elements such as plasmids and transposons is another matter because of the possibility of resistance spreading to other, more harmful bacteria. Expert panels have indicated that strains harbouring transferable antibiotic resistance genes are not suitable for use as probiotics (Salminen *et al*., 2001). In this context, the specific risks related to each probiotic strain must be carefully considered. Although several studies have documented the presence of antibiotic resistances in bifidobacteria (Matteuzi *et al*., 1983; Lim *et al*., 1993; Charteris *et al*., 1998b; Yazid *et al*., 2000; Moubareck *et al*., 2005), none of these were able to detect conjugative plasmids or transposons carrying resistance determinants.

- Finally, it is important not only to evaluate the risks directly associated with the probiotic strain itself, also the **risks associated with the target population** as well as possible **microbe-host interactions** must be taken into consideration. Likewise, the **pharmacokinetics** of the specific probiotics such as survival within the gastro-intestinal tract, colonization and translocation properties, as well as the fate of their metabolic products, need to be determined to predict the potential safety risks associated with a probiotic strain.

Table 4. Analysis of bifidobacteria for safety related properties (based on Guiemonde *et al*., 2004 and Ouwehand *et al*., 2004)

Safety aspect	Data documented for bifidobacteria	Reference(s)
Toxicity:		
Acute	*B. longum* BB536: the median lethal dose (LD_{50}) in mice is > 50 g/kg/day; *B. lactis* HN019: LD50 in mice is > 50 g/kg/day	Momose *et al*., 1979; Zhou *et al.*, 2000
Chronic	No toxicity after repeated oral administration of *B. longum* BB536	Momose *et al*., 1979
Metabolic activity:		
Excessive mucus degradation	Mucus degradation was not observed by *B. bifidum*	Ruseler-Van Embden *et al*., 1995
Excessive bile salt deconjugation	Bile salt deconjugation is a general characteristic of bifidobacteria	Grill *et al*., 1995, 2000; Tanaka *et al.*, 1999
Resistance to human serum mediated killing	Resistance was witnessed, but was similar for all three tested groups of *Bifidobacterium* strains	Ouwehand *et al.*, 2004
Haemolysis	No α– nor β-haemolytic activity of human blood	Ouwehand *et al.*, 2004
Induction of respiratory burst in peripheral blood mononucleocytes	Respiratory burst induced by the tested pathogens (*E. coli, Y. enterocolita, S. typhimurium* and *S. enteritidis*)	Ouwehand *et al.*, 2004
	did not differ from that induced by the tested bifidobacteria	Ouwehand *et al.*, 2004
Adhesion to extracellular matrix proteins	Low level of adhesion to collagen and fibrinogen for all three tested groups of *Bifidobacterium* strains	Ouwehand *et al.*, 2004
Adhesion to intestinal mucus	Dairy strains exhibited the tendency to adhere at higher levels to intestinal mucus than clinical strains	Ouwehand *et al.*, 2004
Phosphatidylinositol-specific phospholipase C (PI-PLC)	No PI-PLC activity was detected for any of the tested bifidobacteria	Ouwehand *et al.*, 2004

1.3.3. Functional aspects

Undoubtedly, one of the most important aspects reflecting the functionality of a probiotic culture concerns its ability to promote human health at the site of action (see § 1.3.1). However, prior to achieving this, probiotic cultures must survive the transit through the gastro-intestinal tract after oral consumption. Although dead bacteria have been shown to mediate a number of beneficial effects, the majority of the health promoting benefits associated with probiotics require viable microorganisms (Ouwehand *et al*., 1998). Consequently, resistance to gastric acidity and bile toxicity is a first major functional requirement.

- The pH in the stomach may be as low as 1.5 (Waterman and Small, 1998), and one of the first challenges encountered by probiotics following ingestion is the ability to **survive** in highly **acidic conditions**. Resistance to these adverse conditions has been investigated in several studies, indicating large variation between strains and species. In general, *Bifidobacterium* cultures are less acid tolerant than *Lactobacillus* cultures, particularly when exposed to human gastric juice (Dunne *et al*., 2001).

- When selecting a probiotic candidate, the ability of bacteria to **resist the effects of bile** is generally considered an important property for survival in the small intestine. Following synthesis from cholesterol and secretion into the duodenum, conjugated bile salts undergo extensive chemical modifications in the colon due to microbial activity. Conjugated bile salt hydrolysis is an important bile salt modification liberating the amino acid moiety from the deconjugated bile acid. Although both forms exhibit anti-bacterial activity, deconjugated bile salts are more inhibitory to anaerobes (Grill *et al*., 1995). Accordingly, most *Bifidobacterium* strains have shown to be bile-sensitive (Kociubinski *et al*., 1999). However, since bile salt resistance can differ considerably among strains of a certain species (Gilliland, 2002), a profound selection of the most resistant strain is necessary.

Following survival of the gastro-intestinal transit, **adhesion** of probiotic strains to the intestinal surface and temporary **colonization** of the human GI-tract have been suggested as important prerequisites for probiotic action. Although several probiotic strains have been noted to temporarily persist in the human GI-tract (Fukushima *et al*., 1998; Johansson *et al*., 1998; Alander *et al*., 1999; Donnet-Hughes *et al*., 1999), adherent strains are likely to persist

longer in the intestinal tract than non-adherent strains and may therefore enhance their health-promoting potential in the GI-tract. Conversely, adhesion to the intestinal mucosa can also be the first step in bacterial pathogenesis (Finlay and Falkow, 1997). However, this concern has recently been shown to be unwarranted as far as bifidobacteria are concerned (Ouwehand *et al*., 2004) (see § 1.3.2).

1.3.4. Technological aspects

Whereas there is little doubt that safety and functional criteria are of paramount importance in the probiotic selection process, it is less well emphasized that also the technological suitability of probiotic cultures is critical to their exploitation (for reviews, see Mattila-Sandholm *et al*., 2002 and Ross *et al*., 2005).

The viability and stability of probiotics has been both a marketing and technological challenge for industrial producers. For successful delivery in foods, probiotics must **survive food processing and maintain a suitable level of viable cells during product maturation and shelf life**. Although adequate strain selection may provide strains with good manufacturing and food technology characteristics, even the physiologically most robust strains are currently limited in the range of food applications to which they can be applied. Additionally, bacteria with exceptional functional health properties are often ruled out due to technological limitations. Intensive research has therefore focussed on protecting the viability of probiotic cultures both during product manufacture and storage (**Table 5**).

Table 5. Overcoming the technological hurdles in the development of probiotic foods (based on Ross *et al.,* 2005)

Selection of probiotic strains for technological properties
The use of protectants
* thermoprotectants during spray-drying
* cryoprotectants during freeze-drying
Micro-encapsulation in carriers
* milk proteins
* complex (prebiotic) carbohydrates (e.g. resistant starch)
Induction of cellular stress responses
* acid tolerance (e.g. F_1F_0-*ATPase*)
* oxygen tolerance (e.g. Osp)
* thermotolerance
Genetic manipulation
e.g. enhanced thermal tolerance due to overexpression of heat shock protein chaperones GroEL and GroES

To maintain confidence in probiotic products it is not only important to demonstrate good survival of the bacteria in consumer products during shelf life, but also to guarantee that the probiotic culture contributes to the **good sensory properties** of the final product. Because the environment within the GI-tract and in a food matrix are quite different, the probiotic is often not suitable as a starter organism (German *et al.*, 1999). Therefore, it is common practice to use probiotics together with other bacteria, e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, in order to obtain the appropriate taste and texture. By selecting an optimal support culture, it is possible to produce fermented probiotic products with excellent sensory properties and good survival of the bacteria (Fondén *et al*., 2000). A recent study by Roy (2005) has elaborated on the technological aspects related to the use of bifidobacteria in dairy products.

1.4. Legislation aspects

Once a probiotic candidate has been carefully selected based on the criteria outlined in § 1.3.2 – 1.3.4 and specific health benefits (see § 1.3.1) have been identified, regulatory and product labelling issues remain to be addressed prior to marketing (Sanders and Huis in 't Veld, 1999). These issues are a complicated matter because they differ for each country, but are likewise critical because they provide the means of communication of the product benefits to the consumer. Accurate information on the content and counts of bacteria in commercial products is one aspect of communication on probiotics. However, the main emphasis of the message addressed to the consumer lies at the level of the health claims. In Europe, commercialisation of probiotics is regulated by the normal legislation on foods, provided that no claims are made related to health, prevention and curing. In Japan, a specific 'FOSHU label' can be requested that grants the producer the permission to put a health claim on a food label, which has been substantiated by scientific evidence. Although regulations are far from unanimous worldwide, efforts are presently being made for the implementation of standards in terms of labelling and use of health claims in United Nations member countries, which are intended to provide the consumer with more useful and precise information (Reid, 2005).

Likewise, the European Food Safety Authority (EFSA) has attempted to develop a qualified generic approval system based on the concept of "qualified presumption of safety" (QPS), defined as the assumption based on reasonable evidence and qualified to allow certain restrictions to apply. Such a system would improve the consistency of safety assessment and at the same time make better use of assessment resources by not requiring a full and arguably unnecessary safety review of organisms with a long history of safe use. Case-by-case safety assessments could be eliminated or restricted to only those aspects that are relevant for the organism in question (i.e. the presence of transmissible antibiotic resistance markers or known virulence factors in a species known to have pathogenic strains) (http://europa.eu.int/comm/food/fs/sc/scf/out178_en.pdf).

Chapter 2

The genus *Bifidobacterium*

The first published reference to the name 'bifidus' dates from 1900, when Tissier isolated an anaerobic bacterium with bifid morphology from the faeces of breast-fed infants, which he named *Bacillus bifidus*. In 1924, Orla-Jensen recognized the existence of the genus *Bifidobacterium* as a separate taxon, explaining that various species of bifidobacteria "doubtless constitute a separate genus, possibly forming a connective link between the lactic acid bacteria and the propionic acid bacteria". However, given their similarities to the genus *Lactobacillus*, bifidobacteria remained included in this genus. Studies on this bacterial group gradually declined thereafter, until in 1957 a separation of bifidobacteria from lactobacilli was accomplished, and the existence of multiple biotypes of *Bifidobacterium* was recognized based on their carbohydrate fermentation patterns (Dehnert, 1957). This was the beginning of an era of taxonomic evolution and knowledge acquisition on the genus *Bifidobacterium*, starting from its initial listing as *Lactobacillus bifidus* in the seventh edition of Bergey's Manual of Determinative Bacteriology (Breed *et al*., 1957) to the 32 current validly described (sub)species.

2.1. Morphology, physiology and metabolism

The genus *Bifidobacterium* consists of Gram-positive, non-spore forming, non-motile anaerobes (some species can tolerate oxygen only in presence of CO_2) that are catalase-negative (except for *B. indicum* and *B. coryneforme* when grown in presence of air). They are pleomorphic, exhibiting a bifid-shaped or multiple-branched cellular morphology, and occur singly or in chains or aggregates (**Figure 2**). Bifidobacteria isolated from humans have been demonstrated to grow optimally at temperatures ranging from 36°C to 38°C. *Bifidobacterium* species isolated from animals, on the other hand, have been shown to grow at higher temperatures (41°C to 43°C), with *B. thermacidophilum* exhibiting a maximum growth temperature of 49.5°C (Dong *et al*., 2000a). The minimum growth temperature for bifidobacteria is generally not below 20°C with the notable exception of *B. psychraerophilum*, which has been shown to grow at 4°C (Simpson *et al*., 2004). The optimum pH at the beginning of growth is between 6.5 and 7.0. No growth has been recorded at pH lower than 4.5 or higher than 8.5 with the exception of *B. thermacidophilum*, which is able to grow at pH 4.0 (Dong *et al*., 2000a).

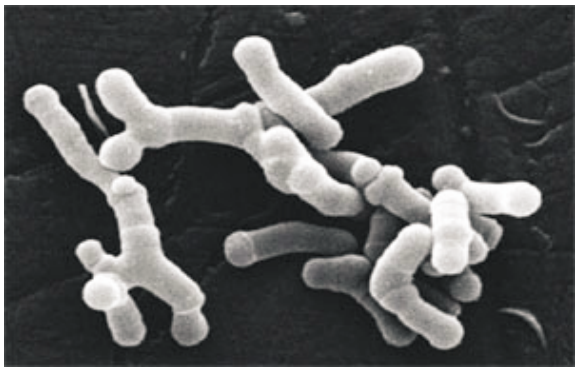

Figure 2. Typical morphology of bifidobacteria

Hexose metabolism of bifidobacteria follows the "fructose-6-phosphate shunt" or "bifidus shunt" (Scardovi and Trovatelli, 1965). The key enzyme of this pathway is fructose-6-phosphate phosphoketolase (F6PPK), which cleaves hexose phosphate into erythrose-4-phosphate and acetyl phosphate. From tetrose and hexose phosphates, through the successive action of transaldolase and transketolase, pentose phosphates are formed which give rise to

lactic acid and acetic acid in the theoretical ratio 1.0:1.5. However, this ratio is scarcely ever found in growing cultures of bifidobacteria. Cleavage of pyruvate to formic acid and acetyl phosphate, and the reduction of acetyl phosphate to ethanol can often alter the fermentation balance to a highly variable extent (Lauer and Kandler, 1976). Different bifidobacterial species produce different amounts of acetic, lactic and formic acid and ethanol under the same conditions. Furthermore, variations of growth conditions, such as type and quantity of the carbon source, may result in the production of varying amounts of fermentation products. During metabolism of hexose, no CO_2 is produced, except during degradation of gluconate.

In order to adapt and compete in an environment with changing nutritional conditions, bifidobacteria possess an array of enzymes that allow them to utilize a great variety of mono- and disaccharides as well as to metabolise complex carbohydrates that are normally not digested in the small intestine. This latter feature should give an ecological advantage to bifidobacterial colonizers of the intestinal environment where complex carbohydrates are present either because of production by the host epithelium or introduction through the diet, which forms the basis of the prebiotic concept.

2.2. Environmental distribution and ecology

The *Bifidobacterium* species described to date can be grouped according to their respective ecological niches: the human intestine, vagina and oral cavity, the animal intestine, the insect intestine and sewage (**Table 6**).

The presence of bifidobacteria in the human gut has stimulated much interest among microbiologists and nutritionists. Many factors, including host development, age, health condition, diet and the adaptability of each bacterial species influences the number and overall composition of microbial populations in different parts of the gastrointestinal tract (Fuller, 1989). In the first few days of life, the gastrointestinal tract of newborns is colonized by coliforms and streptococci, which create a reducing environment favourable to the settlement of anaerobic bacteria such

as *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Lactobacillus*. After 5-7 days, bifidobacteria become the predominant intestinal bacteria in breast-fed infants (Biavati *et al*., 1984), whereas no such predominance has ever been noted in bottle-fed infants. This compositional diversity of the microflora in infants nourished with mothers' or artificial milk is due to the fact that the former contains specific growth factors, the so called 'bifidogenic factors', that stimulate the development of bifidobacteria. After solid food introduction and weaning, the composition of the intestinal microbiota of breast-fed infants evolves to become similar to that of bottle-fed infants. Around the second year of life an adult-like intestinal microbial composition is established in which bifidobacteria are no longer dominant (Conway, 1997), but constitute the third most abundant bacterial group following the genera *Bacteroides* and *Eubacterium* (Finegold *et al*., 1983) reaching numbers of approximately 10^9 CFU per g of faeces (Matsuki *et al*., 2004; Gueimonde *et al*., 2004). Recent studies by Matsuki and co-workers (1999, 2004) using species-specific PCR primers show that *B. catenulatum* and *B. pseudocatenulatum* (i.e. the *B. catenulatum* group) are the most common of the adult intestinal bifidobacterial flora (detected in 44 of 48 samples [92%]), followed by *B. longum* and *B. adolescentis* and that *B. breve*, *B. infantis* and *B. longum* are the predominate bifidobacterial species in the intestinal tracts of infants.

Bifidobacteria have also been located in the human oral cavity. The most common species in this site is *B. dentium* (Scardovi and Crociani, 1974), which seems to be involved in dental plaque formation. In the human vagina, bifidobacteria are considered to play a role in maintaining homeostasis by producing organic acids and bacteriocins antagonistic towards pathogens. It has been estimated that bifidobacteria are present in 22–26% of all healthy women (Werner and Seeliger, 1963; Crociani *et al*., 1973). The species *B. breve* and *B. adolescentis* have been most frequently isolated from the human vagina, whereas *B. longum* and *B. bifidum* appear to be present to a smaller extent (Korschunov *et al*., 1999). Finally, *B. scardovii* is the only species from human origin that has so far only been isolated from clinical sources, i.e. blood, urine and the hip of a female patient. However, its original habitat is not known and the underlying infections, which have lead to the isolation of these organisms, were not discussed in the original description of the species.

A large variety of bifidobacterial species have been isolated from animal faeces. The composition of the bifidobacterial microflora in animals varies with the age, species and diet of the host. Most species are host-specific, and are typical for a given animal habitat, e.g. *B. cuniculi*, *B. magnum* and *B. saeculare* were only isolated from rabbit faeces, *B. gallinarum* and *B. pullorum* were only found in the intestine of chicken, and *B. merycicum* and *B. ruminantium* in cattle rumen. In general, *Bifidobacterium* species are specific either for humans or for animals, with exception of the intestinal microflora of suckling calves and breast-fed infants in which the same *Bifidobacterium* species have been found.

Three *Bifidobacterium* species, namely *B. asteroides*, *B. coryneforme* and *B. indicum*, have been isolated from the hindgut of the honeybee, all of which possess a subtle host dependency. However, the significance of bifidobacteria in the honeybee gut is at present unknown.

Next to human and animal sources, 12 *Bifidobacterium* species have also been isolated from sewage; six are from humans and four from animals, in which case faecal contamination may have been the cause. However, two species, namely *B. subtile* and *B. minimum* have not been found elsewhere, which raises the question of the possible development of bifidobacteria in extra-enteral ecological niches. Similarly, *B. animalis* subsp. *lactis*, formerly classified as *B. lactis*, was originally isolated from fermented milk but was most likely a contamination from another source. *B. thermacidophilum* is one of the more recently described species (Dong *et al*., 2000a) and has been isolated from an anaerobic digester used to treat wastewater from a bean-curd farm.

Table 6. Validly described *Bifidobacterium* (sub)species and their source of isolation (based on Sgorbati *et al*., 1995; The prokaryotes (http://141.150.157.117:8080/prokPUB/index.htm) and the proposal manuscripts)

Species	Source of isolation	Reference(s)
B. adolescentis	Human adult faeces; bovine rumen; sewage	Reuter (1963)
B. angulatum	Human adult faeces; sewage	Scardovi and Crociani (1974)
B. animalis subsp. *animalis*	Faeces of rat	Mitsuoka (1969); Scardovi and Trovatelli (1974); Masco *et al*. (2004)
B. animalis subsp. *lactis*	Fermented milk samples; faeces of human adult and infant, rabbit and chicken; sewage	Meile *et al*. (1997); Masco *et al*. (2004)
B. asteroides	Intestine of honey bee *Apis mellifera* (subsp. *mellifera, ligustica and caucasica*)	Scardovi and Trovatelli (1969)
B. bifidum	Faeces of human adult, infant and suckling calf; human vagina	Tissier (1900); Orla-Jensen (1924)
B. boum	Bovine rumen; faeces of piglet	Scardovi *et al*. (1979)
B. breve	Faeces of infant and suckling calf; human vagina; sewage	Reuter (1963)
B. catenulatum	Faeces of human adult and infant; human vagina; sewage	Scardovi and Crociani (1974)
B. choerinum	Faeces of piglet; sewage	Scardovi *et al.* (1979)
B. coryneforme	Intestine of honey bee *Apis mellifera* subsp. *mellifera* and subsp. *caucasica*	Scardovi and Trovatelli (1969); Biavati *et al*. (1982)
B. cuniculi	Faeces of rabbit	Scardovi *et al.* (1979)
B. dentium	Human dental caries and oral cavity; faeces of human adult; human vagina; human clinical samples	Scardovi and Crociani (1974)
B. gallicum	Human faeces	Lauer (1990)
B. gallinarum	Chicken caecum	Watabe *et al*. (1983)
B. indicum	Intestine of honey bee *Apis indica*	Scardovi and Trovatelli (1969)
B. longum biotype infantis	Faeces of infant and suckling calf; human vagina	Reuter (1963); Sakata *et al.* (2002)
B. longum biotype longum	Faeces of human adult, infant and suckling calf; human vagina; sewage	Reuter (1963); Sakata *et al.* (2002)
B. longum biotype suis	Faeces of piglet	Matteuzi *et al.* (1971); Sakata *et al*. (2002)
B. magnum	Faeces of rabbit	Scardovi and Zani (1974)
B. merycicum	Bovine rumen	Biavati and Mattarelli (1991)
B. minimum	Sewage	Biavati *et al*. (1982)
B. pseudocatenulatum	Faeces of human adult, infant and suckling calf; sewage	Scardovi *et al*. (1979)
B. pseudolongum subsp. *globosum*	Faeces of piglet, suckling calf, rat, rabbit and lamb; sewage; bovine rumen and in a single specimen of human infant faeces	Biavati *et al*. (1982); Yaeshima *et al*. (1992)
B. pseudolongum subsp. *pseudolongum*	Faeces of pig, chicken, dog, bull, calf, rat and guinea pig	Mitsuoka (1969); Yaeshima *et al*. (1992)
B. psychraerophilum	Pig caecum	Simpson *et al*. (2004)
B. pullorum	Faeces of chicken	Trovatelli *et al*. (1974)
B. ruminantium	Rumen of cattle	Biavati and Mattarelli (1991)
B. saeculare	Faeces of rabbit	Biavati *et al.* (1991)
B. scardovii	Human clinical sources	Hoyles *et al*. (2002)
B. subtile	Sewage	Biavati *et al*. (1982)
B. thermacidophilum subsp. *porcinum*	Faeces of piglet	Zhu *et al*. (2003)
B. thermacidophilum subsp. *thermacidophilum*	Waste water of a bean-curd farm in Beijing	Dong *et al*. (2000); Zhu *et al*. (2003)
B. thermophilum	Faeces of pig, chicken and suckling calf; bovine rumen; sewage	Mitsuoka (1969)

2.3. Clinical relevance

In general, bifidobacteria are not clinically relevant. They are rarely associated with infections (Hata *et al*., 1988; Nakazawa *et al*., 1996; Ha *et al*., 1999), which most likely have an opportunistic nature and are caused by bifidobacteria from the patient's own microbiota (Wang et al., 1996; MacFie et al., 1999) or by contact with contaminated material (Ha *et al*., 1999). Only *B. dentium*, isolated from dental caries and plaques, appears to have pathogenic potential in cariogenic processes. However, the role played by bifidobacteria in this pathology is still unclear. Furthermore, the isolation of *B. scardovii* from human sterile sites, including blood, urine and a female hip (Hoyles *et al*., 2002), may imply that this species has clinical relevance. However, since its description, no additional evidence for this assumption has been reported.

2.4. Phylogeny and taxonomic composition of the genus *Bifidobacterium*

Based on their high DNA G+C content (55-67 mol%) and 16S rDNA sequence data, bifidobacteria constitute a phylogenetically coherent unit within the family Bifidobacteriaceae, exhibiting over 93% 16S rDNA sequence similarity, as part of the *Actinobacteria* branch of Gram-positive bacteria. Although generally considered as lactic acid bacteria (LAB) based on a number of common metabolic features and because of their widespread use in the food industry, typical LAB (e.g. *Lactobacillus*, *Lactococcus* and *Pediococcus*) are characterised by a DNA G+C content of less than 50 mol% and belong to the *Clostridium* branch of the Gram-positive bacteria. Phylogenetically, these typical LAB genera are thus only distantly related to members of the genus *Bifidobacterium* (**Figure 3**). Within the phylogenetic family of Bifidobacteriaceae, there also reside the species *Parascardovia denticolens*, *Scardovia inopinata*, *Aeriscardovia aeriphila* and *Gardnerella vaginalis*, which are also known to exhibit F6PPK activity. Although it is reported that the G+C content of *G. vaginalis* (i.e. 42 mol%) is significantly lower than that of *Bifidobacterium*, it is difficult to differentiate *Gardnerella* from *Bifidobacterium* based on 16 rRNA gene

sequences. In contrast, when performing phylogenetic analysis based on partial gene sequences of the 60 kDa heat-shock protein (HSP60), *G. vaginalis* constitutes a well-separated branch within the HSP60 tree (Jian *et al*., 2001). At present, the phylogenetic position of *G. vaginalis* is still under discussion and awaits further studies.

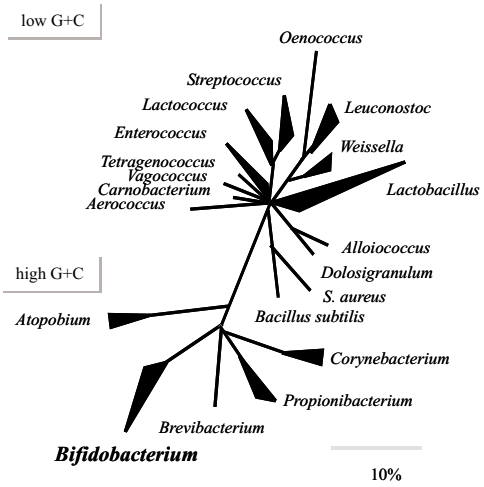

Figure 3. Phylogenetic tree of Gram-positive bacteria (Schleifer and Ludwig, 1995)

Although 16S rRNA gene sequence similarity analysis is demonstrated to be a valuable tool in bacterial phylogeny (Stackebrandt and Goebel, 1994), its value to discriminate species within the genus *Bifidobacterium* is fairly limited. Several closely related species groups are known within which no differentiation is possible even when based on the complete sequence of the 16S rRNA gene. These groups include the *B. catenulatum*/*B. pseudocatenulatum* group (similarity 99.5%), the *B. indicum*/*B. coryneforme* group (similarity 99.1%), the *B. longum*/*B. infantis*/*B. suis* group (similarity 99.1-99.2%), the *B. gallinarum*/*B. pullorum*/*B. saeculare* group (similarity 99.3-99.9%) and the *B. animalis*/*B. lactis* group (similarity 98.9%) (Miyake *et al*., 1998) (**Figure 4**). In recent years, several protein-encoding genes have been proposed as alternative phylogenetic markers for the 16S rRNA gene in *Bifidobacterium*, such as *rec*A (Kullen *et al*., 1997), the HSP60 gene (Jian *et al*., 2001) and *tuf* gene (Ventura *et al*., 2003a) (see § 2.5.2.1., Table 7). All these genes encode housekeeping

functions and are common for all bifidobacteria and thus fulfil the prerequisites for suitable phylogenetic markers. Although some discrepancies have been reported, the use of one or more protein-encoding gene sequences in bifidobacterial phylogeny is considered to be a valuable alternative or complementary approach to the 16S rRNA gene sequencing to unravel the phylogenetic relationships in *Bifidobacterium*. In addition, some of these markers display higher divergence rates and consequently have more discriminatory power than 16 rDNA to differentiate closely related *Bifidobacterium* species (see § 2.5.2.1.).

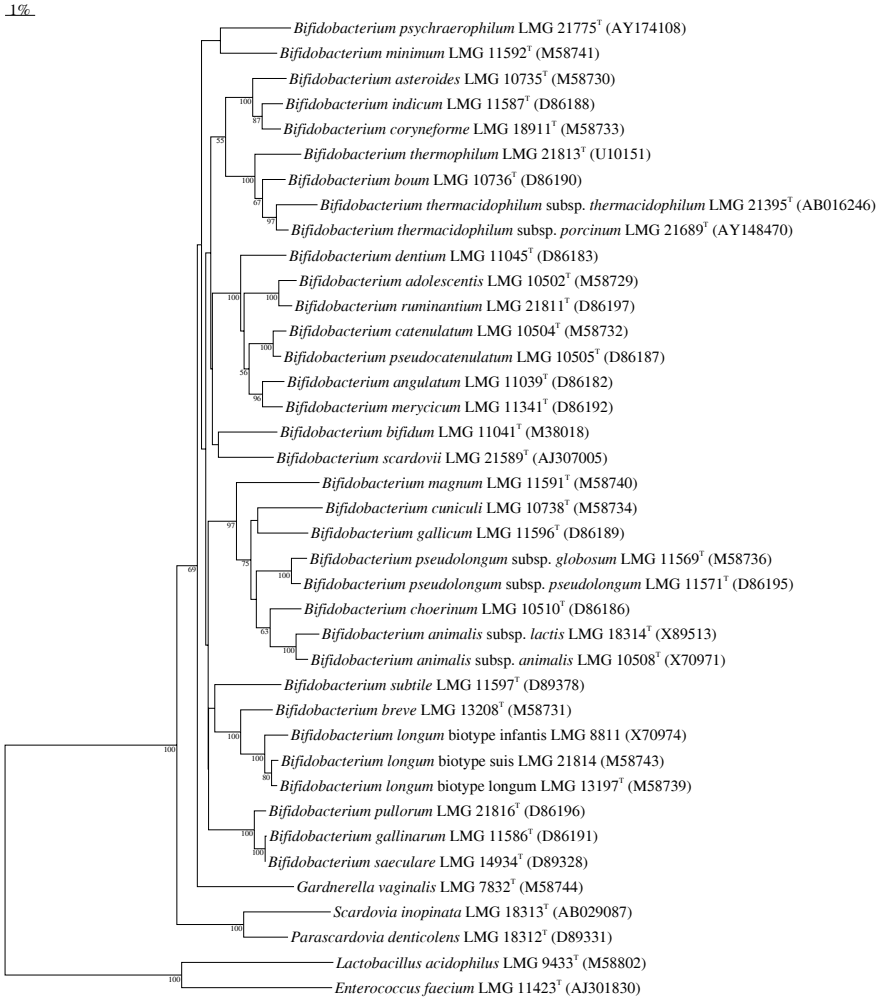

Figure 4. Tree showing the phylogenetic relationship of members of the genus Bifidobacterium and some related species. The tree was constructed using the neighbour-joining method. Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points.

Based on polyphasic taxonomic studies, the classification and nomenclature of the *B. longum*/*B. infantis*/*B. suis* group (Sakata *et al*., 2002) and the *B. animalis*/*B. lactis* group (Masco *et al*., 2004) have been recently updated. The species *B. longum* and *B. infantis* are known to belong to one single DNA hybridisation group together with *B. suis* (Lauer and Kandler, 1983). Substantiated by new taxonomic evidence from carbohydrate fermentation patterning, ribotyping, RAPD-PCR and additional DNA-DNA hybridisation experiments, Sakata and co-workers (2002) proposed to unify *B. longum*, *B. infantis* and *B. suis* into the single species *B. longum* in which three biotypes are delineated, i.e. biotypes longum, infantis and suis, respectively. A similar situation of taxonomic confusion has existed for many years, concerning the taxonomic affiliation between the probiotic species *B. lactis* (Meile *et al*., 1997) and *B. animalis* (Scardovi and Trovatelli, 1974). Many probiotic strains in *Bifidobacterium* have been taxonomically labelled as *B. lactis* although there were several indications that the majority of them actually belonged to the closely related species *B. animalis*. Based on phenotypic characteristics, 16S rRNA gene sequencing, and DNA-DNA hybridisation, Cai and co-workers (2000) proposed to consider *B. lactis* as a junior synonym of *B. animalis*. In contrast, other workers (Ventura and Zink, 2002, Ventura and Zink, 2003; Zhu *et al*., 2003) suggested on the basis of molecular evidence that these two taxa should remain two separate taxonomic entities not at the species level but at the subspecies level. Further work to substantiate the latter proposal was performed in the framework of this Ph.D. study and is reported in Chapter 3.2. of this thesis.

2.5. Identification, typing, detection and quantification of bifidobacteria

At present, members of the genus *Bifidobacterium* are assigned to 32 validly described (sub)species. Although the identification of most of these taxa is relatively straightforward, the taxonomic recognition of closely related species remains problematic (see § 2.4.). The development and evaluation of methods to speciate and type bifidobacteria has mainly focussed on those species that are predominating the human gastro-intestinal tract (GIT). Special attention has also been concentrated on those species that have been claimed

to possess health-promoting properties and of which some have been incorporated in commercial probiotic products. Clearly, the correct identification of bifidobacterial strains that have already been extensively characterized with respect to their probiotic potential is of paramount importance in the functional food industry. As a consequence, a broad range of techniques has been evaluated for the identification of bifidobacteria for human consumption, all displaying differences in discriminatory power, reproducibility and workload.
This part of the chapter provides an overview of the most frequently used culture-dependent and culture-independent methods for the identification, typing, detection and/or quantification of bifidobacteria. It should be noted that the methods discussed below are categorized according to their most straightforward application although several of these techniques can serve multiple purposes. A table summarizing the most important techniques and their possible applications is given at the end of this chapter (**Table 8**).

Note: Despite recently described taxonomic rearrangements (see § 2.4.), the original species names *B. longum*, *B. infantis* and *B. suis*, now classified as *B. longum* biotype longum, biotype infantis and biotype suis, respectively, as well as *B. animalis* and *B. lactis*, now classified as *B. animalis* subsp. *animalis* and subsp. *lactis*, respectively, are still used in the discussion below according to their original citation. Unless specified otherwise, the term 'identification' refers to the classification of isolates at the genus and/or (sub)species level while 'typing' refers to the differentiation of isolates at the strain level .

2.5.1. Culture-dependent methods

Despite their limited taxonomic resolution and high labour intensity, methods based on phenotypic characteristics are still frequently used to identify bifidobacteria. In addition, phenotypic characterization is still indispensable to screen bifidobacterial isolates for interesting probiotic and technological indicators such as inhibitory capacity, resistance to bile and low pH, etc. Nevertheless, the past two decades have witnessed the development of a large series of DNA-based identification and detection methods. Undoubtedly, one of the main advantages of these methods is their independence of variation in growth conditions of the

microorganisms. However, also genotypic characterization techniques are not without limitation (cost, equipment, databases). Therefore, in order to obtain a robust classification and differentiation, a polyphasic or combined approach is usually recommended.

2.5.1.1. Phenotypic methods

In most laboratories involved in industrial and applied microbiology, phenotypic tests are still the principle tools for the identification of (food-associated) bacteria. The most direct and reliable assignment of a bacterial strain to the genus *Bifidobacterium* is based on the presence of F6PPK, the key enzyme of bifidobacterial hexose metabolism. However, this approach does not allow identification at the species level. One of the very first identification schemes developed for *Bifidobacterium* species was based on a simple carbohydrate fermentation pattern (Mitsuoka, 1969). This method is still in use, but data obtained from fermentation tests cannot be considered conclusive. Next to carbohydrate fermentation patterning, identification of bifidobacterial isolates has also been based on cell wall analysis. Extensive studies of peptidoglycan types in bifidobacterial cell walls conducted by Kandler and Lauer (1974) and Lauer and Kandler (1983) revealed a considerable variety of peptidoglycan types within the genus *Bifidobacterium* and was therefore proposed as a taxonomic marker. In general, the use of biochemical and physiological tests for identification of bifidobacterial species is limited because of relative poor reproducibility and a low taxonomic resolution. In this respect, chemotaxonomic methods analysing one specific cellular compound have proven to be much more powerful. Sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins, i.e. protein profiling, has been successfully applied for the discrimination of *B. adolescentis*, *B. bifidum*, *B. breve*, *B. dentium* and *B. longum* isolated from adult faeces, and for the identification of *Bifidobacterium* isolates (mainly *B. lactis*) from European probiotic products (Reuter *et al*., 2002; Temmerman *et al*., 2003a). Furthermore, protein profiling has shown good correspondence with the classification of bifidobacteria based on DNA-DNA hybridisations (Biavati *et al*., 1982) as well as with results obtained from 16S rRNA gene sequence

analysis (Kim *et al*., 2005). Within species, strains that have 80% or greater DNA homology have identical or nearly identical protein profiles, with *B. animalis* being the only exception; within this species, protein patterns of strains isolated from rats appear to differ from those of strains isolated from sewage, rabbits and chickens (Biavati *et al*., 1982), which is in accordance with the recent separation of these strains at the subspecies level (Masco *et al*., 2004). Provided that highly standardized conditions of cultivation and electrophoresis are used throughout the procedure, computer-assisted numerical comparisons of protein patterns is possible, and a database can be created for identification purposes. This allows large numbers of strains to be compared and grouped in clusters of closely related homology. Among the most recently developed physicochemical identification methods, Fourier transform infrared (FT-IR) spectroscopy has been used for the speciation of bifidobacterial isolates from foods and human faeces (Mayer *et al*., 2003). However, differentiation between *B. infantis* and *B. longum* and between *B. animalis* and *B. lactis* remained difficult.

2.5.1.2. Genotypic methods

In contrast to phenotypic methods, genotypic identification is based on primary information obtained from the genome or from specific genes rather than on the products of their expression. DNA-DNA hybridisation, ribotyping, southern hybridisation and sequence analysis of the 16S rRNA gene are amongst the first genotypic methods used to identify bifidobacteria isolated from commercial products and gastro-intestinal tract (GIT) samples. Later, a new generation of genotypic methods emerged that allowed the identification of bifidobacteria up to the genus, species and strain level.

Molecular studies were initiated by Scardovi and co-workers (1971). Methodologies of **DNA-DNA hybridisation** and the value of mol% G+C reconfirmed species that had initially been described only on the basis of phenotypic characteristics. Although DNA-DNA hybridisation is regarded as the 'golden standard' for the description of *Bifidobacterium* species, it cannot be routinely carried out in most laboratories. A few studies have used

(microscale) DNA-DNA hybridisations to assess the taxonomic identity of bifidobacteria, usually in combination with other techniques. Herreman and colleagues (1994) verified the identity of three industrial starter strains of *Bifidobacterium* by DNA-DNA hybridisation with type strains of commercially used *Bifidobacterium* species. In another study (Yaeshima *et al*., 1996), a collection of *Bifidobacterium* strains isolated from dairy products such as yoghurt, cultured milk, butter and cheese were characterized on the basis of phenotypic characteristics followed by determination of DNA similarities with a microplate hybridisation method. The same approach was used by Gavini and Beerens (1999) in order to identify 47 strains of bifidobacteria previously isolated from meat and meat products.

Many genotypic methods are based on the principle of **Polymerase Chain Reaction (PCR)**, which enables the selective amplification of specifically targeted DNA fragments through the use of oligonucleotide primers. Detailed analysis of the 16S rRNA region as well as the 16S-23S internally transcribed spacer (ITS) region has revealed several nucleotide signatures with specificity at different taxonomic levels. This has been employed to generate oligonucleotide primers as well as nucleic acid probes for detection and identification of bifidobacteria to the genus, species or strain level. Although the majority of these primers were evaluated for their ability to identify pure cultures, they can also be used in the culture-independent detection of bifidobacteria in food and faecal samples (see § 2.5.2). Genus-specific primers targeting sequences of the **16S rRNA gene** have been elaborated for bifidobacteria (Kaufmann *et al*., 1997), which enables their detection within the complex microflora of the human gut as well as in a food matrix. Recently, an additional set of *Bifidobacterium* genus-specific primers has been designed that do not cross react with *Propionibacterium*, but then again these primers produced amplicons with *Gardnerella vaginalis* (Matsuki *et al*., 2002). Beyond genus level, 16S rRNA based species- and group-specific primers have been designed for species commonly found in the human gastrointestinal tract, i.e. *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. dentium*, *B. gallicum*, the *B. catenulatum* group (*B. catenulatum* and *B. pseudocatenulatum*) and the *B. longum* group (*B. longum/B. infantis*) (Matsuki *et al*., 1998; Matsuki *et al*., 1999; Dong *et al*., 2000b). One limitation of species-specific primers is that in large-scale studies, the analysis of the species composition requires multiple PCR rounds with different sets of primers. To avoid this inconvenience, **multiplex PCR**

strategies have been developed. By combining species-specific PCR primers that target different sites of the 16S rRNA gene with a genus-specific reversed primer (Kaufmann *et al*., 1997), *B. bifidum*, *B. adolescentis*, *B. infantis*, *B. longum* and *B. breve* can be simultaneously identified (Dong *et al*., 2000b). Mullié and co-workers (2003) focussed on the simultaneous detection of *B. bifidum*, *B. breve* and *B. infantis*, *B. angulatum*, *B. catenulatum/B. pseudocatenulatum* continuum, *B. dentium* and *B. longum*, and *B. adolescentis*, *B. scardovii* and *B. gallicum*. However, cross-reaction of *B. suis* with both *B. infantis* and *B. longum* primers as well as amplification of *B. catenulatum* with *B. angulatum* primers was witnessed under multiplex conditions. Although most PCR primers based on bifidobacterial 16S rRNA genes are developed mainly for the identification and detection of specific *Bifidobacterium* taxa, some workers have also attempted to design primers that target individual strains. For instance, Kok and colleagues (1996) reported the use of three strain-specific 16 rRNA gene-targeted primers for the detection of the probiotic *Bifidobacterium* strain LW420 in infant faeces and for rapid quality control of this strain in culture.

Next to its value in studies on bifidobacterial phylogeny, the **16S-23S internally transcribed spacer (ITS) sequence** has also proven useful for the determination of intraspecific relationships (LeblondBourget *et al*., 1996). Based on ITS sequences, strain-specific primers were designed to trace *Bifidobacterium* strains, incorporated in a pharmaceutical probiotic product (VSL-3), in faecal specimens of patients that were taking VSL-3 (Brigidi *et al*., 2000). The 16S-23S spacer sequences can also be used for the differentiation of closely related bifidobacterial taxa. As such, ITS sequences of the closely related taxa *B. animalis* and *B. lactis* allowed a clear separation of these taxa in two distinct clusters (Ventura and Zink, 2002). Notably, two large insertions in the ITS sequence were identified in *B. animalis* but not in *B. lactis*, which provided a suitable PCR target for reliable separation of these taxa.

The limited resolution of bifidobacterial 16S rRNA gene sequences to distinguish between certain pairs of taxa, e.g. *B. animalis* and *B. lactis*, has initiated the search for **alternative universal taxonomic markers** with higher divergence rates and thus a more pronounced discriminatory power (**Table 7**).

Table 7. Alternative molecular markers for 16S rDNA in *Bifidobacterium*

Molecular marker	Reference
16S-23S Internally Transcribed Spacer (ITS) sequence	LeblondBourget *et al* ., 1996
transaldolase gene encoding transaldolase	Requena *et al* ., 2002
ldh encoding L-lactate dehydrogenase	Roy and Sirois, 2000
rec A encoding a protein for DNA strand exchange and renaturation	Kullen *et al* ., 1997
tuf encoding elongation factor Tu	Ventura *et al* ., 2003
grp E encoding GrpE chaperone	Ventura *et al* ., 2005
dna K encoding DnaK chaperone	Ventura *et al* ., 2005
atp D encoding the β-subunit of F_1F_0-ATPase	Ventura *et al.* , 2004a
gro EL encoding GroEL chaperone	Ventura *et al* ., 2004b
pyruvate kinase gene encoding pyruvate kinase	Vaugien *et al* ., 2002
HSP60 gene encoding 60kDa Heat Shock Protein	Jian *et al* ., 2001
xfp encoding xylulose-5-phosphate/fructose-6-phosphate phosphoketolase	Berthoud *et al* ., 2005

Genes encoding transaldolases were shown to be a suitable target for bifidobacterial detection and differentiation (Requena *et al*., 2002). PCR amplification of a 301 bp transaldolase gene sequence and subsequent comparison of the relative migration of the resulting amplicons in Denaturing Gradient Gel Electrophoresis (DGGE) allowed the differentiation of all human *Bifidobacterium* species tested, except for *B. catenulatum* and *B. angulatum* of which transaldolase amplicons co-migrated in the DGGE gel. Sequence analysis of the conserved *ldh* gene encoding the L-lactate dehydrogenase makes it possible to distinguish between *B. infantis* and *B. longum* (Roy and Sirois, 2000) but not between *B. animalis* and *B. lactis*. Kullen and co-workers (1997) described a PCR-based method targeting a 300 bp fragment of the *rec*A gene for the identification of six intestinal *Bifidobacterium* species. For the separation of *B. longum* and *B. infantis*, the *rec*A-based method allowed a more reliable differentiation (96.9% sequence similarity) compared to assays based on the 16S rRNA gene (>98.5% sequence similarity). Later, Ventura and Zink (2003) demonstrated that the *rec*A gene sequence also allows to disciminate between *B. animalis* and *B. lactis*. Other genes that have proven to be promising taxonomic markers for investigating evolutionary distances and

to discriminate between closely related bifidobacteria include the gene encoding for the elongation factor Tu (Ventura *et al*, 2003; Ventura and Zink, 2003), the *dna*K and *grp*E genes (Ventura *et al*., 2005), the *atp*D (Ventura *et al*., 2004a), *gro*EL (Ventura *et al*., 2004b) and pyruvate kinase gene (Vaugien *et al*., 2002). Also, the analysis of partial HSP60 gene sequences has proven to be very useful for the differentiation of *Bifidobacterium* species (Jian *et al*., 2001; Zhu *et al*., 2003). The sequence similarities of the HSP60 gene have been determined at various taxonomic levels: 99.4-100% at the intraspecific level, 96% at the subspecific level, and 73-96% (mean 85%) at the interspecific level. Sequence similarities ranged from 91-93% between *B. catenulatum* and *B. pseudocatenulatum*, from 98-100% between *B. longum*, *B. infantis* and *B. suis* and from 98.9-100% between *B. animalis* and *B. lactis.* In contrast, the 16S rRNA sequence similarities of all these species are above 98,5%. In a recent study conducted by Berthoud and co-workers (2005), a new identification method for *Bifidobacterium* species based on partial sequencing of the xylulose-5-phosphate/ fructose-6-phosphate phosphoketolase gene (*xfp*) was evaluated. Using sequences of approximately 500 bp from 68 different strains including 34 type strains, all bifidobacterial species could be discriminated with an accuracy higher than that of 16S rDNA sequence analysis. Interestingly, *xfp* sequence analysis even allowed to distinguish *B. longum* biotype infantis from *B. longum* biotype longum and *B. longum* biotype suis, as well as *B. animalis* from *B. lactis*.

In addition to taxon-specific PCR primers, also short **oligonucleotide probes** have been developed that are directed to rRNA regions known to be genus- (Langendijk *et al*., 1995; Kaufmann *et al*., 1997) or species-specific (Yamamoto *et al*., 1992; Mangin *et al*., 1995). These probes can be used to screen presumptive bifidobacterial colonies for the presence of a specific DNA sequence using a labeled probe (see § 2.5.2).

Triggered by the growing insights in the taxonomic divergence of ribosomal RNA gene sequences in *Bifidobacterium*, **ribotyping** was one of the first DNA fingerprinting techniques to be used for the speciation of bifidobacteria. Starting from conventional **restriction fragment length polymorphism (RFLP)** analysis of genomic DNA, a subset of the restriction fragments are selected and visualized after electrophoresis by southern hybridisation with an

rDNA probe in order to obtain a less complex pattern that is easier to interpret. To a large extend, the discriminatory power of ribotyping depends on the size and specificity of the probe as well as on the restriction enzyme used. By using probes derived from 16S or 23S rDNA sequences, this method has been applied for the intra- and interspecies differentiation of industrial and culture collection strains as well as of human faecal isolates of *Bifidobacterium* (Mangin *et al*., 1994, 1996, 1999; McCartney and Tannock, 1995; Mättö *et al*., 2004). In corroboration with Randomly Amplified Polymorphic DNA (RAPD), this approach also allowed the recognition of three biotypes (i.e. longum, infantis and suis) in the species *B. longum* (Sakata *et al*., 2002).

The concept of another ribosomal fingerprinting technique, i.e. **Amplified Ribosomal DNA Restriction Analysis (ARDRA)** essentially relies on the restriction enzyme analysis of 16S rDNA PCR amplicons. Although less discriminatory than ribotyping, ARDRA has shown significant potential to identify *Bifidobacterium* strains at the species level. A first study by Roy and Sirois (2000) demonstrated the differentiation of *B. breve*, *B. bifidum* and *B. adolescentis* and confirmed the close relatedness between *B. longum* and *B. infantis*. However, the restriction patterns of *B. lactis* and *B. animalis* were identical. Later, the ARDRA identification scheme described by Ventura and co-workers (2001) allowed the species-specific detection of a number of ecologically diverse species including *B. catenulatum* and *B. pseudocatenulatum*. However, the closely related taxa *B. animalis* and *B. lactis* and *B. longum* and *B. suis* could not be distinguished with this method. A study by Venema and Maathuis (2003) described the use of ARDRA to differentiate between all *Bifidobacterium* species found in the human alimentary tract as well as *B. animalis* and *B. lactis*. Although this technique is labour-intensive and time-consuming, it is generally regarded as a robust and reproducible molecular identification tool for human *Bifidobacterium* species. Provided that more than one restriction profile is analysed and compared, this method allows the differentiation of *B. longum* and *B. infantis*, *B. catenulatum* and *B. pseudocatenulatum*, and *B. animalis* and *B. lactis*.

Of all conventional fingerprinting techniques, **Pulsed Field Gel Electrophoresis (PFGE)** is generally considered to afford the greatest differentiation, primarily because the PFGE profile generated represents the whole genome. PFGE employs an alternating field of electrophoresis to allow separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes. A number of studies have demonstrated the usefulness of PFGE to monitor changes in the predominant bifidobacterial populations in humans, both within individuals over time and between individuals (McCartney *et al*., 1996; Kimura *et al*., 1997). Other workers have shown the ability of PFGE to characterize and differentiate commercial and faecal *Bifidobacterium* strains (Roy *et al*., 1996; Engel *et al*., 2003; Mättö *et al.*, 2004). Although PFGE is generally not considered for identification purposes, Grand and co-workers (2003) used this method to verify the identity of bifidobacterial isolates from probiotic milk products by comparison of their macro-restriction profiles with those of reference strains from producer companies.

DNA fingerprinting techniques that solely rely on PCR include **Randomly Amplified Polymorphic DNA (RAPD)** and **repetitive sequence based (rep)-PCR.** Both techniques encompass the whole genome and thus exhibit a higher discriminatory power than techniques based on highly conserved rRNA genes. RAPD analysis makes use of short arbitrary primers and low-stringency conditions to randomly amplify DNA fragments, which are then separated electrophoretically to produce a fingerprint. The flexibility in primer choice and PCR conditions allows its application for the differentiation of bifidobacteria at different taxonomic levels, although it also makes this technique prone to poor reproducibility. RAPD analysis has been used for differentiation (Vincent *et al*., 1998; Fanedl *et al*., 1998; Sakata *et al*., 2002; Mättö *et al*., 2004) and monitoring purposes (Fujiwara *et al*., 2001; Alander *et al*., 2001). Superior to the reproducibility of RAPD, fingerprinting methods based on the PCR amplification of repetitive elements (rep-PCR) targeting ERIC (enterobacterial repetitive intergenic consensus) (Shuhaimi *et al*., 2001; Ventura *et al*., 2003b) or BOX (Zavaglia *et al*., 2000) elements are reported to be suitable for both the speciation and the intra-specific differentiation (subtyping) of bifidobacteria.

2.5.2. Culture-independent methods

Traditionally, analysis of bifidobacterial communities (e.g. faeces or food) has been achieved by combining a conventional isolation strategy with culture-dependent identification. However, besides being time-consuming, this approach has been impaired by the lack of suitable media for the selective isolation of bifidobacteria (Roy, 2001). In addition, results may also be biased by a poor viability or low concentration present of the target organism, resulting in an inaccurate reflection of the bifidobacterial composition of the sample. As a result, culture-independent techniques have been promoted as alternative and/or complementary approaches to study the microbial ecology of the GIT and to trace bifidobacterial strains in probiotic products and environmental samples.

The fastest culture-independent approach for the genus, species or strain specific analysis of bifidobacterial populations is based on the use of specific primers (§ 2.5.1.2.) for the PCR-based detection of bifidobacteria in bacterial community DNA extracted from a sample. However, one of the major drawbacks of this approach is the prerequisite for prior knowledge of the bacterial content of the sample, making such PCR assays of limited value in the analysis of highly complex ecosystems or samples showing variable or unknown species composition. In these cases, community fingerprinting techniques, such as DGGE and T-RFLP provide worthy alternatives. **Denaturing Gradient Gel Electrophoresis (DGGE)** is a PCR-based technique that allows the sequence-dependent separation of a mixture of amplified DNA fragments, all identical in size, on an acrylamide gel containing a well-defined gradient of denaturing components. By combining genus- or group-specific PCR with DGGE, a fingerprint can be obtained from the bifidobacterial community, which allows to monitor its taxonomic complexity as well as its temporal and spatial changes. Furthermore, individual members of the community can be identified either by cloning and sequencing of the PCR fragments or by comparing the obtained DGGE band positions with an identification database. The nested-PCR DGGE methodology developed by Temmerman and colleagues (2003b) allowed the reliable taxonomic characterization of 32 (sub)species of *Bifidobacterium*, including *B. longum, B. infantis* and *B. suis*, and representatives of *B. animalis* and *B.*

lactis. Only *B. indicum* and *B. coryneforme* could not be distinguished. Several applications of DGGE analysis of 16S rDNA (Satokari *et al*., 2001a and b; Fasoli *et al*., 2003; Favier *et al*., 2003; Temmerman *et al*., 2003b; Burton *et al*., 2003) or transaldolase gene (Requena *et al*., 2002) amplicons have been reported for the identification, detection and monitoring of *Bifidobacterium* species prominent in probiotic products and human faecal and vaginal flora. Another community fingerprinting method, i.e. **terminal restriction fragment length polymorphism (T-RFLP)**, has been used to assess the diversity of the human faecal bifidobacteria and rapid comparison of the bifidobacterial community structure among human individuals (Sakamoto *et al*., 2003; Hayashi *et al*., 2004; Sakata *et al*., 2005).

Although highly valuable for qualitative detection purposes, community fingerprinting methods generally do not yield quantitative information and need to be combined with culture-based techniques to obtain total bifidobacterial counts. **Real-time PCR** (or quantitative PCR) enables the simultaneous detection and quantification of microorganisms by measuring the relative amount of amplicon generated throughout the PCR reaction using a combination of specific primers and intercalating dyes or specific fluorescently labeled probes. Several real-time PCR approaches targeting either the 16S rRNA gene (Vitali *et al*., 2003; Matsuki *et al*., 2004; Penders *et al*., 2005; Bartosch *et al*., 2005), 16S-23S ITS sequence (Haarman and Knol, 2005) or transaldolase gene (Requena *et al*., 2002) have been used for the detection and enumeration of bifidobacteria in faecal samples as well as for strain-specific detection in probiotic products. The application of a real-time PCR based method for the qualitative analysis of bifidobacterial populations implies that each species requires a separate probe or primer set, which may result in an enormous increase of cost and workload. For such purposes, the combined use of real-time PCR and DGGE analysis can provide quantitative as well as qualitative data (Requena *et al*., 2002).

Unlike conventional PCR primers, **hybridisation probes** are linked to a radioactive or fluorescent label enabling the visual detection of the target after hybridisation under controlled conditions. Labelled oligonucleotide probes can be employed in a variety of assays including colony, dot blot and *in situ* hybridisations. **Colony hybridisation** involves probing of bacterial

colonies that have been transferred onto membranes (e.g. nitrocellulose membranes). This technique has proven successful in tracking down bifidobacterial indicators of human faecal pollution (Lynch *et al*., 2002; Nebra *et al*., 2003), in detecting specific probiotic *Bifidobacterium* strains (Su *et al*., 2005) and in analysing human faecal samples (Kaufmann *et al*., 1997; Kaneko and Kurihara, 1997). **Dot blot** assays involve probing DNA extracts from samples and have been used to study the binding of *Bifidobacterium* strains to amylomaize starch granules (O'Riordan *et al*., 2001).

The most frequently applied method that makes use of oligonucleotide probes is **Fluorescent In Situ Hybridisation (FISH),** which enables the direct enumeration of whole bacterial cells in samples using either fluorescence microscopy or flow cytometry. Probes targeting 16S ribosomal RNA sequences specific for *Bifidobacterium* have been applied for microscopic analysis of bifidobacteria in human faecal samples (Langendijk *et al*., 1995; Welling *et al*., 1997). Recently, Takada and colleagues (2004) used a multi-color FISH method to detect seven *Bifidobacterium* species predominant in human faeces in one single assay. Flow cytometry is a rapid and sensitive technique that can determine cell numbers and measure various physiological characteristics of each individual cell using appropriate fluorescent dyes (e.g. SYTO9 and propidium iodide for LIVE/DEAD analysis) (Bunthof and Abee, 2002). Flow cytometry has been applied to evaluate the effect of bile salt on the viability of bifidobacteria (Ben Amor *et al*., 2002) as well as for the analysis of the composition of microbial communities, including bifidobacteria, in human faeces (Rigottier-Gois *et al*., 2003). One of the most recent technological developments is the integration of a multiple oligonucleotide probe approach in an ELISA-based system. This approach has been used to monitor the fluctuation of nine bifidobacterial species in faecal samples during and after a human feeding trial (Malinen *et al*., 2002).

Miniaturization of DNA hybridisation techniques has led to the development of DNA chips or **DNA microarrays**. With these tools, an array of probes can be immobilized on a small glass slide in such a way that one sample can be tested simultaneously against a large number of probes. This miniaturized approach overcomes one of the major limitations associated with probe-based methods, namely the restricted number of bacteria that can be targeted in a single analysis. Recently, DNA microarrays containing 20 16S rDNA probes of the predominant

intestinal bacterial species, including bifidobacteria, have been developed to monitor the populations of anaerobic bacteria in human faecal samples (Wang *et al*., 2002). Although probe-based techniques are reliable and relatively easy to use, probe design and detection limit remain the major bottlenecks in the construction of a microarray.

Table 8. Overview of the most frequently used techniques for the identification, typing, detection and/or quantification of bifidobacteria

	Identification	Phylogeny	Intraspecific differentiation	Typing	Detection	Quantification
Method						
Culture-dependent						
Phenotypic characterization	X					
SDS-PAGE	X		X			
DNA-DNA hybridisation	X	X				
16S rRNA gene:						
Sequence analysis	X	X	x			
Specific primers	X		X	X	X	
16S-23S ITS:						
Sequence analysis	X	X	X			
Specific primers	X		X	X	X	
Housekeeping genes:						
Sequence analysis	X	X	x			
Specific primers	X		X	X	X	
RFLP	X		X			
Ribotyping	X		X			
ARDRA	X		x			
PFGE				X		
RAPD	X		X			
rep-PCR	X		X			
Culture-independent						
DGGE	X		X		X	
Real-Time PCR					X	X
Hybridisation probes	X		X	X	X	
FISH					X	X

x: Applicable under certain circumstances

2.6. Genomics of bifidobacteria

Genomic work on bifidobacteria has only started relatively recent. The early work of Sgorbati and coworkers (1982) demonstrated the presence of plasmids in several members of the genus *Bifidobacterium* including *B. longum*, *B. pseudolongum* subsp. *globosum*, *B. asteroides* and *B. indicum*. The investigated strains of *B. infantis*, which is most closely related to *B. longum*, did not carry plasmids. Later studies also reported the presence of plasmids in *B. breve* (Iwata and Morishita, 1989; Bourget *et al*., 1993). Recently, the first genome sequence of a *Bifidobacterium* strain, i.e. *B. longum* NCC2705, has become publicly available (Schell *et al*., 2002). This strain has a genome of 2,256,646 bp with a G+C content of 60,1% and contains four nearly identical rrn operons. Complete pathways for the biosynthesis of the majority of amino acids, all nucleotides and some vitamins (folic acid, thiamine, and nicotinate) have been identified. Furthermore, sequence analysis revealed the capacity of *B. longum* NCC2705 to encode a rich arsenal of proteins (more than 8% of the total predicted proteins) that are probably involved in the catabolism of a variety of oligosaccharides. This finding might be reflected by the ability of this microorganism to grow and persist in the colon. Genome analysis not only enhances our understanding of bifidobacterial physiology, it also provides useful information in understanding the processes underlying speciation and evolution. By comparing genome sequences (i.e. comparative genomics), patterns of similarity or variability are obtained indicating physiological plasticity and various evolutionary processes. Unfortunately, to date, only the *B. longum* genome sequence is publicly available. However, in this respect, the DNA microarray technology allows a global comparative analysis of gene content between different bifidobacterial isolates of a given species without the necessity of sequencing many strains (i.e. genomotyping). Recently, a *B. longum* NCC2705-based DNA microarray has been developed to compare the genomes of a number of bifidobacterial strains in order to evaluate the genetic variability at intra-specific and inter-specific level (Rezzonico *et al*., 2003). However, genomic comparison with microarrays does not respect the syntheny of the bacterial genome. The genomic comparison of two genomes that have very similar gene contents but that are organized differently will not reveal genetic differences in microarray analysis. Nevertheless,

while awaiting additional bifidobacterial genome sequence data, microarrays provide a highly powerful, high-throughput means to characterize strains and are expected to complement other techniques in a polyphasic taxonomic approach.

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Part 3
Experimental work

SOURCE: http://www.hygiene.ruhr-uni-bochum.de/hygiene/pict/publications.gif

Probiotic products claiming to contain *Bifidobacterium*
Microbial analysis
Culture-dependent
Isolation
Identification: BOX-PCR fingerprinting
Typing: Pulsed-Field Gel Electrophoresis
Culture-independent
Qualitative
Detection: Denaturing Gradient Gel Electrophoresis
Quantitative
Enumeration: Real-Time PCR
Product isolates
+
Human type- and reference strains
= *Bifidobacterium* strain set
Strain-specific properties
Safety: Antimicrobial susceptibility
Functionality:
- Immunomodulatory characteristics
- GI-tract transit tolerance

Chapter 3: Classification and identification of *Bifidobacterium* species

1.1. Identification of *Bifidobacterium* species using rep-PCR fingerprinting

Rep-PCR fingerprinting was evaluated for the differentiation of a broad taxonomical range of bifidobacteria and subsequently used for the establishment of an identification framework using *Bifidobacterium* type and reference strains.

1.2. Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of B*ifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov.

In this study, the taxonomic position of the species *B. lactis* and *B. animalis* was investigated using a polyphasic approach which resulted in a proposal to unify both taxa at the species level but to differentiate them at the subspecies level.

Chapter 4: Culture-dependent and culture-independent microbial analysis of probiotic products claiming to contain bifidobacteria

4.1. Culture-dependent and culture-independent qualitative analysis of probiotic products claiming to contain bifidobacteria

This study describes the isolation of bifidobacteria from 58 worldwide collected probiotic products, followed by their identification using BOX-PCR fingerprinting and strain typing using PFGE. In parallel, DGGE was used for the culture-independent detection of *Bifidobacterium* species in these products.

Table A of the addendum compiles the data obtained in the studies described in §3.1-4.1 and gives an overview of the origin and taxonomic diversity of *Bifidobacterium* strains studied in this work.

4.2. Evaluation of real-time PCR targeting the 16S rRNA and *recA* genes for the enumeration of bifidobacteria in probiotic products

For a subset of 29 probiotic products, real-time PCR targeting the multicopy 16S rRNA gene and the single copy *recA* gene was evaluated for the culture-independent enumeration of bifidobacteria.

Chapter 5: Safety assessment of product isolates and reference strains of *Bifidobacterium*

5.1. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products

The research in this section investigates the phenotypic susceptibility of *Bifidobacterium* strains to 15 antimicrobial agents. For a subset of strains, the genotypic basis of tetracycline resistance was further characterized.

Table B of the addendum summarizes the tetracycline resistance properties of *Bifidobacterium* strains tested in this study.

Chapter 6: Functionality assessment of product isolates and reference strains of *Bifidobacterium*

6.1. *Bifidobacterium* strains induce *in vitro* cytokine production by human peripheral blood mononuclear cells in a strain-specific way

This study describes the use of an *in vitro* method to study the effect of human and probiotic *Bifidobacterium* strains on cytokine production by human peripheral blood mononuclear cells.

6.2. *In vitro* assessment of the gastrointestinal transit tolerance of human reference strains and probiotic isolates of *Bifidobacterium*

A selection of human and probiotic *Bifidobacterium* isolates, representing 9 species was evaluated towards GI-tract survival capacity using a microplate scale fluorochrome assay.

Table C of the addendum provides an overview of the immunological and survival properties of *Bifidobacterium* strains tested in this study.

Chapter 3

Classification and identification of *Bifidobacterium* species

3.1. Identification of *Bifidobacterium* species using rep-PCR fingerprinting

Masco L., Huys G., Gevers D., Verbrugghen L. and Swings J. (2003). *Systematic and Applied Microbiology* **26**: 557-563.

Summary

The aim of the present study was to evaluate the use of repetitive sequence-based PCR fingerprinting (rep-PCR) for the taxonomic discrimination among the currently described species within the genus *Bifidobacterium*. After evaluating several primer sets targeting the repetitive DNA elements BOX, ERIC, $(GTG)_5$ and REP, the BOXA1R primer was found to be the most optimal choice for the establishment of a taxonomic framework of 80 *Bifidobacterium* type and reference strains. Subsequently, the BOX-PCR protocol was tested for the identification of 48 unknown bifidobacterial isolates originating from human faecal samples and probiotic products. In conclusion, rep-PCR fingerprinting using the BOXA1R primer can be considered as a promising genotypic tool for the identification of a wide range of bifidobacteria at the species, subspecies and potentially up to the strain level.

Keywords: *Bifidobacterium*, Identification, BOX, ERIC, $(GTG)_5$, REP, faecal isolates, probiotic products

Introduction

Bifidobacteria are Gram-positive, non-spore forming, non-motile rod-shaped anaerobes. Although most bifidobacteria are known to reside within the animal intestine (Matteuzi *et al*., 1971; Scardovi and Zani, 1974; Biavati and Mattarelli, 1991) or in the oral cavity, intestine and vagina of humans (Reuter, 1963; Scardovi and Crociani, 1974; Lauer, 1990), they have also been isolated from various other environments such as wastewater (Scardovi and Trovatelli, 1974), anaerobic digesters (Dong *et al*., 2000) and fermented milk (Meile *et al*., 1997). Members of the genus *Bifidobacterium* dominate the indigenous microflora of infants and as humans age bifidobacteria become the third most abundant bacterial group following the genera *Bacteroides* and *Eubacterium* (Holzapfel *et al*., 1998). Based on suggested probiotic functions, *Bifidobacterium* strains from a number of species are added as living cultures to milk products, pharmaceutical preparations and animal feed.

Currently, over 30 species are recognised within the genus *Bifidobacterium* and the taxonomic position of several of these species has been controversial for many years. Bifidobacteria belong to the class of the *Actinobacteria* (Stackebrandt *et al*., 1997), characterised by a high guanine plus cytosine (G+C) content, i.e. 55 to 67 mol%. Bifidobacteria have been identified by physiological and biochemical methods, which are often time-consuming, laborious and do not always allow the differentiation of closely related species. Consequently, molecular techniques based on restriction fragment analysis (Ventura *et al*., 2001) and/or the polymerase chain reaction (PCR) (Matsuki *et al*., 1999) have recently been evaluated for the identification of bifidobacteria.

Repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting is a genotypic technique using outwardly facing oligonucleotide PCR primers complementary to interspersed repetitive sequences, which enable the amplification of differently sized DNA fragments lying between these elements. Examples of evolutionarily conserved repetitive sequences are BOX, ERIC, REP and $(GTG)_5$. Rep-PCR fingerprinting is considered to be a valuable tool for classifying and typing of a wide range of Gram-negative and several Gram-positive genera (Versalovic *et al*., 1994). To our knowledge, the use of the rep-PCR fingerprinting technique for the identification or typing of bifidobacteria has not been fully

evaluated. Shuhaimi and co-workers (2001) conducted a study to determine whether ERIC-like sequences were present in the genomes of bifidobacteria, whereas Gómez Zavaglia and colleagues (2000) evaluated rep-PCR fingerprinting using the BOXA1R primer for the identification of *Bifidobacterium* isolates from newborns' faeces.

The aim of the present study was to assess the applicability of rep-PCR fingerprinting for the differentiation of a broad range of bifidobacteria. For this purpose, several primer sets targeting different repetitive DNA elements were evaluated on a subset of reference strains. The most suitable primer set was used to establish a taxonomic framework of type and reference strains representing all of the currently described *Bifidobacterium* species. This framework was subsequently tested for the identification of bifidobacterial isolates from different environments.

Materials and methods

Bacterial strains and cultivation

Type and reference strains were obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm/lmg.htm) (**Figure 1**). Faecal bifidobacterial isolates (CFPL) were kindly provided by M.-B. Romond and C. Mullié, Université de Lille 2 (Lille, France). Bifidobacterial isolates R-3933 ® R-3940 are from faecal origin whereas probiotic isolates are numbered R-20204 ® R-20229. All bifidobacterial strains as well as the strains belonging to the genus *Propionibacterium* were grown overnight at 37°C under anaerobic conditions (84% N_2, 8% H_2, 8% CO_2) on modified Columbia agar comprising 23 g special peptone (L72, Oxoid, Drongen, Belgium), 1 g soluble starch, 5 g NaCl, 0.3 g cystein-HCl-H_2O (C-4820, Sigma, Bornem, Belgium), 5 g glucose and 15 g agar dissolved in 1 litre of distilled water (BCCM™/LMG, Medium 144). *Gardnerella vaginalis* strains were grown on Columbia Agar Base (211124, BD, Erembodegem, Belgium) supplemented with 5% defibrinated horse blood (355-6642, Bio-Rad, Nazareth-Eke, Belgium), and were incubated aerobically at 37°C with 5% CO_2 enrichment.

Total DNA extraction

Extraction of total bacterial DNA was based on the method of Pitcher and co-workers (1989) with slight modifications regarding the concentration of lysozyme and an additional step involving RNase at the end of the procedure. Cells grown overnight were harvested and washed in 500 µl TE-buffer (1 mM EDTA pH 8.0; 10 mM Tris-HCl pH 8.0) after which the cell suspension was centrifuged for 2 min at 13000rpm. Following the removal of the supernatant, the resulting pellet was frozen at –20°C for at least one hour to facilitate the rupture of the Gram-positive cell wall. The thawed pellet was then resuspended in 150 µl lysozyme-solution [5 mg lysozyme (28262, Serva, Zandhoven, Belgium) in 150 µl of TE buffer] followed by an incubation step at 37°C during 40 min. The remaining steps of the procedure were performed according to Pitcher and co-workers (1989). Finally, the resulting DNA pellet was dissolved in 200 µl TE-buffer and kept overnight at 4°C. An RNA digesting step was then performed by adding 2 µl of an RNase solution [10 mg RNase (R6513, Sigma) dissolved in 1 ml MQ water] followed by an incubation step of 90 min. at 37°C. Quality of the DNA samples was verified by spectrophotometric measurements at 260/280/234 nm. The DNA was then diluted to a working concentration of 50 ng/µl. The integrity of the DNA was checked by gel electrophoresis in 1% agarose in 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA) buffer. The gel was visualised after staining with ethidium bromide under ultraviolet light.

Rep-PCR fingerprinting

The repetitive sequence-based oligonucleotide primers (Sigma-Genosys, Cambridge, UK) that have been evaluated in this study are ERIC (ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3', ERIC2: 5'-

AAGTAAGTGACTGGGGTGAGCG-3'), BOX (BOXA1R: 5'-CTACGGCAAGGCGACGCTGACG-3'), GTG_5 (GTG_5: 5'-GTGGTGGTGGTGGTG-3') and REP (REP1R: 5'-IIIICGICGICATCIGGC-3', REP2I: 5'-ICGICTTATCIGGCCTAC-3') each with their specific annealing temperature (Versalovic *et al*., 1994). A universal PCR reaction mix was used for all rep-PCR assays in which only the primer was changed. Each 25 µl PCR reaction contained 5 µl 5x Gitschier buffer (83 mM $(NH_4)_2SO_4$, 335 mM Tris-HCl (pH 8.8), 33.5 mM $MgCl_2$, 32.5 µM EDTA (pH 8.8) and 150 mM ˜-mercapto-ethanol), 160 µg/ml BSA, 10% DMSO, 1.25 mM of each of 4 dNTPs (dATP, dCTP, dGTP and dTTP), 0.3 µg/µl oligonucleotide primer, 2 units of Red Goldstar DNA polymerase (Eurogentec, Seraing, Belgium) in MQ water containing 50 ng of template DNA. PCR amplifications were performed in a DNA thermal cycler (Perkin Elmer 9600) with an initial denaturation step (95°C, 7 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (variable temperature, 1 min) and extension (65°C, 8 min), and a single final extension step (65°C, 16 min). The amplified fragments were fractionated on a 1.5% agarose gel during 16 h at a constant voltage of 55 V in 1x TAE at 4°C. The rep-PCR genomic fragments were visualised after staining with ethidium bromide under ultraviolet light, followed by digital capturing of the image using a CCD camera and storage as a tiff file. The resulting fingerprints were analysed using the BioNumerics V2.5 software package (Applied Maths, Kortrijk, Belgium).

Results

Evaluation of the different primer sets

Two single oligonucleotide primers, BOXA1R and $(GTG)_5$, and two oligonucleotide primer pairs, REP1R-I/REP2-I and ERIC1R/ERIC2, were tested for their ability to differentiate a subset of 35 strains belonging to 7 *Bifidobacterium* species, namely *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. lactis* and *B. longum*, previously identified by protein profiling (data not shown). Fingerprints generated with the ERIC and REP primers contained less than 20 bands, with an average of 19.8 and 14.8, respectively. The use of BOX and $(GTG)_5$ resulted in banding patterns containing more than 20 bands, with an average of 25.5 and 29.9 bands, respectively. The highest discriminatory power was obtained using the BOXA1R primer, which generated banding patterns that displayed a much higher inter-strain heterogeneity compared to $(GTG)_5$-generated banding patterns, which were too complex. The size of the amplicons generated with the BOXA1R primer ranged from 250bp to 6000bp, the broadest range witnessed after amplification with each of the 4 primer sets. For these reasons, we decided that rep-PCR fingerprinting using the BOXA1R primer (BOX-PCR) was the method of choice for the further construction of a taxonomic framework for identification of bifidobacteria.

Identification of bifidobacteria using BOX-PCR

A total of 80 type and reference strains, belonging to 32 *Bifidobacterium* (sub)species, were subjected to BOX-PCR fingerprinting for the establishment of a taxonomic framework. The results of the numerical analysis of the generated BOX-PCR banding patterns are shown in a dendrogram (**Figure 1**). Three strains of the closely related species *Gardnerella vaginalis* that were subjected to BOX-PCR fingerprinting produced banding patterns containing less than 7 bands (**Figure 1**). Three strains belonging to the genus *Propionibacterium*, another member of the *Actinobacteria* branch of the Gram-positive bacteria, were also included. The resulting amplification profiles grouped into a single BOX-PCR cluster within the framework of bifidobacteria (**Figure 1**). When clusters were delineated at a correlation level of 50%,

type and reference strains of *Bifidobacterium* taxa that were represented by two or more strains usually grouped in clusters according to their taxonomic designation. In fact, only the representatives of *B. pseudolongum* subsp. *pseudolongum* and *B. asteroides* did not group in a single BOX-PCR cluster, respectively.

In order to evaluate the applicability of BOX-PCR for the identification of unknown *Bifidobacterium* isolates, three different sets of isolates were subjected to BOX-PCR fingerprinting (**Figure 2**). A first set comprised 14 bifidobacterial isolates from faecal origin, previously identified by means of Multiplex PCR including different species-specific primers (C. Mullié, personal communication). The results of numerical analysis of the generated BOX-PCR banding patterns confirmed the identification results obtained with Multiplex PCR, except for one. The isolates could be identified as *B. adolescentis*, *B. bifidum*, *B. breve* and *B. longum*. A second set consisted of 26 new bifidobacterial isolates originating from different kinds of probiotic products. These were subjected to BOX-PCR and the resulting banding patterns were clustered together with the reference framework. The newly isolated bifidobacteria were assigned to the species *B. lactis*, *B. longum* and *B. bifidum*. A third set comprising 8 strains from faecal origin, previously identified by means of protein profiling (data not shown), were also added to our reference frame. The strains could be assigned to the species *B. adolescentis*, *B. catenulatum* and *B. pseudolcatenulatum*. No pronounced effect was observed from the addition of these isolates on the stability of the cluster analysis based on the BOX-PCR banding patterns of the reference strains.

The reproducibility of each PCR run was evaluated by the inclusion of reference strain LMG 10733. All runs were performed with the same thermal cycler. Throughout this study, a similarity range from 92.5% to 97% was found for repeated BOX-PCR banding patterns of strain LMG 10733 (data not shown). These variations were mainly due to changes in band intensity rather than to qualitative differences, i.e. the presence or absence of a band. Overall, these variations did not significantly affect the stability of the cluster analysis.

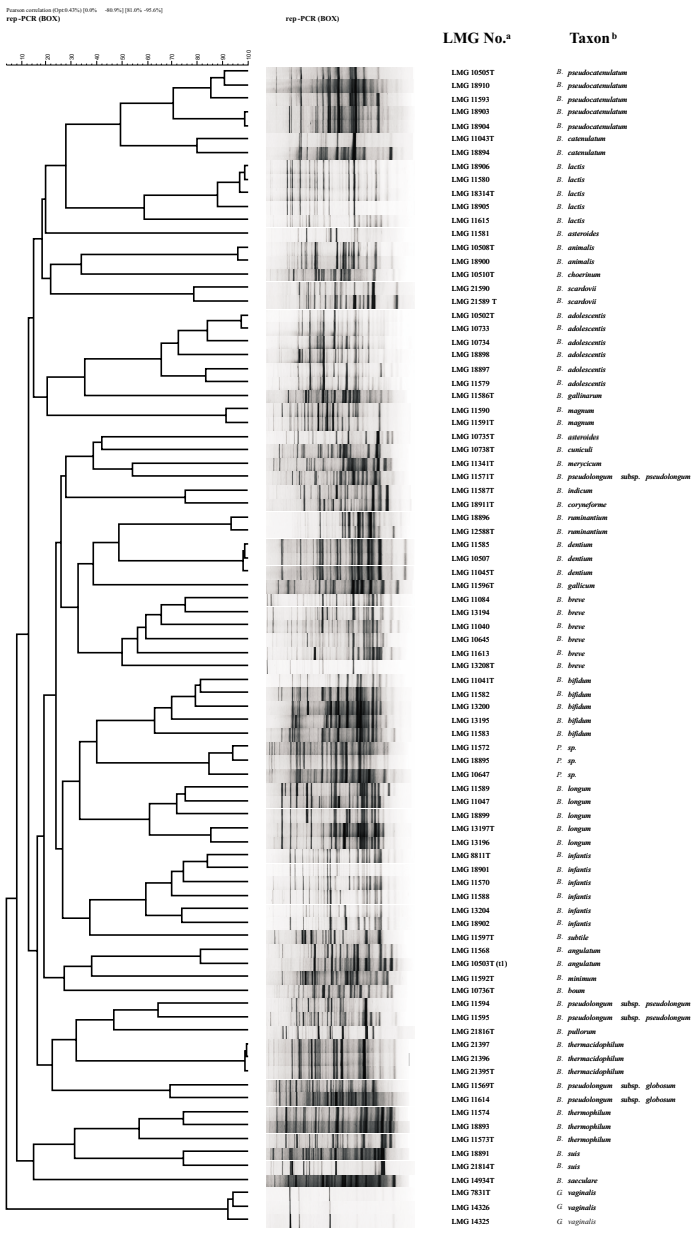

Figure 1. Dendrogram generated after cluster analysis of digitized BOX-PCR fingerprints of type and reference strains. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficiënt.

[a] LMG: BCCM™/LMG Bacteria Collection; T: type strain; t1: type of colony 1

[b] *B.*: *Bifidobacterium*; *P.*: *Propionibacterium*; *G.*: *Gardnerella*

Discussion

Based on the discriminatory power, complexity of the patterns and the taxonomic correlation, the BOXA1R primer was found to be the most suitable rep-PCR primer for the identification of bifidobacteria. Furthermore, the resulting BOX-PCR patterns were sufficiently complex to allow discrimination at the subspecies level whilst the absence or presence of one or more bands within a cluster resulted in a heterogeneity among strains which was far more pronounced than with any other primer set. Consequently, BOX-PCR fingerprinting is not only suitable for identification purposes but can also be used for the pre-grouping of isolates. In this way, only those isolates that display highly similar if not identical BOX-PCR banding patterns may need further typing.

For many years, members of the genus *Bifidobacterium* were considered as lactic acid bacteria (LAB) based on some common metabolic features and because of their widespread use in the food industry. However, based on their high DNA G+C content and from 16S rDNA sequence data, bifidobacteria appear to constitute a phylogenetically coherent unit, i.e. the family *Bifidobacteriaceae* within the *Actinobacteria* class which also comprises the genus *Propionibacterium*. Within this taxonomic unit, there resides only one other organism, namely *Gardnerella vaginalis*. The typical LAB, such as *Lactobacillus*, *Lactococcus* and *Pediococcus* are characterised by a DNA G+C content of less than 50 mol% and therefore belong to the *Clostridium* branch of the Gram-positive bacteria. Because of this phylogenetic position, LAB are only distantly related to members of the genus *Bifidobacterium*. Accordingly, BOX-PCR profiles of LAB typically contain merely up to 6 bands (Gevers *et al*., 2001) whereas *Bifidobacterium* profiles usually comprise more than 20 bands. Due to its close relatedness to the genus *Bifidobacterium* we also included 3 strains of the species *Gardnerella vaginalis* in this BOX-PCR fingerprinting study. Representatives of *G. vaginalis* are known to exhibit fructose-6-phosphate phosphoketolase activity and display a 16S rDNA sequence similarity of 93.1% with the type species *Bifidobacterium bifidum* (Van Esbroeck *et al*., 1996). Nevertheless, the BOX-PCR banding patterns of the three *G. vaginalis* strains contained as little as 7 bands and could therefore be clearly differentiated from those of bifidobacteria. The *Propionibacterium* strains included in this study grouped in a separate

cluster within the *Bifidobacterium* reference framework (**Figure 1**). Unlike the *Gardnerella* strains, however, the BOX-PCR profiles of the *Propionibacterium* strains displayed a similar number and distribution range of bands compared to typical *Bifidobacterium* banding patterns.

In cluster analysis, *Bifidobacterium* type and reference strains grouped according to their taxonomic designation except for representatives of *B. pseudolongum* subsp. *pseudolongum* and *B. asteroides*. In the former case, the 2 reference strains LMG 11594 (28T) and LMG 11595 (29 Sr-T) and the type strain LMG 11571^T (PNC-2-9G) did not group in a single cluster (**Figure 1**). Interestingly, previous taxonomic studies have indicated that the species *B. pseudolongum* is phenotypically and genomically heterogeneous. Mitsuoka (1969) proposed four biotypes (a, b, c and d) in *B. pseudolongum* on the basis of differences in the fermentation of four sugars. Later, in the proposal of Yaeshima and co-workers (1992) on the unification of *B. pseudolongum* and *B. globosum*, four groups of strains were distinguished based on G+C content and DNA-DNA hybridisation values. The authors assembled strains similar to the type strain of *B. pseudolongum* and strains similar to the type strain of *B. globosum* in two new subspecies of *B. pseudolongum*, while the remaining strains were divided in 2 intermediate groups (I and II), which were more closely related to *B. globosum* than to *B. pseudolongum*. Furthermore, it was shown that strains belonging to biotype a, consist of *B. pseudolongum* and *B. globosum*, whereas strains belonging to biotype b and c consist of intermediate group II and biotype d consists of intermediate group I (Yaeshima *et al*., 1992). Correspondingly, the reference strains LMG 11594 and LMG 11595, both belonging to intermediate group II (biotype b and c) display banding patterns that are distinct from the banding pattern of the type strain LMG 11571^T, which belongs to biotype a, and that are more similar to the banding patterns of the *B. pseudolongum* subsp. *globosum* strains. Collectively, our BOX-PCR data confirm previous observations that strains belonging to intermediate group I and II (represented here by LMG 11594 and LMG 11595) are distinct from the type strains of both subspecies of *B. pseudolongum*. Further studies including more isolates affiliated to these intermediate groups should be conducted in order to determine their taxonomic position.

The two strains of *B. asteroides* included in this study, LMG 10735^T and LMG 11581, also did not group in the same BOX-PCR cluster (**Figure 1**). In a previous study,

Lauer and Kandler (1983) performed DNA-DNA hybridisations between representatives of the *B. indicum*/*B. coryneforme*/*B. asteroides* group, all inhabitants of the intestine of the honeybee. The type strains of *B. indicum* and *B. coryneforme* showed 100% DNA-DNA homology to each other. The type strain (C51^T = LMG 10735^T) and one reference strain (C3 = LMG 11581) of *B. asteroides* showed only about 65% DNA-DNA homology to each other and were only moderately related to *B. indicum* and *B. coryneforme* at a level of 35% DNA-DNA homology. Also from BOX-PCR fingerprinting, the profiles of type strains of *B. indicum* and *B. coryneforme* were relatively closely related at a similarity level of 75.5%. In contrast, the banding patterns of the two representatives of *B. asteroides* were quite different from each other and from those of *B. indicum* and *B. coryneforme*. Possibly, our BOX-PCR results corroborate with the phenotypic heterogeneity in *B. asteroides*, reflected by the high number of different isoenzymes of transaldolase (Scardovi et al., 1979) and carbohydrate fermentation patterns (Scardovi and Trovatelli, 1969). However, the study of Lauer and Kandler (1983) seems to indicate that the type strain of *B. asteroides* does not fit the original description, in contrast to the reference strain LMG 11581. Therefore, as mentioned in the minutes of the meetings of the International Committee on Systematic Bacteriology (Biavati, 2001), our BOX-PCR results support the proposal that the original species description of *B. asteroides* has to be emended because of a lack of accordance with the type strain LMG 10735^T.

The taxonomic standing of *B. lactis* has been much debated since its description by Meile and co-workers (1997). In fact, there has been a proposal from Cai and colleagues (2000) to consider *B. lactis* as a subjective synonym of the earlier described *B. animalis* (Mitsuoka, 1969; Scardovi and Trovatelli, 1974) as well as to reclassify *B. lactis* as a subspecies of *B. animalis*, namely *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* (Ventura and Zink, 2002). As shown in **Figure 1**, reference strains of *B. lactis* and *B. animalis* grouped in two separate clusters. These results suggest that future taxonomic proposals on the unification of these two species should take into account their pronounced genotypic heterogeneity displayed by BOX-PCR fingerprinting. Likewise, Sakata and co-workers (2002) recently suggested to unify the species *B. longum*, *B. infantis* and *B. suis*, whereas in our

study strains belonging to each of these three species could be easily differentiated with BOX-PCR fingerprinting.

A good correlation was obtained when comparing the BOX-PCR identification results of faecal isolates with protein profiling and Multiplex-PCR, respectively. As an exception, one isolate identified by Multiplex-PCR as *B. infantis* clustered together with representatives of *B. longum* within the BOX-PCR reference frame, which may be a reflection of the phylogenetic relatedness of these 2 species (Sakata *et al*., 2002). Overall, no pronounced effect was observed from the addition of unknown isolates on the stability of the cluster analysis based on the BOX-PCR banding patterns of the reference strains. However, it is important to realise that the continuous addition of new isolates encompassing a large taxonomical and/or geographical diversity may lead to a minor shift of the reference framework.

In conclusion, rep-PCR fingerprinting using the BOXA1R primer is a rapid, easy to perform and reproducible method that is suitable for a high throughput of *Bifidobacterium* strains. It is a highly discriminatory technique that permits differentiation at the species, subspecies and potentially up to the strain level. In our opinion, this technique is a promising tool for the identification of bifidobacteria originating from all kinds of environments.

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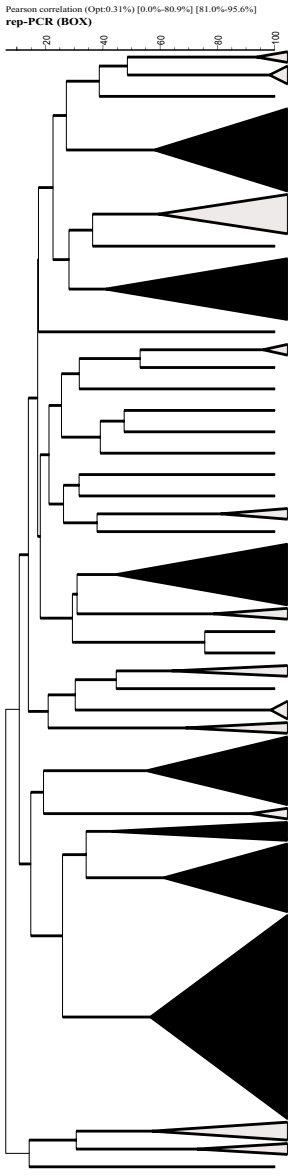

Figure 2. Dendrogram generated after the cluster analysis of digitized BOX-PCR fingerprints of the 80 *Bifidobacterium* type and reference strains and 48 bifidobacterial isolates. The dendrogram was constructed using the unweighted pair-group method using arethmetic averages with correlation levels expressed as a percentage values of the Pearson correlation coefficient.
CFPL: Collection de la Faculté de Pharmacie de Lille
R: Research collection, Laboratory of Microbiology, Ghent University

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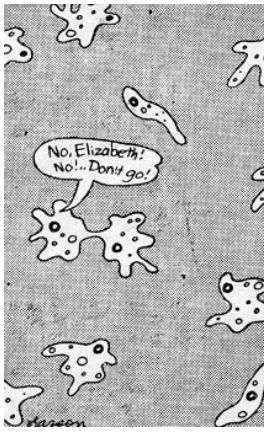

SOURCE: www.umanitoba.ca/.../Images/cartoons/petri.jpg

3.2. Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov.

Masco L., Ventura M., Zink R., Huys G. and Swings J. (2004). *International Journal of Systematic and Evolutionary Microbiology* **54**, 1137-1143.

Summary

The taxonomic standing of *Bifidobacterium lactis* and *Bifidobacterium animalis* was investigated using a polyphasic approach. Sixteen representatives of both taxa were found to be phenotypically similar and shared more than 70 % DNA-DNA relatedness (76-100 %), which reinforces the conclusions of previous studies in which *B. lactis* and *B. animalis* were considered as one single species. However, the results of protein profiling, BOX-PCR fingerprinting, Fluorescent Amplified Fragment Length Polymorphism (FAFLP) and *atp*D and *gro*EL gene sequence analysis demonstrate that representatives of *B. animalis* and *B. lactis* constitute two clearly separated subgroups. This subdivision was also phenotypically supported based on the ability to grow in milk. Given the fact that *B. lactis* Meile *et al*. 1997 has to be considered as a junior synonym of *B. animalis* (Mitsuoka, 1969) Scardovi and Trovatelli 1974, our data indicate that the latter species should be split up in two new subspecies, i.e. *Bifidobacterium animalis* subsp. *animalis* subsp. nov. (type strain R101-8^T = LMG 10508^T = ATCC 25527^T = DSM 20104^T = JCM 1190^T) and *Bifidobacterium animalis* subsp. *lactis* subsp. nov. (type strain UR1^T = LMG 18314^T = DSM 10140^T = JCM 10602^T).

Keywords: *Bifidobacterium animalis*, *Bifidobacterium lactis*, polyphasic taxonomy, reclassification

Introduction

The taxonomic standing of the species *Bifidobacterium lactis* has been much debated since its description by Meile *et al*. (1997), and several studies have investigated its affiliation with the closely related but earlier described *Bifidobacterium animalis* (Scardovi and Trovatelli, 1974). Based on phenotypic characteristics, 16S rDNA sequence analysis and DNA-DNA hybridisation, Cai *et al*. (2000) proposed that *B. lactis* should be considered as a junior synonym of *B. animalis*. However, new genotypic evidence, recently reported by Ventura and Zink (2002, 2003) and Zhu *et al.* (2003), suggested that *B. lactis* and *B. animalis* should still be considered as two separate taxonomic entities, not at the species level but at the subspecies level.

Compared to *B. animalis*, strains of *B. lactis* exhibit an elevated oxygen tolerance, which is a remarkable trait within the bifidobacteria that allows organisms to reach high numbers in commercial products under non-anaerobic conditions. Because of this, *B. lactis* strains are frequently applied in probiotic dairy products, food supplements and pharmaceutical preparations (Prasad *et al*., 1998). In order to guarantee the quality and the label correctness of such products, it is thus very important that the taxonomic position of this industrially applied microorganism is well clarified.

The aim of this polyphasic study was to investigate the taxonomic relationship between *B. animalis* and *B. lactis* on the basis of DNA-DNA hybridisation, mol% G+C determination, sugar fermentation patterns, the ability to grow in milk, protein profiling, BOX-PCR and Fluorescent Amplified Fragment Length Polymorphism (FAFLP) fingerprinting and *atp*D and *gro*EL gene sequence analysis.

Materials and methods

Bacterial strains and cultivation

The 16 *Bifidobacterium* strains used in this study, namely *B. animalis* LMG 10508^T, LMG 18900 and NCC 273 and *B. lactis* LMG 18314^T, LMG 11615, LMG 18906, LMG 11580, NCC 239, NCC 282, NCC 311, NCC 330, NCC 362 (= Bb12, Chr. Hansen, Denmark), NCC 363, NCC 383, NCC 387 and NCC 402 were obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm/lmg.htm) or from the Nestlé Culture Collection (NCC), Nestlé Research Centre, Lausanne, Switzerland (additional descriptive data are available as supplementary Table 1). All strains were grown overnight at 37 °C under anaerobic conditions (84 % N_2, 8 % H_2, 8 % CO_2) on modified Columbia agar comprising 23 g special peptone (Oxoid), 1 g soluble starch, 5 g NaCl, 0.3 g cystein-HCl-H_2O (Sigma), 5 g glucose and 15 g agar dissolved in 1 litre of distilled water (BCCM™/LMG, Medium 144).

Phenotypic characterization

Strains were phenotypically characterized using the AN MicroPlate™ system (Biolog) according to the instructions of the manufacturer. Cells were subcultured twice on modified Columbia agar, after which the MicroPlates were incubated under a hydrogen-free anaerobic atmosphere (100 % CO_2) during 24 h. The MicroPlates were spectrophotometrically read using the Biolog Micro Station™-reader. The ability to ferment starch was tested separately by inoculation of the strains on modified Columbia agar depleted of the usual carbon sources and subsequently supplemented with an equal amount (w/v) of soluble starch. After incubation under anaerobic conditions at 37 °C during 72 h, a lugol solution (0.5 % I_2 + 1 % KI in distilled water) was poured on the growth zone and visually checked for a hydrolysis halo.

DNA-DNA hybridisation and mol% G+C determination

High molecular weight-DNA for DNA-DNA hybridisations and mol% G+C determination was prepared using a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989), as described by Goris *et al*. (1998). DNA base compositions were determined by the method of Mesbah *et al.* (1989). DNA was enzymatically digested into deoxyribonucleosides and separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent used was 0.02 M $NH_4H_2PO_4$ pH 4.0 with 1.5 % acetonitrile. Unmethylated lambda phage DNA (Sigma) was used as the calibration reference. DNA-DNA hybridisations were performed with biotin-labelled probes in microplate wells (Ezaki *et al.* 1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 45 °C in the presence of 50 % formamide. Reciprocal experiments were performed for every pair of strains.

Fingerprinting techniques

Sodium Dodecyl Sulphate-Polyacrylamide Gelelectrophoresis (SDS-PAGE) analysis of whole-cell proteins, using standardized conditions for comparison with the laboratory-based protein pattern database, was performed according to Pot *et al.* (1994).

Microscale DNA extraction was based on the method of Pitcher *et al*. (1989) with slight modifications as described earlier (Masco *et al*., 2003). Microscale DNA extracts were used for BOX-PCR and FAFLP fingerprinting. Repetitive sequence-based (rep-) PCR fingerprinting using the BOXA1R primer was carried out as described previously (Masco *et al*., 2003). FAFLP template preparation was carried out essentially as described by Thompson *et al*. (2001) with slight modifications. High-molecular DNA was digested with *Taq*I (Westburg) and *Eco*RI (Amersham Pharmacia Biotech). For the pre-selective PCR the E00 primer (5'-GACTGCGTACCAATTC-3', 1 µM) and T00 primer (5'-CGATGAGTCCTGACCGA-3', 5 µM) (Sigma-Genosys) were used. The initial denaturation step was performed at 94 °C. In the selective PCR, the E01-6FAM primer (5'-6FAM-GACTGCGTACCAATTCA-3', 1 µM) and T01 primer (5'-CGATGAGTCCTGACCGAA-3', 5 µM) (Sigma-Genosys) were used. The selective PCR products were separated on a denaturing polyacrylamide gel (10.6 % v/v acrylamide, 36 % w/v urea, 1 % w/v resine and 10 % v/v 1x TBE in HPLC water) in 1x TBE buffer. Numerical analysis was performed with BioNumerics V2.5 software (Applied Maths).

Sequencing of the *atp*D and *gro*EL genes

For sequencing of the *atp*D and *gro*EL genes, DNA was prepared as previously described (Ventura *et al*., 2001). An 1133 bp fragment of *atp*D and an 1158 bp fragment of *gro*EL were amplified using oligonucleotide primers atp-1 (5'-CACCCTCGAGGTCGAAC-3', position 180 of *B. longum* NCC 2705) and atp-2 (5'-CTGCATCTTGTGCCACTTC-3', position 1313 of *B. longum* NCC 2705), and gro-1 (5'-GACCATCACCAACGATG-3', position 138 of *B. longum* NCC 2705) and gro-2 (5'-GCTCCGGCTTGTTGGC-3', position 1296 of *B. longum* NCC 2705), respectively. Each PCR mixture (50 ˜l) contained 20 mM Tris-HCl, 50 mM KCl, 200 ˜M of each deoxynucleoside triphosphate, 50 pmol of each primer, 1.5 mM of $MgCl_2$ and 1 U of *Taq* DNA polymerase (Gibco BRL). The PCR cycling profile consisted of an initial denaturation step of 3 min at 95 °C, followed by amplification for 30 cycles as follows: denaturation (30 sec at 95 °C), annealing (30 sec at 50 °C) and extension (2 min at 72 °C), and completed with an elongation phase (10 min at 72 °C). The resulting amplicons were separated on a 1 % agarose gel followed by ethidium bromide staining. PCR fragments were purified using the PCR purification kit (Qiagen) and were subsequently cloned in the pGEM-T Easy plasmid vector (Promega) following supplier's instructions. Nucleotide sequencing of both strands from cloned DNA was performed using the fluorescent-labelled primer cycle-sequencing kit (Amersham Buchler) following supplier's instructions. The primers used were: atp-1, atp-2 and gro-1, gro-2 labelled with IRD800 (MWG Biotech). Sequence alignment was done using the MultiAlign program and the Clustal W program. Dendrograms from gene

sequences were drawn using the Clustal X program. All *atp*D and *gro*EL gene sequences reported in this study have been deposited at Genbank and their accession numbers are indicated in **Figure 3**.

Growth performance in milk

The ability to grow in milk was checked by measuring changes in the impedance of the milk medium using the Rapid Automated Bacterial Impedance Technique (Don Whitley System). This system measures the transformation of polar uncharged lactose into charged lactic acid via changes in the electric conductivity. The changes vs incubation time curve is proportional to the acidification of the medium that was measured with a pH electrode. The bacterial growth was measured as cfu ml^{-1}. To determine the cell yield, fermentations were performed using Skim milk (Difco) medium. Anaerobic fermentations were conducted in duplicate and samples were taken periodically during fermentation and analysed for viable counts using duplicate MRS-cysteine agar plates.

Results and Discussion

According to the DSMZ Bacterial Nomenclature Up-to-date site (http:// http://www.dsmz.de/bactnom/bactname.htm), *B. lactis* is considered as a heterotypic synonym of *B. animalis* based on the proposal of Cai *et al.* (2000). In spite of this proposal, both names are still regularly used. In the period from January 2001-August 2003 following the proposal of Cai *et al.* (2000) to unify *B. lactis* and *B. animalis*, the species name *B. lactis* has been cited in at least 37 papers. A recent genotypic study of Ventura and Zink (2002) supported this unification, but also concluded from their Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR fingerprinting and 16S–23S Internally Transcribed Spacer (ITS) analysis that strains formerly classified as *B. lactis* should be allocated in a subspecies of *B. animalis*.

The current study was initiated to collect more polyphasic evidence in support of the subdivision of *B. animalis* at the subspecies level. DNA base compositions were determined which ranged from 60.3 mol% to 61.4 mol%, with averages of 61.3 mol% and 61.0 mol% for representatives of *B. animalis* and *B. lactis*, respectively (additional data are available as supplementary Table 2). DNA-DNA hybridisations were performed using seven strains of which some were also included in the study of Cai *et al.* (2000). Consistent with their findings, all DNA-DNA reassociation values were above 70 % ranging from 76 to 100 % and the type strains of *B. lactis* and *B. animalis* displayed at least 90 % DNA relatedness. This is in contrast with the findings of Meile *et al.* (1997) who found only 27 % DNA homology between the type strain of *B. lactis* and *B. animalis* using a rather unusual technique based on hybridization of uniformly labeled *Eco*RI-restricted chromosomal DNA of the *B. lactis* type strain followed by southern hybridization with the same amounts of *Eco*RI-restricted DNA of other *Bifidobacterium* strains. Based on the narrow % G+C range and the high DNA reassociation values, our data reinforce the proposal of Cai *et al*. (2000) to join *B. lactis* and *B. animalis* in one single species for which the name of the oldest description, i.e. *B. animalis*, should be maintained according to Rule 42 of the Bacterial Code (1990 Revision) (Lapage *et al*., 1992).

Following a polyphasic approach, all *B. animalis* and *B. lactis* strains were subjected to a number of techniques that have the potential to unravel relationships at the subspecific level which included protein and DNA (BOX-PCR and FAFLP) fingerprinting as well as *atp*D and *gro*EL gene sequence typing. Furthermore, the ability of some strains to grow in milk was determined. As further discussed below, the overall result of this approach showed that each of these methods allowed the unambiguous separation of *B. animalis* from *B. lactis*. In case of SDS-PAGE protein profiling, BOX-PCR and FAFLP, the resulting *B. lactis* and *B. animalis* clusters exhibited similarity levels that were comparable to those between clusters of other *Bifidobacterium* species (data not shown). The results of the numerical analysis of the SDS-PAGE protein patterns are shown in **Figure 1**. After numerical comparison of the digitized protein electrophoretic fingerprints, two well-delineated clusters were observed which corresponded to strains previously assigned to *B. animalis* and *B. lactis*, respectively. Given the fact that protein profiling displays a lower taxonomic resolution compared to genotypic techniques such as rep-PCR and FAFLP, these findings indicate that both species are distinct from each other on a phenotypic basis. As shown previously by BOX-PCR fingerprinting (Masco *et al*., 2003), reference strains of *B. lactis* and *B. animalis* group in two separate clusters indicating their pronounced genotypic heterogeneity. In the present study, BOX-PCR was performed on additional strains from the NCC, which demonstrated the robustness of these genotypic subgroups (data not shown). Recently, Ventura and Zink (2002) reported that rep-PCR targeting the ERIC element also allowed to differentiate between type and reference strains of *B. animalis* and *B. lactis*, respectively. FAFLP exhibits a slightly higher resolution than BOX-PCR fingerprinting and is considered, along with Pulsed-Field Gel Electrophoresis (PFGE), as the most discriminating genotypic technique. Clustering of the FAFLP banding patterns of 14 strains studied, resulted in two clusters representing *B. animalis* and *B. lactis* at a cut-off level of 59 % (**Figure 2**).

The partial nucleotide sequences of the *atp*D and *gro*EL genes from *Bifidobacterium* strains belonging to *B. lactis* and *B. animalis* species were determined and phylogenetic trees based on these data were constructed. The topology of the *atp*D and *gro*EL-based trees was highly comparable (**Figure 3**). In these trees, *Bifidobacterium* strains were grouped into two clusters. Cluster I contained only the type strain of *B. animalis* and the reference

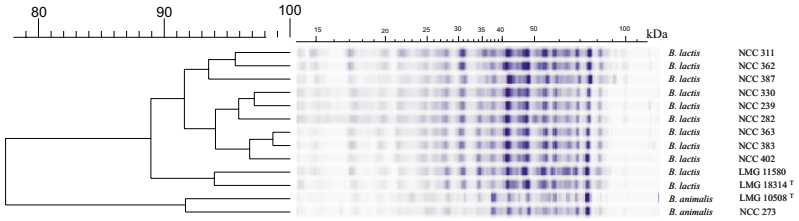

Figure 1. Dendrogram of protein profiles calculated by the unweighted pair-group method using arithmetic averages for 13 strains investigated. Correlation levels are expressed as percentage values of the Pearson correlation coefficient.

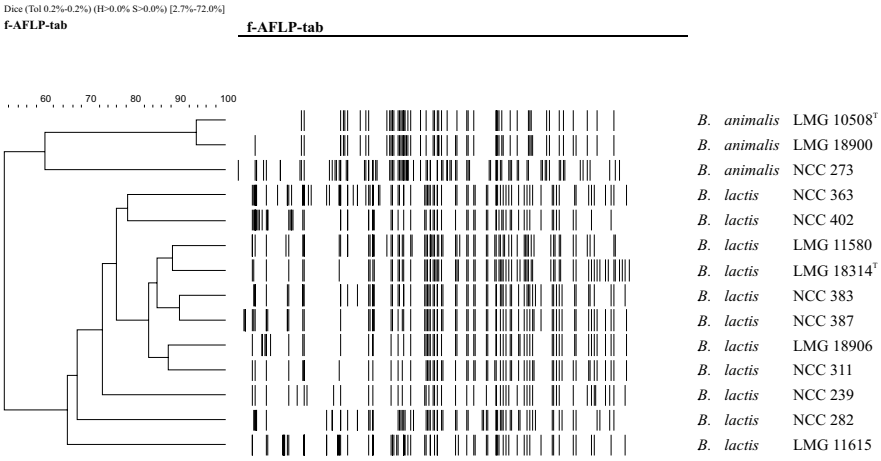

Figure 2. Dendrogram generated after cluster analysis of digitized FAFLP fingerprints of *B. animalis* and *B. lactis* strains. A band based (Dice) cluster analysis (UPGMA) was used. The threshold for cluster delineation was 59 %.

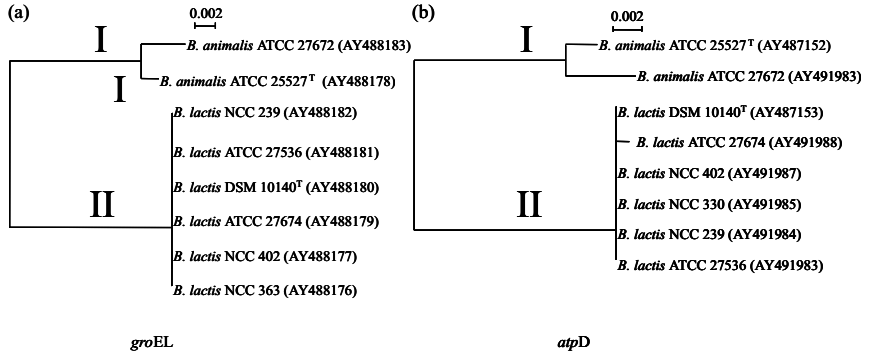

Figure 3. Phylogenetic trees of *B. animalis* and *B. lactis* strains drawn using the Clustal X program. (a) Tree based on partial *gro*EL sequences (1158 base positions). (b) Tree based on partial *atp*D sequences (1133 base positions). Accession numbers are given in parentheses. Bars indicate evolutionary distance.

strain ATCC 27672, whereas cluster II contained six representatives of *B. lactis* including its type strain. Twenty-eight nucleotide substitutions were observed between the *atp*D gene sequences of *B. lactis* DSM 10140^T and *B. animalis* ATCC 25527^T. Likewise, 31 synonymous nucleotide substitutions were noticed between the *gro*EL gene sequences of the two type strains. The phylogenetic distances calculated from the nucleotide substitution ratios at synonymous positions in the *atp*D and *gro*EL genes were examined for all possible combinations of these *Bifidobacterium* genes. A significant correlation between the phylogenetic distances in the *atp*D genes and those in the *gro*EL genes was observed. This result was not unexpected, because it has been demonstrated that a synonymous substitution rate is constant for many chromosomal genes in many organisms, and can thus serve as a molecular clock of their evolution (Lawrence *et al*., 1991). Noteworthy, a clear separation of *B. animalis* ATCC 25527^T and *B. lactis* DSM 10140^T was not possible based on 16S rDNA sequence analysis since their sequences displayed at least 98.8 % homology (Cai *et al*., 2000).

Twelve *B. lactis* and *B. animalis* strains used in this work were tested for their ability to grow on a milk based medium during which growth was monitored by measuring changes in conductance. When the milk medium was inoculated with 10^6 cfu ml^{-1}, only *B. lactis* strains DSM 10140^T, NCC 363, NCC 383, NCC 311, NCC 387, NCC 402, NCC 239, ATCC 27673, ATCC 27674, and ATCC 27536 showed an increase in conductivity whereas *B. animalis* ATCC 25527^T and ATCC 27672 did not reveal any changes in the impedance values of the milk medium. All *B. lactis* strains maintained viable counts greater than 2x 10^8 cfu ml^{-1} throughout the 24 h of fermentation and displayed differences in growth of 1.5 log (**Figure 4a**). On the other hand, *B. animalis* ATCC 25527^T and ATCC 27672 did not reveal any significant growth and their viable counts dropped steadily to below the value of 5x 10^7 cfu ml^{-1} with small differences (below 0.5 log) in relative growth (**Figure 4b**). Collectively, these findings indicate that only *B. lactis* has the potential to grow in milk or milk-based media.

As could be expected from the results of Cai *et al*. (2000), the carbohydrate-fermentation patterns of *B. animalis* and *B. lactis* were very similar based on the examination of 95 different carbon sources. As some characters varied from strain to strain, it was not possible to define a species-specific pattern for representatives of *B. animalis* and *B. lactis*. This is clearly illustrated by the fact that, of all tested carbon sources, only dextrin, α-D-glucose, maltose, maltotriose, D-raffinose and sucrose were fermented by all tested strains. At the individual strain level, only strains NCC 311 and NCC 362 displayed an identical fermentation behavior. In addition to the AN MicroPlate™ characterization, the ability to ferment starch was verified based on the formation of a hydrolysis halo on M144-medium depleted of the usual sugars and supplemented with 0.6 % soluble starch. Meile *et al.* (1997) asserted that the non-utilisation of starch by *B. lactis* was a major difference between both species. However, consistent with the findings of Cai *et al.* (2000) and Lauer and Kandler (1983), we observed that both species were unable to use this carbon source.

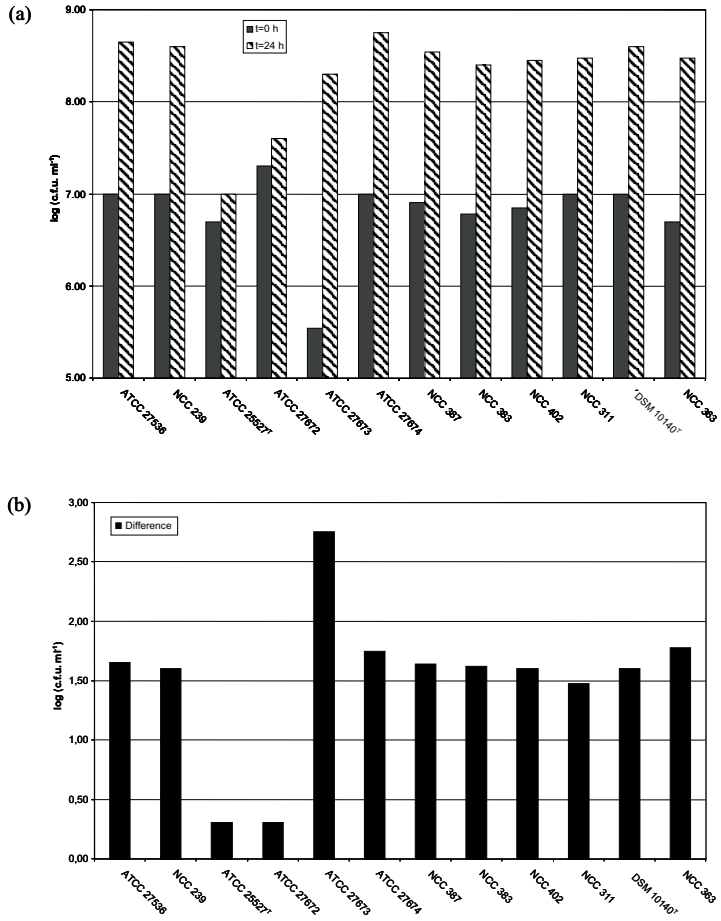

Figure 4. (a) Bar diagram representing viable counts at t = 0 and t = 24 h; (b) Bar diagram reflecting the difference in viable counts after 24 h of incubation.

In support of the proposal of Cai *et al*. (2000), the DNA-DNA hybridisation data and phenotypic results reported in this study evidence that *B. animalis* and *B. lactis* belong to one single species. However, results of protein profiling, genotypic analyses and growth evaluation in milk indicate that both these taxa are clearly different. Based on the fact that members of both species share more than 70 % DNA-DNA relatedness, *B. lactis* should be

reclassified as *B. animalis*, as required by Rule 42 of the Bacterial Code (1990 Revision) (Lapage *et al.*, 1992). Taking into account that strains formerly assigned to *B. animalis* and *B. lactis* can be clearly distinguished at the intraspecific level, we propose to create two subspecies in *B. animalis*, for which the names *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* are suggested, respectively.

The following descriptions are based on data obtained from the present study and on previously reported data (Scardovi and Trovatelli, 1974; Meile *et al*., 1997).

Description of *B. animalis*

Strains display the following characteristics typical for the genus *Bifidobacterium*: Gram-positive, non-motile, non-spore forming, irregular rod-shaped anaerobes. Glucose is fermented using the characteristic enzyme fructose-6-phosphate phosphoketolase in the so-called Bifidus-shunt. Dextrin, α-D-glucose, maltose, maltotriose, D-raffinose and sucrose are fermented, starch is not fermented.

Description of *B. animalis* subsp. *animalis* subsp. nov.

Strains display characteristics typical for the species *B. animalis* as reported above. The optimum growth temperature is 39 to 41 °C. No growth occurs in slants incubated in air or in air enriched with carbon dioxide. No growth occurs in milk or milk-based media. Lactate and acetate are produced in a molar ratio of 1:3.6 ± 0.3. Strains originate from the faeces of rats. The G+C content of the DNA is 61.3 ± 0.0 %.

Type strain: *Bifidobacterium animalis* subsp. *animalis* R101-8 (LMG 10508^T = ATCC 25527^T = DSM 20104^T = JCM 1190^T).

Description of *B. animalis* subsp. *lactis* subsp. nov.

Strains display characteristics typical for the species *B. animalis* as reported above. The optimum growth temperature is 39 to 42 °C. No growth occurs on agar-plates exposed to air, but *B. animalis* subsp. *lactis* tolerates 10 % of oxygen in the headspace atmosphere above liquid media. Growth occurs in milk or milk-based media. The molar ratio of acetate to

lactate from glucose metabolism is about 10 to 1 under anaerobic conditions, e.g. lactate production is replaced by formate production. Strains have been isolated from fermented milk samples, human and infant faeces, rabbit and chicken faeces and from sewage. The G+C content of the DNA is 61.0 ± 0.5 %.

Type strain: *Bifidobacterium animalis* subsp. *lactis* UR1 (LMG 18314^T = DSM 10140^T = JCM 10602^T).

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Supplementary data

Supplementary Table 1. Strains used of the species *B. animalis* and *B. lactis*

Species	Strain designation*	Biological origin	PFGE fingerprint type	Reference
B. animalis	LMG 10508^T = ATCC 25527^T = DSM 20104^T = JCM 1190^T	Rat feces	I	Scardovi and Trovatelli, 1974
B. animalis	LMG 18900	Unknown	I	Masco *et al.*, 2003
B. animalis	NCC 273 = ATCC 27672	Rat feces	II	Scardovi and Trovatelli, 1974
B. lactis	LMG 18314^T = DSM 10140^T= JCM 10602^T	Yoghurt	III	Meile *et al.*, 1997
B. lactis	LMG 11615	Unknown	V	Masco *et al.*, 2003
B. lactis	LMG 18906 = ATCC 27674 = JCM 7117	Rabbit feces	III	Scardovi and Trovatelli, 1974
B. lactis	LMG 11580 = ATCC 27536 = DSM 20105 = JCM 1253	Chicken feces	III	Scardovi and Trovatelli, 1974
B. lactis	NCC 239	Human feces	III	Ventura and Zink, 2002
B. lactis	NCC 282 = ATCC 27673	Sewage	VI	Scardovi and Trovatelli, 1974
B. lactis	NCC 311	Human feces	IV	Ventura and Zink, 2002
B. lactis	NCC 330	Unknown	III	This study
B. lactis	NCC 362 = Bb12	Yoghurt	III	Cai *et al*., 2000
B. lactis	NCC 363	Human feces	III	Ventura and Zink, 2002
B. lactis	NCC 383	Yoghurt	III	Ventura and Zink, 2002
B. lactis	NCC 387	Infant feces	III	Ventura and Zink, 2002
B. lactis	NCC 402	Yoghurt	III	Ventura and Zink, 2002

* ^{T}, type strain; LMG, BCCM^{TM}/LMG Bacteria Collection; ATCC, American Type Culture Collection; NCC, Nestlé Culture Collection

Supplementary Table 2. DNA base compositions and levels of DNA relatedness of *B. lactis* and *B. animalis*.

Species	Strain	G+C content (mol%)	% DNA-DNA reassociation*						
			LMG 10508^T	LMG 18900	LMG 18314^T	LMG 11615	LMG 18906	LMG 11580	LMG 15132^T
B. animalis	LMG 10508^T	61.3	100	95	90	76	91	94	1
	LMG 18900	61.3	100†	100	91	80	96	91	
B. lactis	LMG 18314^T	60.3	90	93	100	89	100†	100†	1
	LMG 11615	61.1	97	91	100†	100	100†	100†	
	LMG 18906	61.4	85	83	93	85	100	97	
	LMG 11580	61.3	89	90	94	86	100†	100	
Lb. kefirgranum	LMG 15132^T		1		1				100

* Results are expressed as means of four determinations. Reciprocal hybridisations showed a maximum standard deviation of 7 %.

† Values over 100 % were cut down to 100 %

Chapter 4

Culture-dependent and culture-independent microbial analysis of probiotic products claiming to contain bifidobacteria

4.1. Culture-dependent and culture-independent qualitative analysis of probiotic products claiming to contain bifidobacteria

Masco L., Huys G., De Brandt E., Temmerman R. and Swings J. (2005). *International Journal of Food Microbiology* **102**:221-230.

Summary

A total of 58 probiotic products obtained worldwide, which were claimed to contain *Bifidobacterium* strains (including 22 yoghurts, 5 dairy fruit drinks, 28 food supplements and 3 pharmaceutical preparations) were investigated in parallel using a culture-dependent and a culture-independent approach. Three isolation media previously reported as selective for *Bifidobacterium* were evaluated for their suitability in the quality analysis of these products. Subsequently, possible bifidobacterial colonies were picked from the best medium and identified by means of rep-PCR fingerprinting using the BOXA1R primer (BOX-PCR). *Bifidobacterium animalis* subsp. *lactis,* formerly classified as *Bifidobacterium lactis*, was most frequently found, but strains belonging to *Bifidobacterium longum* biotypes longum and infantis, *Bifidobacterium bifidum*, and *Bifidobacterium breve* were recovered also. In parallel, all products were also subjected to culture-independent analysis which involved a nested-PCR step on total bacterial DNA extracted directly from the product, followed by separation of the amplicons by Denaturing Gradient Gel Electrophoresis (DGGE) and subsequent identification of species from the band patterns. By conventional cultivation, 70.7 % of the products analysed were found to contain culturable bifidobacteria whereas by culture-independent DGGE analysis, members of the genus *Bifidobacterium* could be detected in 96.5 % of the analysed products. Genotypic characterization of a number of bifidobacterial isolates at the strain level by means of Pulsed-Field Gel Electrophoresis (PFGE) revealed a relatively high degree of genomic homogeneity among the *Bifidobacterium* strains currently used in the probiotic industry.

Keywords: *Bifidobacterium*, probiotic products, DGGE, BOX-PCR, PFGE

Introduction

Together with *Lactobacillus* species, bifidobacteria are the most commonly used group of lactic acid bacteria (LAB) in the production of human probiotics. In particular, strains of *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium longum* biotypes infantis and longum are often implemented in probiotic products in combination with other LAB. Despite the fact that the use of probiotics to improve human health has gained widespread popularity in recent years, there is a growing concern on the side of consumers and consumer organisations regarding the quality and the labelling of commercial probiotic products. A number of studies have demonstrated that the recovery of the incorporated probiotic organisms including bifidobacteria is often poor and that more attention should be paid to describe the identity, safety and functionality of these microorganisms more accurately (Hamilton-Miller *et al*., 1999; Temmerman *et al*., 2003a).

Ideally, microbial analysis of *Bifidobacterium*-containing products for taxonomic purposes requires standardized and accurate procedures for both the isolation and the identification of the implemented bifidobacterial strains. In practice, however, enumeration and isolation of probiotic bifidobacteria from a product is still problematic since there is no standard medium available that allows selective differentiation of bifidobacteria from other LAB. Currently available isolation media are usually insufficiently selective or elective, which in both cases results in inaccurate and/or irreproducible quantitative results (Roy, 2001), and many of them are not commercially available and hence laborious to prepare. Nevertheless, plate count methods are still routinely used in the quality control assessment of probiotic products often being followed by identification of a limited number of isolates at the species level. A variety of methods mainly based on DNA fingerprinting (Masco *et al*., 2003; Ventura *et al*., 2003) or on sequence analysis of ribosomal and housekeeping genes (Matsuki *et al*., 1999; Zhu *et al*., 2003) are currently available for the reliable speciation of bifidobacteria. In contrast, very few studies have attempted to differentiate bifidobacterial isolates from probiotic products at the individual strain level although this is an important tool in functionality and safety assessments.

The combination of a conventional isolation strategy with culture-dependent identification usually renders microbial analysis of probiotic products relatively time consuming, and results may be biased by poor viability or low densities of the target organism. For this reason, culture-independent analysis has recently been promoted as an alternative and/or complementary approach for quality control measurements of probiotic products (Temmerman *et al*., 2003b, c). Essentially, this cultivation-independent detection and identification is based on extraction of total bacterial DNA from the probiotic product followed by selective amplification of a specific part of the bacterial 16S rRNA gene and separation of the resulting amplicons by Denaturing Gradient Gel Electrophoresis (DGGE).

In the present study, we compared the performance of a culture-dependent and a culture-independent approach to study the taxonomic composition of 58 worldwide collected probiotic products labeled as containing *Bifidobacterium*. Culture-dependent analysis involved the evaluation and use of *Bifidobacterium*-selective media, followed by repetitive sequence-based (rep-) PCR fingerprinting and Pulsed-Field Gel Electrophoresis (PFGE) of a selection of isolates. In parallel, all products were also subjected to a culture-independent analysis based on DGGE analysis of 16S rDNA nested-PCR products.

Material and methods

Evaluation of isolation media

Three media developed for the isolation of bifidobacteria, namely Dicloxacillin-Propionic acid medium (DP), *Bifidobacterium* medium (BFM) and Arroyo, Martin and Cotton agar (AMC), were prepared as described previously (Bonaparte *et al*., 2001; Nebra and Blanch, 1999; Payne *et al*., 1999). Their ability to support growth of probiotic *Bifidobacterium* strains and to inhibit growth of yoghurt starter cultures and probiotic non-bifidobacteria was assessed using the organisms listed in **Table 1**. The capacities of the selected *Bifidobacterium* strains to grow on the three selective media were evaluated quantitatively against their growth on a reference medium, Modified Columbia Agar [MCA; 23 g special peptone (L72, Oxoid, Drongen, Belgium), 1 g soluble starch, 5 g NaCl, 0.3 g cystein-HCl-H_2O (C-4820, Sigma, Bornem, Belgium), 5 g glucose and 15 g agar dissolved in 1 litre of distilled water]. Depending on the result obtained with each of the type strains using the spread or pour plate technique, the other reference strains of the corresponding species were grown using the best plating technique. All cultures were incubated overnight at 37 °C under anaerobic conditions (84 % N_2, 8 % H_2, 8 % CO_2) on MCA. Subsequently, a suspension of each strain with an optical density (600nm) (OD_{600}) of approximately 0.2 was prepared in 10 ml Modified Columbia Broth (MCB). A serial dilution was prepared in peptone physiological saline solution [PPS, 0.1 % (w/v) Bacteriological Peptone (L37, Oxoid) and 0.85 % (w/v) NaCl in distilled water] of which the 10^{-4} – 10^{-6} dilutions were plated in triplicate on each of the three test media and on MCA. For spread plating, 50 µl of each dilution was plated whereas in case of the pour plate technique, 1 ml of each dilution was pipetted in a petri dish to which medium cooled to 55 °C was added. All plates were subsequently incubated anaerobically at 37 °C. After 72 h of incubation, colonies were counted and the number of CFU ml^{-1} was determined.

In a second part of the growth evaluation the performances of the three *Bifidobacterium* specific selective media were tested for a selection of yoghurt starter cultures, probiotic non-bifidobacteria and strains of *Bifidobacterium animalis* subsp. *lactis*. Bifidobacterial isolates were grown in MCB and incubated anaerobically at 37 °C, whereas non-bifidobacteria were inoculated into MRS broth and incubated as recommended (http://www.belspo.be/bccm/lmg.htm). From an overnight grown culture, 1 ml was plated using the pour plate technique after which the plates were incubated anaerobically at 37 °C for 72 h. Growth was scored as positive or negative based on the visible presence or absence of colonies, respectively.

Probiotic products

The 58 probiotic products collected from 13 countries comprised 22 yoghurts, 28 food supplements, 5 dairy fruit drinks and 3 pharmaceutical preparations. After purchase, the recommended storage conditions were maintained. All products were analysed before the end of their shelf-life.

Bacterial isolation

Depending on the type of product, 1 ml of product was dissolved in 9 ml PPS (dairy products) or a 10^{-1} dilution was prepared by adding the appropriate amount of PPS to a weighted amount of product (food supplements and pharmaceutical preparations). A 10-fold dilution series was prepared in PPS and pour plated using the most suitable isolation medium, i.e. DP and/or BFM. All plates were incubated anaerobically at 37 °C for 72 h. Subsequently, 10 colonies/product/isolation medium were picked and purified on MCA. Products, which yielded no colonies were subjected to a second isolation procedure after enrichment in MCB.

DNA extraction

Extraction of total genomic DNA from pure cultures was based on the method of Pitcher *et al*. (1989) with slight modifications as described by Masco *et al.* (2003). Extraction of total bacterial DNA from the probiotic product was also based on the method of Pitcher *et al*. (1989) with slight modifications depending on the product type as described by Temmerman *et al*. (2003b).

Nested PCR, DGGE analysis and gel processing

Nested PCR amplification using *Bifidobacterium*-specific 16S rDNA primers (Kaufmann *et al*., 1997) generated amplicons, which served as templates in the second reaction using primers that multiply the V3 region of the bacterial 16S rRNA gene. The resulting V3 amplicons were analysed on 50-70 % DGGE gels. Every six lanes a reference DNA ladder was loaded (Temmerman *et al*., 2003c) which allowed normalization of the gels by reference to the standard pattern. Subsequently, every band position within a lane was compared with those of identified reference strains present in a user-generated BioNumerics database (Applied Maths, Sint-Martens-Latem, Belgium) (Temmerman *et al*., 2003c).

Identification of bacterial isolates

Isolates were first identified as members of the genus *Bifidobacterium* by subjecting DNA obtained from alkaline extraction to PCR amplification using genus-specific primers (Kaufmann *et al*., 1997). After agarose gel electrophoresis, three confirmed bifidobacterial isolates per product and isolation medium were retained for further identification at the species level, using BOX-PCR fingerprinting, as described previously (Masco *et al*., 2003).

Typing of bifidobacterial isolates

The genotypic relatedness among the isolates was assessed by means of Pulsed-Field Gel Electrophoresis (PFGE) typing. The preparation of genomic DNA for PFGE was performed *in situ* in agarose blocks, according to the method of Hung and Bandzulius (1990) with slight modifications

(Neysens *et al*., 2003). Genomic DNA was digested overnight at 37 °C with restriction endonuclease *Spe*I (20U) (Promega, Madison WI, US). Macrorestriction fragments were separated on a 1.1 % PFGE-certified agarose gel (Bio-Rad, Nazareth Eke, Belgium) in 0.5x TBE (10x TBE: 108 g Tris-base; 55 g Boric acid; 9.3 g Na_2 EDTA $2H_2O$) using the CHEF Mapper pulsed-field electrophoresis system (Bio-Rad) with the two-state algorithm program (current: 5.3 V cm^{-1}; run time: 24 h; included angle: 120 °; switch time: 2-30 s at 14 °C).

Phenotypic characterization of bifidobacterial isolates

Strains were phenotypically characterized using the AN MicroPlate™ system (Biolog) according to the instructions of the manufacturer. Cells were subcultured twice on MCA, after which the MicroPlates were incubated under a hydrogen-free anaerobic atmosphere (100 % CO_2) during 24 h. The MicroPlates were spectrophotometrically read using the Biolog Micro Station™-reader.

Results

Evaluation of isolation media

The DP medium was developed for the enumeration of bifidobacteria in fermented milks (Bonaparte *et al*., 2001) and is based on Columbia agar supplemented with dicloxacillin for inhibition of streptococci and lactococci (Sozzi *et al*., 1990) and with propionic acid to stimulate bifidobacterial growth (Beerens, 1991). DP medium was able to sustain growth of all tested strains of *Bifidobacterium animalis* subsp. *animalis*, *Bifidobacterium breve, Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum* biotype longum but it was not able to support growth of *Bifidobacterium adolescentis*, *Bifidobacterium bifidum* and *Bifidobacterium longum* biotype infantis. However, this medium inhibited growth of several non-bifidobacteria, such as the yoghurt starter cultures, as well as the (sub)species *Lactobacillus acidophilus*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactococcus lactis* subsp. *lactis* (**Table 1**).

BFM is antibiotic-free and includes lactulose as the main carbon source (Nebra and Blanch, 1999) as well as propionic acid and the inhibitory agents methylene blue and lithium chloride. BFM was able to sustain growth of all tested strains except for the type strain of *Bifidobacterium bifidum* and strain LMG 11084 of *Bifidobacterium breve*. Overall, colonies obtained on BFM tended to be very small and hence difficult to pick. BFM did not allow growth of the yoghurt starter cultures and *Lactobacillus acidophilus* (**Table 1**).

AMC is based on the commercially available Reinforced Clostridial Agar (CM151, Oxoid) to which a number of supplements (i.e. lithium chloride, sodium propionate, iodoacetate and 2,3,5 trifenyltetrazolium) and antibiotics (nalidixic acid, polymyxin B sulphate and kanamycin sulphate) are added. AMC supported growth of all tested *Bifidobacterium* strains, but failed to inhibit growth of several of the most important non-bifidobacteria such as *Streptococcus thermophilus*. Thus, none of the media were completely selective for the isolation of *Bifidobacterium* in the presence of non-bifidobacteria. The DP and BFM media were moderately selective and, more importantly, they were complementary in supporting growth of all tested *Bifidobacterium* strains. Therefore, for the isolation of bifidobacteria from probiotic products, DP and/or BFM were used, depending on the species claimed on the product

label. The pour plate technique was the method of choice for isolation of bifidobacteria from probiotic products.

Culture-dependent analysis of probiotic products

Analysis of 58 probiotic products yielded 626 isolates of which 434 were confirmed as bifidobacteria with the genus-specific PCR test. From 15 food supplements (53.6 %), one pharmaceutical preparation (33.3 %) and one yoghurt (4.6 %), no bifidobacteria could be recovered, even after a second round of isolation following enrichment in MCB. Because none of the isolation media used in this study were totally selective, it was not possible to obtain specific bifidobacterial counts. The total counts obtained either on DP or BFM from yoghurts and dairy fruit drinks were in the range of 10^3-10^8 CFU ml^{-1} and 10^4-10^6 CFU ml^{-1}, respectively; whereas food supplements and pharmaceutical preparations yielded, respectively, 10^2-10^9 CFU g^{-1} and 10^7 CFU g^{-1} of product.

Of 434 confirmed bifidobacterial isolates, 154 were identified to the species level with BOX-PCR fingerprinting (**Table 2**). *Bifidobacterium animalis* subsp. *lactis* (in 80 % of the products) and *Bifidobacterium breve* (20 %) were the main species isolated from the dairy fruit drinks, whereas from the yoghurts mainly *Bifidobacterium animalis* subsp. *lactis* (90.9 %) was isolated except for one product, which yielded *Bifidobacterium longum* biotype longum isolates (4.55 %). From food supplements, *Bifidobacterium animalis* subsp. *lactis* (28.6 %), *Bifidobacterium bifidum* (7.1 %) and *Bifidobacterium longum* biotype longum (17.9 %) were isolated, whereas from the pharmaceutical preparations *Bifidobacterium bifidum* (33.3 %) and *Bifidobacterium longum* biotype infantis (33.3 %) were the only two species retrieved.

Culture-independent analysis of probiotic products

Except for one product, the bifidobacterial compositions of the dairy fruit drinks and the yoghurts as determined by culture-dependent and culture-independent approaches were comparable. From one product (i.e. Yoghurt natural), no bifidobacteria could be isolated on DP or BFM whereas DGGE revealed the presence of two *Bifidobacterium* species. In contrast, several discrepancies between the methodologies were found with some of the food

supplements and pharmaceutical preparations. For these products, three types of relationship between culture-dependent and culture-independent analyses could be distinguished. For 13 products (41.9 %), the results of the analyses coincided. This group included two products in which no bifidobacteria could be detected using DGGE, which is in agreement with the finding that bifidobacteria were not recovered on DP or BFM. For 17 products (54.9 %), DGGE analysis detected more species than were recovered by isolation. For one product (i.e. Friendly Bifidus), two species were isolated from the product whereas only one was detected with DGGE.

Typing of bifidobacterial isolates

From the 154 *Bifidobacterium* isolates subjected to BOX-PCR, a subset of 48 isolates (mainly corresponding to one isolate/species/product) was included for PFGE typing, to study the genotypic diversity of these commercially used strains. This selection included 39 *Bifidobacterium animalis* subsp. *lactis* isolates, six *Bifidobacterium longum* biotype longum isolates and three *Bifidobacterium bifidum* isolates (**Table 2**). Within the *Bifidobacterium animalis* subsp. *lactis* subset, four PFGE types could be distinguished. The majority of the isolates gave highly similar or identical macrorestriction patterns with *Spe*I digestion, whereas the three remaining isolates gave unique PFGE fingerprints. To verify the robustness of these clusters, an additional restriction enzyme (i.e. *Xba*I) was used with a subset of 11 strains representing all four PFGE types obtained after *Spe*I restriction analysis. Although the four PFGE types were not readily distinguishable as with the *Spe*I restriction patterns, comparable clusters were obtained. Furthermore, the 6 *B. longum* biotype longum isolates grouped in two separate clusters, represented by respectively 5 and one isolate, which was in contrast with the three *B. bifidum* isolates each of which gave a unique PFGE fingerprint.

Table 1. List of species used for the evaluation of three *Bifidobacterium* -specific isolation media

Species (no. of strains)[a]	AMC[b]	BFM[b]	DP[b]
B. adolescentis (n=6)	+	+	-
B. animalis subsp. *animalis* (n=2)	+	+	+
B. bifidum (n=5)	+	+, except for LMG 11041^T	-
B. breve (n=4)	+	+, except for LMG 11084	+
B. longum biotype infantis (n=6)	+	+	-
B. animalis subsp. *lactis* (n=8)	+	+	+
B. longum biotype longum (n=5)	+	+	+
Bacillus sp. (n=1)	+	+	+
E. faecium (n=6)	+	+	+
L. acidophilus (n=7)	-	-	-
L. amylovorus (n=1)	+	+	-
L. delbrueckii subsp. *bulgaricus* (n=3)	-	-	-
L. crispatus (n=1)	-	-	-
L. johnsonii (n=5)	+	+	-
L. lindneri -like (n=1)	+	+	+
L. paracasei subsp. *paracasei* (n=3)	+	+	-
L. plantarum (n=2)	+	+	+
L. reuteri (n=2)	+	+	+
L. rhamnosus (n=6)	+	+	+
Lc. lactis subsp. *lactis* (n=4)	+	+	-
P. acidilactici (n=2)	+	+	+
S. thermophilus (n=13)	+	-	-

[a] *B.: Bifidobacterium; L.: Lactobacillus; Lc.: Lactococcus; S.: Streptococcus; E.: Enterococcus; P.: Pediococcus*

[b] AMC: Arroyo, Martin and Cotton agar; BFM: *Bifidobacterium* medium; DP: Dicloxacillin-Propionic acid medium;

+: growth; -: no growth; LMG: BCCM™/LMG Bacteria Collection

Table 2. Overview of culture-dependent and culture-independent analysis of 58 probiotic products

Product name	Producing country	Microbial claim on product label	Culture-dependent analysis		Culture-independent analysis
			BOX-PCR identification	PFGE type[a]	Nested-PCR DGGE identification
YOMO	Italy	Bifidobacterium	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Crema actidrink	Germany	Bifidobacterium Lactis (BB12)	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Nutrigen	Malaysia	Bifidobacterium	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bio drink	Spain	Bifidobacterias	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Yakult bifiel	Japan	B. breve Yakult strain	*B. breve*	ND	*B. breve*
Actilus	France	Bifidobacterium	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Activia	France	Bifidus essensis	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
B'A (citron)	France	Bifidus Actif	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
B'A (vanille)	France	Bifidus Actif	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Benecol	UK	Active bifidus	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bifidus 1	Belgium	Bifidus	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bifidus 2	Belgium	Bifidus	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bifidus 3	Belgium	Natural bifidus	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bifidus nature	France	Bifidobacterium	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bifidus y acidophilus	Spain	Bifidobacterium	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bio con bifido activo	Spain	Bifidobacterias	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Bio Life	UK	No bifidobacteria claimed	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Joghurt Gold	Germany	B. bifidum	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
KYR	Italy	Bifidobacterium Bb12	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
MIO	France	Bifidus BL	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Nature bifidus & acidophilus	Switzerland	Bifidusbakterien	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Natural bio yoghurt	UK	Bifidobacteria	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Teddi	Italy	Bifidobacterium	*B. animalis* subsp. *lactis*	I and II	*B. animalis* subsp. *lactis*
Vitality	Germany	Bifidobacterium sp.	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Yogosan	Germany	Bifidobacterium BB12	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Lactoferrin yoghurt	Japan	Bifidus BB536	*B. longum* biotype longum	I	*B. longum* biotype longum
Yoghurt natural	Malaysia	Bifido bacterium	NB		*B. animalis* subsp. *lactis*, *B. bifidum*
Culturelle	South-Africa	Bifidobacterium longum	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Debaflor	Belgium	Lactobacillus bifidus	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Ecoflor	The Netherlands	Bifidobacterium Lactis	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Hygiaflora	France	B. bifidum	*B. animalis* subsp. *lactis*	III	*B. animalis* subsp. *lactis*, *B. longum* biotype longum
Proflora	Belgium	Bifidobacterium	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Pronopal plus	Belgium	No bifidobacteria claimed	*B. animalis* subsp. *lactis*	I	*B. animalis* subsp. *lactis*
Biodophilus	UK	Bifidobacterium Bifidum-bacteriën stam, INT9	*B. animalis* subsp. *lactis*, *B. bifidum*	I and III	*B. animalis* subsp. *lactis*, *B. bifidum*
Friendly bifidus	Malaysia	*Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium lactis, Bifidobacterium bifidum*	*B. animalis* subsp. *lactis*, *B. bifidum*	IV and II	*B. animalis* subsp. *lactis*
Beneflora	Belgium	Bifidobacterium species Bifidobacterium longum	*B. longum* biotype longum	I	*B. animalis* subsp. *lactis*, *B. longum* biotype longum
Combiforte	South-Africa	Bifidobacterium longum, Bifidobacterium bifidum	*B. longum* biotype longum	I	*B. longum* biotype longum
Infantiforte	South-Africa	Bifidobacterium infantis	*B. longum* biotype longum	I	*B. longum* biotype longum

Table 2. *Continued*

Product name	Producing country	Microbial claim on product label	Culture-dependent analysis		Culture-independent analysis
			BOX-PCR identification	PFGE type[a]	Nested-PCR DGGE identification
Lactoferrin active	Japan	*B. longum* BB536	*B. longum* biotype longum	I	*B. longum* biotype longum
Lola	Japan	Bifidus	*B. longum* biotype longum	II	*B. longum* biotype longum
Bifibiol	Malaysia	Bifidobacterium bifidum	NB		*B. animalis* subsp. *lactis, B. bifidum, B. longum* biotype longum
Bioprotus	France	Bifidobacterium longum Bifidobacterium bifidum	NB		*B. animalis* subsp. *lactis, B. longum* biotype longum, *B. bifidum*
Decoflor	Belgium	L. bifidus	NB		*B. animalis* subsp. *lactis, B. bifidum*
Lacto Ca	Canada	L. Bifidus	NB		*B. animalis* subsp. *lactis, B. bifidum*
Lactofer-200	Italy	Lactobacillus bifidus	NB		/
Natural factors	Canada	B. bifidum	NB		*B. bifidum*
Neolactoflorene Capsule	Italy	Bifido bacterium bifidum	NB		*B. animalis* subsp. *lactis*
Neolactoflorene Flacon	Italy	Bifido bacterium bifidum	NB		*B. animalis* subsp. *lactis*
Platte buik	France	Bifidobacterium bifidum	NB		*B. animalis* subsp. *lactis*
Swiss 3	Canada	L. Bifidus	NB		*B. animalis* subsp. *lactis, B. bifidum*
Junior	Australia	bifido bacteria	NG		*B. animalis* subsp. *lactis*
Platte buik	France	Bifidobacterium bifidum	NG		/
Prunellines	France	Bifidobacterium bifidum	NG		*B. animalis* subsp. *lactis, B. longum* biotype longum
Transiphyt	France	Bifidobacterium bifidum	NG		*B. animalis* subsp. *lactis, B. bifidum*
Vivaflore	France	Bifidobactérium bifidum	NG		*B. animalis* subsp. *lactis*
Infloran Berna	Italy	Bifidobacterium bifidum	*B. bifidum*	I	*B. bifidum*
Probiotical	Belgium	Bifidobacterium infantis, Bifidobacterium lactis	*B. longum* biotype infantis	ND	*B. longum* biotype infantis, *B. bifidum*
Yovis	Italy	Bifidobacteria (B. breve, B. infantis, B. longum)	NB		*B. animalis* subsp. *lactis*

NB: no bifidobacteria identified by genus-specific 16S rDNA PCR; NG: no growth.

[a] Based on macrorestriction analysis with *Spe* I

Discussion

Despite the fact that many media have been described for the elective, selective or differential enumeration and isolation of bifidobacteria (Roy, 2001), the recovery of these organisms from food or other matrices remains problematic. For the purpose of isolating bifidobacteria from probiotic products, three previously described media were evaluated. Overall, the DP medium produced the highest colony yield of these three despite the fact that we found it to be too selective. In contrast to the complex compositions of BFM and AMC, the DP medium is relatively easy to prepare, which makes it more suitable for routine enumeration of bifidobacteria from probiotic products. However, to guarantee the isolation of the entire taxonomic range of currently used probiotic bifidobacteria, the complementary use of a second medium was indispensable. These findings reinforce the opinion of Pacher and Kneifel (1996) that reliable enumeration of bifidobacteria can only be achieved successfully when the particular *Bifidobacterium* strain used in the product is known. Furthermore, it has also been shown that the plating methodology can influence the accuracy of bifidobacterial counts (Payne *et al*., 1999.). In the present study, the pour plate technique was superior to spread plating for growth of bifidobacteria, presumably because pour plating is more effective for creating the anaerobic conditions favorable for these organisms. Additionally, pour plating facilitated the development of separate colonies.

From both the culture-dependent and culture-independent methodologies, it is clear that the microbial qualities of the freeze-dried products are not as good as those of the dairy products. From 16 of the freeze-dried products (51.6 %) it was not possible to isolate bifidobacteria despite the fact that all but two of these products were found (by nested 16S rDNA-DGGE analysis) to contain at least one *Bifidobacterium* species. These findings may point to the fact that the lyophilization and encapsulation processes significantly reduced the recovery on DP or BFM media of some *Bifidobacterium* strains. Furthermore, it is likely that some of the products no longer contain viable bacteria, which must raise questions about their claimed probiotic effects. Despite the fact that some of these effects might also be exerted by dead bacteria, it can be assumed that most of the beneficial effects of probiotics, such as stimulation of the immune system, anti-tumor activity and reduction of faecal enzyme activity

require metabolically active cells (Ouwehand and Salminen, 1998). In contrast, only one yoghurt did not yield viable bifidobacteria despite the fact that DGGE analysis indicated the presence of *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*. In the majority of the cases (i.e. 96,8 %), DGGE was able to detect all or more species than the culture-dependent approach. However, DGGE analysis does not provide information regarding the viability of the microorganisms, which highlights the continuing need for culture-dependent analysis for quality control in the production of probiotic products. In one case, more species than could be detected by DGGE were isolated. Presumably, the undetected species was present in numbers beyond the DGGE detection limit of 10^4 CFU ml^{-1} (Temmerman *et al*., 2003b).

At present, the (sub)species *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* biotypes infantis and longum and *Bifidobacterium animalis* subsp. *lactis*, formerly classified as *Bifidobacterium lactis* (Masco *et al*., 2004) represent the *Bifidobacterium* taxa that are most frequently used as probiotics. After identification of 154 isolates with BOX-PCR fingerprinting, *Bifidobacterium animalis* subsp. *lactis* proved to be the most frequently found species in yoghurts and fruit drinks. It was also present, to a lesser extent, in food supplements. This was not unexpected since strains belonging to this subspecies are known to be relatively oxygen tolerant (Meile *et al*., 1997), which favours the maintenance of a high number of viable bifidobacteria in a commercial product. In contrast, *Bifidobacterium adolescentis* was not found in the present study although strains of this species have been reported as potential probiotics (Holzapfel *et al*., 1998).

For each of the detected *Bifidobacterium* species macrorestriction analysis with *Spe*I indicated that each was represented by a small number of unique types. This was particularly the case for *Bifidobacterium animalis* subsp. *lactis* and the *Bifidobacterium longum* biotype longum isolates, most of which (respectively 92.3 % and 83.3 %) gave identical PFGE profiles. The high level of genotypic relatedness among *B. animalis* subsp. *lactis* was also reflected phenotypically by AN MicroPlate™ (Biolog) analysis of a subset of eight product isolates belonging to PFGE types I-IV (data not shown). Interestingly, one of these isolates was somewhat atypical by its inability to ferment glucose and its ability to ferment lactose which was in contrast with all other isolates tested, even the ones belonging to PFGE types II, III

and IV. Although PFGE is often considered as the gold standard of bacterial typing, this finding illustrates that caution is needed in the interpretation of such results. Nevertheless, our PFGE typing data clearly indicate that the number of unique *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum* biotype longum strains used as commercial probiotics is surprisingly low in view of the great diversity of *Bifidobacterium*-claiming products analyzed in this study.

In conclusion, this is one of the first studies combining the aspects of isolation, molecular identification and typing to characterize the taxonomic contents of a worldwide collection of *Bifidobacterium*-claiming probiotic products. In agreement with previous product quality studies (Hamilton-Miller *et al*., 1999; Temmerman *et al*., 2003a), our data indicate that a large number of dried probiotic products inadmissibly lack the presence of any viable microorganisms and that a rather high percentage of probiotic products are incorrectly labeled with respect to the identity of the incorporated strains. DGGE proved to be a fast and reproducible culture-independent approach for taxonomic analysis of probiotic products and had a greater detection potential than conventional culture-dependent analysis. Nevertheless, DGGE is unable to provide information on the metabolic status or strain diversity of the incorporated microorganisms, which makes cultivation indispensable for reliable qualitative analyses. Demonstration of the presence of viable probiotic microorganisms is only the first step in the assessment of product quality. Quantitative measurements and assessments of functionality and safety are other requirements for validation of the quality of a given probiotic product.

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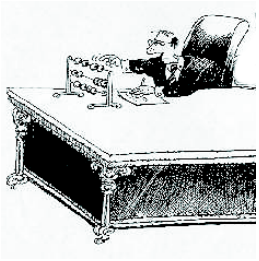

SOURCE: http://www.invention-ifia.ch/images/Cartoon_Syria_Counting_Farzat.jpg

4.2. Evaluation of real-time PCR targeting the 16S rRNA and *recA* genes for the enumeration of bifidobacteria in probiotic products

Masco L., Vanhoutte T., Temmerman R., Swings J. and Huys G. Submitted to International Journal of Food Microbiology

Summary

The application of real-time PCR targeting the multicopy 16S rRNA gene and the single copy *recA* gene was evaluated for the enumeration of bifidobacteria in 29 probiotic products claimed to contain these organisms. Both assays relied on the use of genus-specific primers and the non-specific SYBR Green I chemistry. For both applications, the calibration curve was constructed using the type strain of *Bifidobacterium animalis* subsp. *lactis*. Upon correction with a factor corresponding to the 16S rRNA gene copy number, both assays generally produced comparable enumeration results. Only in exceptional cases, differences between both gene targets were found in probiotic products containing low amounts of bifidobacteria in which case the quantification of the multicopy 16S rRNA gene turned out to be more sensitive than the *recA*-based assay. On the other hand, the use of the latter single copy gene in real-time PCR quantification offers the advantage that no prior knowledge of bacterial content is required when using genus-specific primers, since no correction for multiple gene copies has to be performed. Only 11 of the analysed products (38%), including one dairy based product and ten dried products, contained a minimal *Bifidobacterium* concentration of 10^6 CFU per ml or g of product. Depending on the application, both assays proved to be rapid and reproducible alternatives for culture-based detection and quantification of bifidobacteria in probiotic products.

Keywords: Real-time PCR, 16S rRNA gene, *recA* gene, *Bifidobacterium*, probiotic products

Introduction

Bifidobacteria are natural inhabitants of the human gastrointestinal tract and are known to contribute to a balanced intestinal microflora. Because of their probiotic potential, interest in the commercial exploitation of selected bifidobacterial strains in the functional food industry is growing rapidly. In parallel, this evolution has stimulated the need for advanced methods to perform qualitative and quantitative control measurements of newly developed probiotic products. In terms of quantification, traditional culture-dependent methods are still frequently used despite the fact that they are labour intensive and time consuming. In case of bifidobacteria, the reliability of many enumeration procedures is compromised by the lack of suitable media for the selective isolation of these organisms from probiotic products, which often also contain other lactic acid bacteria such as lactobacilli and *Streptococcus thermophilus* (Hamilton-Miller *et al*., 1999; Temmerman *et al*., 2003; Masco *et al*., 2005). Triggered by these shortcomings, culture-independent methods such as enzymatic-colorimetric assays (Bibiloni *et al*., 2000) and real-time PCR (Vitali *et al*., 2003) have been developed for the enumeration of bifidobacteria in probiotic products that overcome the limitations of conventional cultivation. Real-time PCR has been successfully applied for the detection and quantification of a variety of microorganisms in food, including pathogens (McKillip and Drake, 2004) and lactic acid bacteria (Pinzani *et al*., 2004; Furet *et al*., 2004). However, when it comes to enumerating bifidobacteria, most studies using real-time PCR have mainly focussed on the quantification of these organisms in faecal samples (Requena *et al*., 2002; Queimonde *et al*., 2004; Matsuki *et al*., 2004). For the construction of the calibration curve, being one of the most critical aspects of real-time PCR-based quantification, most methodologies traditionally rely on plate counting for the determination of the initial number of bifidobacteria. Whereas this approach may be suitable for the enumeration of bacteria represented by low numbers, it is difficult to extrapolate towards high bacterial concentrations. At present, the majority of the real-time PCR applications are based on the quantification of the 16S rRNA gene. However, the fact that 16S rRNA genes can be present in multiple copies in the bifidobacterial chromosome may compromise the interpretation of quantitative data obtained by real-time PCR.

This study describes the development and evaluation of a real-time PCR methodology for the culture-independent quantitative analysis of 29 probiotic products claimed to contain bifidobacteria. Prior to real-time PCR analysis, five DNA extraction methods were evaluated. A standard curve was generated by determining the bacterial concentration of a dilution series of the *Bifidobacterium animalis* subsp. *lactis* type strain by plate counting and by flow cytometry. This standard curve formed the basis for quantification of bifidobacteria in the product matrix by real-time PCR analysis. For this purpose, the use of two primer sets targeting either the multicopy 16S rRNA gene or the single copy *recA* gene, respectively, was evaluated.

Materials and methods

Strain and sample collection

The type strain of *B. animalis* subsp. *lactis* (LMG 18314^T) that was used for the optimisation of real-time PCR, and strains *B. adolescentis* LMG 10502^T, *B. animalis* subsp. *animalis* LMG 10508^T, *B. bifidum* LMG 11582 and *B. longum* biotype longum LMG 13197^T used for the determination of the ribosomal RNA (rrn) operon copy number were obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm/lmg.htm). All nonbifidobacterial strains used in this study were obtained from our research collection and included 13 strains representing five lactic acid bacteria (LAB) genera (i.e. *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Lactococcus* and *Streptococcus*). Bifidobacterial strains were grown overnight at 37 °C under anaerobic conditions (84 % N_2, 8 % H_2, 8 % CO_2) on modified Columbia agar (MCA) comprising 23 g special peptone (Oxoid), 1 g soluble starch, 5 g NaCl, 0.3 g cystein-HCl-H_2O (Sigma), 5 g glucose and 15 g agar dissolved in 1 litre of distilled water. All LAB strains were grown overnight at 30 °C on MRS agar (Oxoid). Extraction of total bacterial DNA was performed as described by Pitcher *et al*. (1989) with slight modifications as described by Masco *et al*. (2003). A total of 29 probiotic products were collected from 11 countries worldwide, including 19 food supplements (FS1 –FS19), five yoghurts (Y1 – Y5), two dairy drinks (D1 – D2) and three pharmaceutical preparations (PH1 – PH3) (**Table 1**). After purchase, the recommended storage conditions were used. All products were analysed before the end of their shelf life.

Evaluation of total DNA extraction methods

For a subset of three probiotic products including one yoghurt, one food supplement and one dairy drink, five different total DNA extraction methods were evaluated on the basis of spectrophotometric determination of $OD_{260/280}$ ratios and performance in real-time PCR analysis. Unless indicated otherwise, the product sample consisted of 1 ml yoghurt, 1 ml dairy drink or of 1 ml of a 10^{-1} dilution of the food supplement or the pharmaceutical preparation in peptone physiological saline solution (PPS, 0.1 % (w/v) Bacteriological Peptone (Oxoid) and 0.85 % (w/v) NaCl in distilled water). Subsequently, product samples were centrifuged at 13000 rpm during 10 min. after which the supernatant was removed and the remaining pellet was subjected to five different extraction protocols: (i) the method of Pitcher and co-workers (1989) with slight modifications as described by Masco *et al*. (2003) and hereafter referred to as the modified Pitcher method; (ii) the phenol-chloroform method as described by Gevers *et al.* (2001); (iii) the alkaline extraction method during which 1 ml of lysis buffer (25 µl 10 % SDS and 50 µl 1N NaOH dissolved in 925 µl MQ) was added to the product pellet and heated during 15 min. at 95 °C. After cooling on ice, the lysed suspension was centrifuged for a few seconds at 13000 rpm and 9 ml of MQ was added followed by a final centrifugation step (5 min. at 13000 rpm) and removal of the supernatant; (iv) the

QIAamp® DNA stool mini kit (Qiagen) and (v) the NucleoSpin® food kit (Macherey-Nagel) were used following the instructions of the respective manufacturers. DNA pellets were dissolved in 100 µl TE-buffer (1 mM EDTA pH 8.0; 10 mM Tris-HCl pH 8.0).

Real-time PCR

Real-time PCR amplification reactions were performed with the LightCycler™ (Roche diagnostics) using two different primer sets. The first set of primers, g-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and g-Bifid-R (5'-GGTGTTCTTCCCGATATCTACA-3'), described by Matsuki *et al.* (2002), targets a 596 bp region of the 16S rRNA gene. The second set of primers was designed using the Kodon™ (version 1.0) software (Applied Maths) and amplified a 203 bp fragment of the single copy gene *recA* (the sequence corresponds to the nucleotide numbers 338-541 of the *recA* sequence of *E. coli*), i.e. recA-F (5'-CGTYTCBCAGCCGGAYAAC-3') and recA-R (5'-CCARVGCRCCGGTCATC-3'). Specificity of both primer sets was tested for *B. adolescentis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* biotype longum and infantis and for 13 non-bifidobacterial strains representing the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Lactococcus* and *Streptococcus*. The optimal $MgCl_2$ concentration for each primer set was determined using the 10^{-1} and 10^{-2} dilutions of strain LMG 18314^T using the PCR program described below. Target DNA was amplified in the presence of different $MgCl_2$ concentrations, ranging from 1-5 mM, obtained by altering the amounts of $MgCl_2$ and PCR water in the PCR reaction mix. After determination of the optimal $MgCl_2$ concentration the composition of reaction mix per sample was formulated as follows: 9.6 µl PCR water, 2.4 µl $MgCl_2$ (25mM), 2 µl of each primer (0.5 µM), 2 µl template DNA and 2 µl of FastStart DNA Master SYBR Green I (Roche) which includes *Taq* polymerase, reaction buffer, a deoxynucleotide triphosphate mixture, SYBR Green I dye and Hot Start antibody. The PCR programme consisted of an initial denaturation and anti-*Taq* DNA polymerase antibody-inactivation step (10 min. 95°C), an amplification step (40 cycles of 0 sec at 95 °C, 5 sec at 65 °C for g-Bifid-R/g-Bifid-F or at 60 °C for recA-F/recA-R and 23 sec for g-Bifid-R/g-Bifid-F or 9 sec for recA-F/recA-R at 72 °C) and a melting-curve determination step (from 70 °C for g-Bifid-R/g-Bifid-F or 65 °C for recA-F/recA-R to 95 °C at a transition rate of 0.1 °C sec^{-1}). Measurement of SYBR green fluorescence was performed at the end of each amplification step and continuously during the melt-curve analysis.

Standard curve

For quantification of bifidobacteria in an unknown sample, a standard curve was generated and used in subsequent analyses. A tenfold serial dilution of an overnight grown culture of strain *B. animalis* subsp. *lactis* LMG 18314^T, which represents the most frequently isolated *Bifidobacterium* species from probiotic products (Temmerman *et al*., 2003; Masco *et al*., 2005), was prepared in a particle-free sterile saline solution after which triplicate aliquots of 50 µl of each dilution were spread-plated on MCA medium. After 72 h of anaerobic incubation at 37 °C, colonies were counted. In parallel, the dilution series

was also quantified using the CyAn (DakoCytomation) flow cytometer. Initially, live/dead analysis was performed of the 10^{-2} and 10^{-3} dilutions in order to determine the relationship between both fractions. A two-color nucleic acid fluorescence assay was performed using the LIVE/DEAD® *Baclight*™ Bacterial Viability kit (Molecular probes), which consists of a mixture of the green-fluorescent SYTO® 9 for total cell staining and the red-fluorescent propidium iodide for staining cells with damaged membranes. As a result, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. First, 500 µl of a 10^{-1} dilution of the staining mixture was added to 500 µl of each sample dilution and incubated in the dark at room temperature during 15 min. before measurement. The actual cell number was subsequently determined by mixing 100 µl of each dilution (10^{-1}-10^{-5}) with 900 µl saline solution and 0.5 µl SYTO® 16 green-fluorescent nucleic acid stain (Molecular Probes), followed by incubation for 1h at 4 °C, and analysis of each dilution after addition of 10^5 beads per ml.
Finally, DNA extracted from the initial dilution series was analyzed using the real-time PCR protocol as described above. Quantitative data from MCA plating and flow cytometric analysis obtained within the most suitable dilution range were subsequently used to generate a standard curve in which the bacterial cell numbers ml^{-1} was plotted against the C_T value, being the minimal cycle number at which the fluorescence signal exceeds the threshold level.

Quantification

Each probiotic product was analyzed in triplicate using both the 16S rRNA gene and the *recA* targeting primers. As a standard calibration point, each run included one DNA sample from the dilution series originally used to create the standard curve. Following each real-time assay, the LightCycler™ software adjusts the standard curve using this point and calculates the bacterial cell numbers ml^{-1} or g^{-1} on the basis of the C_T value of each sample. This protocol was subsequently evaluated on 29 probiotic products claiming to contain bifidobacteria.

Southern hybridization

For determination of the 16S rRNA gene copy number, single strains of *B. adolescentis*, *B. animalis* subsp. *animalis* and subsp. *lactis*, *B. bifidum* and *B. longum* biotype longum were subjected to Pulsed-Field Gel Electrophoresis (PFGE) fingerprinting as described previously (Masco *et al*., 2005). Two different macrorestriction enzymes, *Spe*I and *Xba*I (Promega), were used. Probe labeling and southern hybridization were performed using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences) according to the manufacturer's instructions. A 1417 bp genus-specific 16S rRNA gene PCR product (Kaufmann *et al*., 1997) was used as probe.

Results and Discussion

Although still a matter of debate, it is assumed that a minimal concentration of 10^6 CFU per ml or g of product is needed for probiotic bacteria to exert a health-promoting effect (Kailasapathy and Chin, 2000). Consequently, the correct enumeration of probiotic bacteria in commercial products on a routine basis is indispensable in the process of delivering a functional product. Due to the lack of suitable isolation media, culture-dependent methods are intrinsically limited in reliability and sensitivity for the selective detection and enumeration of bifidobacteria. In the present study, a new method based on real-time PCR was evaluated for its use as a more rapid and sensitive culture-independent alternative for the quantification of bifidobacteria in probiotic products.

Evaluation of DNA extraction methods

When performing real-time PCR for quantitative means, differences in DNA extraction efficiency (incl. cell lysis and DNA elution) between different extraction methods may influence the outcome of the results. For this purpose, the performance of five DNA extraction methods was evaluated on the basis of OD measurement and performance in real-time PCR analysis. DNA preparations of sufficient purity (1.8 £ $OD_{260/280}$ £ 2.2) were only obtained using the modified Pitcher method (Masco *et al*., 2003) and the phenol chloroform method (Gevers *et al*., 2001). However, when performing real-time PCR analysis with genus-specific 16S rRNA gene and *recA* primers, only the melting curves resulting from the modified Pitcher DNA extracts indicated that a *Bifidobacterium*-specific product had been amplified. This observation was further substantiated by the fact that the lowest C_T values were observed when quantifying DNA samples extracted using the modified Pitcher method. Based on these results, the latter method was considered the most optimal choice for DNA extraction from pure cultures and probiotic products.

The reproducibility of the modified Pitcher method in real-time PCR applications was assessed for three probiotic matrices (i.e. one dairy based product, one tablet and one capsule) by sampling each product four times a day during two subsequent days after which the resulting DNA samples were analyzed in a single real-time PCR run. In total, eight C_T values were

obtained per product from which the average and standard deviation (SD) were calculated. Whereas the SD values were moderately low for the capsule sample (SD = ±0.31 cycles) and the dairy based sample (SD = ±1.95 cycles), a higher SD of ±6.01 cycles was obtained for the tablet sample. Possibly, the relatively high rate of variation obtained for the latter product type may have partly originated from the additional grinding step necessary to obtain a homogenous sample suspension.

Real-Time PCR

In the real-time PCR assay, the non-selective fluorescent dye SYBR Green I was used in combination with *Bifidobacterium*-specific primers. The SYBR Green I chemistry is considered to be equally sensitive as the Taqman chemistry (Malinen *et al*., 2003) and is particularly useful when there is little or no information on the species-specific content of the sample prior to its analysis. In this case, additional qualitative data can be obtained by analysing the amplicons generated using genus-specific primers by Denaturing Gradient Gel Electrophoresis (DGGE) (Requena *et al*., 2002), without the need for separate primers or probes for each possible *Bifidobacterium* species.

Although frequently used as target molecule for real-time quantification, it is well documented that in many bacteria the 16S rRNA gene can be present in multiple copies (http://rrndb.cme.msu.edu/rrndb/servlet/controller), possibly resulting in an overestimation of the number of bacteria in a product sample. This problem can be solved by determining the copy number of the 16S rRNA gene of the detected species, which allows to correct the raw quantitative data, or by targeting a single copy gene. Both approaches were investigated in this study. By southern hybridisation with a ribosomal probe, the number of 16S rRNA gene operons (n) was determined for the type strains of *B. adolescentis* (n = 3), *B. longum* biotype longum (n = 4), *B. animalis* subsp. *animalis* (n = 3) and subsp. *lactis* (n = 3) and for reference strain *B. bifidum* LMG 11582 (n = 2). The rrn copy number of *B. breve* (n = 3) and *B. longum* biotype infantis (n = 3) were previously determined by Bourget and co-workers (1993). The *recA* gene was selected as an alternative real-time PCR target for the 16S rRNA gene because it is present only in a single copy and it has also been proposed as a phylogenetic marker for the differentiation of bifidobacterial species (Kullen *et al*., 1997).

In the present study, new PCR primers (recA-F and recA-R) were designed for the amplification of a 203 bp *recA* fragment of *B. bifidum*, *B. breve*, *B. longum* biotypes longum and infantis and *B. animalis* subsp. *lactis*. The specificity of the 16S rRNA gene and *recA* targeting primers was tested for 13 non-bifidobacterial strains representing the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Lactococcus* and *Streptococcus*. None of these strains yielded a PCR product with either of the two primer sets. Optimisation of the real-time PCR protocol for both the 16S rRNA gene and *recA* primer sets was performed using the type strain of *B. animalis* subsp. *lactis* LMG 18314^T. $MgCl_2$ titration indicated that 4 mM was the optimal concentration for both PCR mixes based on the observation that the obtained curves exhibited the lowest crossing point, the highest fluorescence intensity and the steepest curve slope.

In order to quantify bifidobacteria in probiotic product samples, a standard curve for each real-time PCR target was generated for the mathematical conversion of C_T values into bacterial cell numbers per ml or g of product. In most real-time PCR studies, the initial number of bacteria necessary for the construction of a standard curve is determined by conventional plate counting. In practice, however, this approach can only be used in the low concentration range, given the fact that colony counting of plated samples $\geq 10^4$ CFU ml^{-1} is unreliable, and cannot be extrapolated towards high bacterial concentrations. For this reason, bacterial cell numbers of a dilution series of reference strain LMG 18314^T, which harbors the relevant PCR targets, were determined both by traditional plate counting and by flow cytometric analysis. The latter method allowed an accurate quantification of the dilution series in the range of 10^4 – 10^8 bacterial cell numbers ml^{-1}. Prior live/dead analysis indicated that the fraction of dead cells (1.07 % ±0.25) was negligible compared to the fraction of live cells (95.18 % ±0.96) resulting in a good correlation between the culture-dependent and culture-independent detection ranges. Hence, the results from both approaches were considered complementary for the construction of a standard curve in the range of 10^1 – 10^8 bacterial cell numbers per ml. The standard curve was generated from real-time PCR analysis of DNA extracts using 16S rRNA and *recA* as target genes. A linear relation between initial bacterial concentration and C_T values was obtained in the range corresponding to 10^2 –10^8 and 10^3 – 10^8 bacterial cell numbers using the protocols based on the 16S rRNA gene ($R^2 = 0,99$) and *recA* gene ($R^2 = 0,98$), respectively.

Using both target genes, 29 probiotic products claimed to contain bifidobacteria were subjected to real-time PCR analysis. For each product sample, the bifidobacterial concentration was determined by comparing the obtained C_T value to the standard curve generated from an included standard calibration point (**Table 1).** Linear regression analysis demonstrated a good correlation ($R^2 = 0.9625$) between the real-time PCR results based on the quantification of the 16S rRNA gene and the *recA* gene. For 23 products (79,3 %), *Bifidobacterium* numbers obtained from real-time PCR analysis, using both gene targets, were in agreement within a range of one log unit. In fact, major differences between the two genes were only found in dried probiotic products in which low amounts of bifidobacteria were detected (10^2 - 10^4 bacterial cell numbers g^{-1}). For the products FS10 and 17, bacterial cell numbers obtained using *recA* primers were two log units higher than when using 16S rRNA gene primers. However, in four other products (i.e. FS3, 11, 18 and 19) real-time PCR analysis using 16S rRNA gene targeting primers was able to detect bifidobacteria down to 10^2 bacterial cell numbers g^{-1} whereas the *recA* primers were not able to detect any bifidobacteria. This may be due to the fact that the use of single-copy genes in real-time PCR such as *recA* may result in a higher detection limit compared to the use of the multi-copy 16S rRNA gene. Based on real-time PCR quantification results obtained for 29 probiotic products, the estimated detection limit was found to be 2.5x 10^2 CFU per weight unit for the 16S rRNA gene assay and 5x 10^3 CFU per weight unit for the *recA* targeting assay. The lower detection limit of the 16S rRNA gene assay makes it somewhat more sensitive than *recA* for bacterial quantification, although the multi-operon effect remains an important drawback of this method when analysing unknown samples using universal primers. In case of the products Y2 and FS7, real-time PCR did not produce a detectable *Bifidobacterium*-specific amplicon using either of the two primer sets. This may be due to the fact that the bifidobacterial concentration in these products was below the detection limit of both assays or to the presence of possible PCR inhibitors.

Independent from the gene target used for real-time PCR quantification, it was striking to see that the 29 products displayed a very broad distribution of bifidobacterial concentrations ranging from 0 – 10^8 CFU g^{-1} or ml^{-1}. In fact, only 11 products (38 %) fulfilled the proposed minimum probiotic concentration of 10^6 CFU g^{-1} or ml^{-1} (Kailasapathy and Chin, 2000) when only bifidobacteria are taken into consideration. Although the scientific basis for defining minimum

probiotic dosages is still a matter of debate, it is indeed unlikely that *Bifidobacterium* strains present in probiotic products at concentrations of 10^1-10^4 CFU per weight unit can contribute to the host's intestinal health. On the other hand, it is clear that also other criteria of probiotic bacteria such as viability in the product, survival capacity through the gastrointestinal tract, colonization potential and immunological properties should be considered in order to deliver a probiotic product that answers to the claimed health-promoting effects.

In the present study, we investigated the applicability of real-time PCR for the quantitative analysis of bifidobacteria in commercial probiotic products. The preliminary results indicate that the method described provides a molecular tool for high throughput quantitative analysis of bifidobacteria in commercial probiotic products. Despite the inherent differences in sensitivity and the requisite for prior knowledge of bacterial content, the real-time PCR assays based on 16S rRNA and *recA* gene detection are promising alternatives for culture-based detection and quantification of probiotic organisms in various product types. Due to the fact that real-time PCR analysis is based on the detection of both living and dead bacteria, however, it is important to keep in mind that this results in an overestimation of the number of intact metabolically active *Bifidobacterium* cells.

Acknowledgements

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Table 1. Results of real-time quantification of bifidobacteria in probiotic products using recA and 16S rDNA targeting primers

Product	*Bifidobacterium* species identified by DGGE [a]	Bifidobacterial concentration determined by real-time PCR based on [b]:	
		recA	16S rDNA
Dairy based products (bacterial cell number ml^{-1})			
Yoghurt:			
Y1	*B. animalis* subsp. *lactis*	7.02 E+04 ±1.31 E+04	1.12 E+05 ±6.39 E+04
Y2	*B. animalis* subsp. *lactis*	0	0
Y3	*B. animalis* subsp. *lactis*	2.40 E+04 ±4.55 E+03	8.29 E+04 ±1.06 E+05
Y4	*B. animalis* subsp. *lactis*	2.17 E+05 ±1.34 E+05	9.00 E+04 ±7.42 E+04
Y5	*B. animalis* subsp. *lactis*	1.55 E+04 ±3.88 E+03	2.59 E+04 ±2.48 E+04
Drink:			
D1	*B. animalis* subsp. *lactis*	1.76 E+05 ±5.12 E+04	1.58 E+05 ±8.28 E+04
D2	*B. breve*	1.08 E+06 ±3.50 E+05	2.90 E+05 ±2.27 E+05
Freeze-dried products (bacterial cell number g^{-1})			
Food supplements:			
Capsule			
FS1	*B. animalis* subsp. *lactis, B. bifidum*	2.19 E+06 ±7.22 E+04	6.74 E+06 ±2.15 E+06[d]
FS2	*B. longum* biotype longum	1.04 E+06 ±3.95 E+05	2.34 E+06 ±1.34 E+06
FS3	*B. animalis* subsp. *lactis, B. bifidum*	0	3.46 E+03 ±1.28 E+03[d]
FS4	*B. animalis* subsp. *lactis*	7.00 E+06 ±2.18 E+06	2.83 E+07 ±9.38 E+06
FS5	*B. longum* biotype longum	1.36 E+05 ±5.57 E+04	4.97 E+05 ±1.90 E+05
FS6	*B. animalis* subsp. *lactis*	3.61 E+05 ±8.52 E+04	4.82 E+05 ±2.14 E+05
FS7	*B. animalis* subsp. *lactis, B. bifidum*	0	0
FS8	*B. bifidum*	3.20 E+06 ±3.08 E+05	3.12 E+06 ±1.41 E+06
FS9	*B. animalis* subsp. *lactis*	1.32 E+06 ±2.85 E+05	1.77 E+06 ±1.01 E+06
FS10	*B. animalis* subsp. *lactis*	1.47 E+04 ±7.62 E+03	7.65 E+02 ±6.81 E+02
FS11	*B. animalis* subsp. *lactis, B. bifidum*	0	9.91 E+03 ±4.73 E+03[d]
Powder			
FS12	*B. animalis* subsp. *lactis, B. bifidum, B. longum* biotype longum	4.84 E+05 ±1.96 E+05	7.55 E+05 ±4.74 E+05[d]
FS13	*B. animalis* subsp. *lactis*	5.23 E+05 ±5.68 E+04	1.46 E+06 ±5.89 E+05
FS14	*B. animalis* subsp. *lactis, B. longum* biotype longum	1.00 E+04 ±0.00[c]	2.51 E+03 ±1.55 E+03[d]
FS15	*B. animalis* subsp. *lactis*	1.06 E+07 ±2.00 E+07	2.21 E+07 ±7.27 E+06
Tablet			
FS16	*B. longum* biotype longum	**4.21 E+03 ±1.62 E+03**	4.80 E+03 ±4.99 E+03
FS17	*B. animalis* subsp. *lactis, B. longum* biotype longum	4.60 E+04 ±6.15 E+03	6.74 E+02 ±1.80 E+02[d]
FS18	*B. animalis* subsp. *lactis, B. bifidum*	0	2.71 E+02 ±1.48 E+02[d]
FS19	*B. animalis* subsp. *lactis*	0	1.31 E+03 ±0.00[c]
Pharmaceutical preparations:			
Capsule			
PH1	*B. longum* biotype infantis, *B. bifidum*	3.07 E+06 ±2.64 E+06	5.71 E+06 ±1.62 E+06[d]
PH2	*B. bifidum*	2.18 E+06 ±2.88 E+05	2.00 E+07 ±6.08 E+06
Powder			
PH3	*B. animalis* subsp. *lactis*	3.82 E+07 ±4.01 E+07	2.22 E+08 ±3.52 E+08

[a] Masco *et al*., 2005

[b] Mean concentrations and standard deviations were calculated from triplicate determinations from the same DNA extract

[c] Single experiment

[d] Corrected concentrations obtained by dividing the original bacterial cell numbers ml^{-1} or g^{-1} values by the average rrn copy number of the included species

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Chapter 5

Safety assessment of potentially probiotic *Bifidobacterium* strains

It was on a short-cut through the hospital kitchens that Albert was first approached by a member of the Antibiotic Resistance.

SOURCE: www.nearingzero.net/ screen_res/nz149.jpg

5.1. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products.

Masco L., Van Hoorde K., De Brandt E., Swings J. and Huys G. *Journal of Antimicrobial Chemotherapy*, accepted

Summary

The aim of this study was to assess the antimicrobial susceptibility of a taxonomically diverse set of *Bifidobacterium* strains to different classes of antimicrobial agents using a recently described medium. The susceptibility of 100 strains encompassing 11 bifidobacterial species originating from humans, animals and probiotic products to 12 antimicrobial agents was tested by agar overlay disc diffusion. Based on these results, one or two strains per species were selected for susceptibility testing to nine antibiotics by broth microdilution using the Lactic acid bacteria Susceptibility test Medium (LSM) supplemented with cysteine. The genotypic basis of atypical tetracycline resistance was further characterized using PCR, Southern blotting and partial sequencing. Based on the distribution of inhibition zone diameters and MIC values, all strains tested were susceptible to amoxicillin, chloramphenicol, erythromycin, quinupristin-dalfopristin, rifampicin and vancomycin. Our data also reinforce earlier observations indicating that bifidobacteria are intrinsically resistant to gentamicin, sulphamethoxazole and polymyxin B. Susceptibility to trimethoprim, trimethoprim/ sulphamethoxazole, ciprofloxacin, clindamycin, tetracycline and minocycline was variable. The *tet*(W) gene was responsible for tetracycline resistance in 15 strains including 7 probiotic isolates belonging to the taxa *B. animalis* subsp. *lactis* and *B. bifidum*. This gene was present in a single copy on the chromosome and did not appear to be associated with the conjugative transposon Tn*B1230* previously found in *tet*(W)-containing *Butyrivibrio fibrisolvens*. The use of the LSM + cysteine medium allowed us to discriminate between intrinsic and atypical resistance properties of bifidobacteria, and sets the scene for future definition of epidemiological cut-off values for all important *Bifidobacterium* species. The presence of an acquired *tet*(W) gene in several probiotic product isolates stresses the need for a minimal safety evaluation during the selection of *Bifidobacterium* strains for probiotic use.

Keywords: *Bifidobacterium*, disc diffusion, MICs, LSM, tetracyclines, *tet*(W), probiotics

Introduction

Bifidobacteria are Gram-positive, bifid-shaped anaerobes, that constitute a major group of the human and animal gastrointestinal microbiota. Because these organisms are known to play a pivotal role in maintaining the microbial balance of a healthy intestinal tract, they are frequently applied as probiotics in health-promoting dairy products and dried food supplements (Gomes and Malcata, 1999). Therapeutic administration of antimicrobial agents is likely to affect the intestinal microbial balance, e.g. by suppressing bacterial groups such as bifidobacteria that are beneficial to the host, and often results in intestinal disorders. In co-administration with antibiotics in order to restore the intestinal health of the host, the presence of antimicrobial resistance in probiotic *Bifidobacterium* strains might be regarded as a desirable trait to allow their survival in the gastrointestinal tract. On the other hand, there is also the growing concern that these antimicrobial resistances, if encoded by genes located on mobile elements, may be potentially transferable from probiotic strains to commensal flora or human opportunists. For this reason, the presence of acquired antimicrobial resistances is one of the first safety criteria to be checked during the selection process of a potentially probiotic strain.

Bifidobacteria are generally considered to be food-grade organisms that do not impose health risks on the consumer or the environment. Nevertheless, it should be noted that rare cases of *Bifidobacterium*-associated gastrointestinal and extra-intestinal infections have been described (Brook and Frazier, 1993; Ishibashi and Yamazaki, 2001). In contrast to susceptibility testing of clinically important bacteria (BSAC, Andrews and the BSAC Working Party on Susceptibility Testing, 2001; CLSI, 2005), no standard procedures are specifically dedicated to the determination of resistance phenotypes in *Bifidobacterium* strains. To date, a large variety of methods and protocols have been described for antimicrobial susceptibility testing of bifidobacteria, including agar (overlay) disc diffusion (Charteris *et al*., 1998; Yazid *et al*., 2000; Moubareck *et al*., 2005), broth dilution (Matteuzzi *et al*., 1983; Lim *et al*., 1993) and agar dilution (Moubareck *et al*., 2005). In addition, various growth media have been used primarily on the basis that they meet the complex growth requirements of bifidobacteria. As opposed to conventional susceptibility test media such as Mueller-Hinton (NLSI) and Iso-Sensitest medium (BSAC), none of these *Bifidobacterium*-specific media are well-defined

in terms of minimal interaction between specific antimicrobial agents and growth medium components.

Recently, a newly defined medium formulation referred to as the Lactic acid bacteria Susceptibility test Medium supplemented with cysteine (LSM + cysteine) was proposed for susceptibility testing of bifidobacteria (Klare *et al*., 2005). The LSM + cysteine medium was tested for a minimal set of *Bifidobacterium* reference strains and was not found to display significant antagonistic effects with any of the tested agents. In the present study, the LSM + cysteine medium was used to determine the susceptibility profile of 100 bifidobacterial isolates to 15 common antimicrobial agents, including inhibitors of cell wall synthesis, protein synthesis, nucleic acid synthesis and cytoplasmic membrane function using the agar overlay disc diffusion method and the broth microdilution method. The bifidobacterial isolates under investigation represent 11 species encompassing strains of human and animal origin, strains previously isolated from probiotic products (Masco *et al*., 2005) as well as strains isolated from dental caries (Scardovi and Crociani, 1974) and clinical sources (Hoyles *et al*., 2002). For a subset of strains, the genotypic basis of tetracycline resistance was characterized.

Materials and methods

Bacterial strains

A total of 100 *Bifidobacterium* strains were investigated in this study, including 50 type and reference strains obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm/lmg.htm) and 50 isolates obtained from a variety of probiotic products (Masco *et al*., 2005). The strain selection included representatives of the following species: *B. adolescentis* (n = 6), *B. angulatum* (n = 2), *B. animalis* subsp. *animalis* (n = 2), *B. animalis* subsp. *lactis* (n = 44), *B. bifidum* (n = 8), *B. breve* (n = 7), *B. catenulatum* (n = 2), *B. dentium* (n = 3), *B. gallicum* (n = 1), *B. longum* biotype infantis (n = 7), *B. longum* biotype longum (n = 11), *B. pseudocatenulatum* (n = 5) and *B. scardovii* (n = 2).

Agar overlay disc diffusion testing

Susceptibility testing was based on the agar overlay disc diffusion (DD) method described by Charteris *et al.* (1998) with slight modifications as described by Huys *et al.* (2002). Initially, 10 strains were used to compare the performance of two complex growth media for DD testing, i.e. LSM + cysteine [i.e. 90 % Iso-sensitest broth, 10 % MRS broth and 15 g/l agar, supplemented with 0.3 g/l L-cystein-HCl (Sigma, C-4820)][10] and Modified Columbia Agar (MCA) [i.e. 23 g special peptone (Oxoid, L72), 1 g soluble starch, 5 g NaCl, 0.3 g L-cystein-HCl, 5 g glucose and 15 g agar dissolved in 1 litre of distilled water]. The 10 strains used for the comparison of both media represented the species *B. animalis* subsp. *animalis* and subsp. *lactis*, *B. bifidum*, *B. breve*, *B. dentium*, *B. longum* biotype infantis and biotype longum and *B. scardovii*. Subsequently, the most suitable medium was used for antimicrobial susceptibility testing of all 100 bifidobacterial strains. Strains were grown overnight in the corresponding broth medium at 37 °C under anaerobic conditions (84% N_2, 8% H_2, 8% CO_2). Cell suspensions with an inoculum density (OD_{590}) of 1.0 ± 0.05 were prepared using a vitalab 10 spectrophotometer (Vital Scientific). Further manipulations were performed as described by Huys *et al*. All plates were subsequently incubated under anaerobic conditions at 37 °C during 24h. In the exceptional case that inhibition zones could not be measured accurately after 24h of incubation, plates were incubated for another 48h. Susceptibility was tested against antimicrobial agents (Oxoid) representing inhibitors of cell wall synthesis (i.e. amoxicillin, AMX10), protein synthesis (i.e. gentamicin, GEN10; tetracycline, TET30; chloramphenicol, CHL30; erythromycin, ERY15; clindamycin, CLI2 and quinupristin/dalfopristin, Q/D15), nucleic acid synthesis (i.e. rifampicin, RIF5; ciprofloxacin, CIP5; sulphamethoxazole, RL100 and trimethoprim, TMP5) and inhibitors of cytoplasmic membrane function (i.e. polymyxin B, PB300). Inhibition zones were measured using digital callipers (Mauser digital 2). Partial inhibition was defined as a slightly turbid inhibition zone close to the disc compared to areas of no inhibition further away from the disc. In these cases, inhibition

zone diameters were measured as far as the turbid zone. *B. animalis* subsp. *animalis* strain LMG 10508^T was included as a control strain in every DD assay.

Determination of the Minimal Inhibitory Concentration (MIC)

Strains were grown overnight in LSM + cysteine broth under anaerobic conditions at 37 °C. Fresh inocula with a density of OD_{590} 0.1 ± 0.01 were prepared using a Biolog™ reader (Biolog). In order to obtain a 1/100 dilution, 100 µl of this suspension was transferred to 9.9 ml LSM + cysteine broth. For each agent, two sterile stock solutions were prepared from which a 2-fold dilution series was prepared in LSM + cysteine broth each encompassing a range of four concentrations. Subsequently, 50 µl of each agent dilution was added to the wells of a microtiter plate and mixed with 50 µl of the 1/100 diluted cell suspension. Each plate also included a well only containing 50 µl LSM + cysteine broth as a negative control. Inoculated plates were incubated for 24h under anaerobic conditions at 37 °C. For a selection of strains, the MIC of the following antimicrobial agents was determined: tetracycline (Sigma, T-3383), minocycline (Sigma, M-9511), clindamycin (Sigma, C-5269), ciprofloxacin (Fluka, 17850), polymyxin B (Sigma, P-4932), vancomycin (Sigma, V-2002), trimethoprim (Sigma, T-0667)), sulphamethoxazole (Sigma, S7507) and sulphamethoxazole/trimetoprim (20/1). The MIC was determined as the lowest concentration of antimicrobial agent at which no visible growth was recorded. The MIC_{90} was defined as the lowest concentration of a given agent that inhibited growth of 90% of the tested strains. For each agent tested, a control strain of which the MIC was located within the concentration range tested, was included for reproducibility assessment.

Molecular detection of *tet* genes and Tn*B1230* in strains showing atypical tetracycline resistance

Total genomic DNA was extracted as described previously (Masco *et al*., 2003). The 50 µl PCR assay mix used for detection of tetracycline resistance genes contained 32.8 µl MQ, 5 µl 10x PCR buffer including 15 mM $MgCl_2$ (Applied Biosystems), 200 µM of each of four dNTPs (dATP, dCTP, dGTP and dTTP), 3 µl oligonucleotide primer (10 pmol/µl) (**Table 1**) and 1 U AmpliTaq DNA polymerase (Applied Biosystems). A 50 ng/µl dilution of total genomic DNA was used as template. All PCR amplifications were performed using a Perkin Elmer 9600 thermal cycler. In a first PCR assay, the presence of tetracycline resistance genes encoding a ribosomal protection (RP) mechanism was investigated with the group-specific degenerate primer pairs DI/DII and Ribo-2-FW/Ribo-2-RV. The following temperature program was used for primer pair DI/DII: initial denaturation (95 °C, 5 min); 35 cycles of denaturation (95 °C, 45 s), annealing (45 °C, 45 s) and extension (72 °C, 1 min); final extension (72 °C, 10 min). For the degenerate Ribo2 primers, a touchdown PCR was performed as follows: initial denaturation (95 °C, 5 min); 22 cycles of denaturation (94 °C, 30 s), annealing (30 s with 1 °C decrements from 72 °C to 50 °C), and extension (72 °C, 30 s); 20 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; final extension (72°C, 7 min). Strains containing an RP-type *tet* gene were subjected to additional PCR assays with primers specific for individual

genes of the RP group, i.e. *tet*(M), *tet*(O), *tet*(S), *tet*(T) and *tet*(W). In addition, strains with atypical resistance for tetracycline were also tested for the presence of the tetracycline efflux genes *tet*(K) and *tet*(L). For the detection of the RPP *tet* genes as well as the tetracycline efflux genes, the following temperature program was used: initial denaturation (95 °C, 5 min), followed by 25 cycles of denaturation (94 °C, 45 s), annealing [primer-specific temperature, 1 min (**Table 1**)], extension (72 °C, 1 min), and a single final extension step (72 °C, 10 min). PCR products were visualized by electrophoresis on a 1 % agarose gel stained with ethidium bromide.

The presence of transposon Tn*B1230* was verified by PCR using primers designed by Dr. Katarzyna Kazimierczak (personal communication) based on the published sequence of Tn*B1230* (accession number: AJ222769) (Melville *et al*., 2004), as well as by hybridisation of the DNA samples of all *tet*(W)-positive strains with a Tn*B1230* specific PCR product, derived from *Butyrivibrio fibrisolvens* (DNA of this organism was kindly provided by Dr. Karen Scott).

Localisation and copy number determination of the *tet*(W) gene

Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay (1983). *B. breve* strain LMG 13194, which is known to possess one plasmid of size 5.6 kb (Bourget *et al*., 1993), was used as a positive control for plasmid DNA extraction. Plasmids were separated after electrophoresis on a 0.7 % agarose gel during 3.5h at 100V and visualized by ethidium bromide staining. Total genomic DNA was prepared *in situ* in agarose blocks and digested with endonucleases *Spe*I and *Xba*I and subsequently separated using Pulsed-Field Gel Electrophoresis (PFGE) as described previously (Masco *et al*., 2005). Probe labeling and southern hybridization were performed using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences) according to the manufacturer's instructions. A 1,100 bp *tet*(W)-specific amplicon generated with PCR primers DI/DII was used as probe.

Partial sequencing of the *tet*(W) gene

The *tet*(W) gene of a selection of strains, including two *B. pseudocatenulatum* strains, two *B. animalis* subsp. *lactis* strains and one *B. adolescentis* strain, was amplified using the degenerate primer pair DI/DII as described above. PCR products were purified using a QIAquick PCR Purification kit (Qiagen) according to the instructions of the manufacturer. Sequence analysis was performed using the Big Dye™ Termination RR Mix V3.1 (Applied BioSystems) on an ABI 3100 automated DNA sequencer (Applied BioSystems). For each sequencing reaction, a 10 µl reaction mixture was prepared containing 0.67 µl Big Dye™, 1.66 µl 5x sequencing buffer (Applied BioSystems), 3 µl DI or DII (5 µM), 3.67 µl sterile Milli-Q water and 1 µl of the purified PCR product. The temperature program consisted of 30 cycles of denaturation (96°C, 15 s), annealing (35°C, 1 s) and extension (60°C, 4 min). PCR products were purified using the Genesis workstation 200 (Tecan Customized Solutions). Sequence analysis was performed using the software package Kodon (Applied Maths) and sequences were blasted against the EMBL sequence database to confirm the *tet*(W) identity of the amplicons.

Table 1. PCR primers and conditions used for detection of *tet* genes

Primer pair	Gene(s) targeted	Sequence[a]	Annealing temp (°C)	Amplicon size (bp)	Positive control plasmids (reference)	Primer reference
DI	RPP type	5'-GAYACNCCNGGNCAYRTNGAYTT-3'	45	1083	pJI3 (Morse *et al*., 1986)	Clermont *et al*., 1997
DII		5'-GCCCARWANGGRTTNGGNGGNACYTC-3'				
Ribo-2-FW	RPP type	5'-GGMCAYRTGGATTTYWTIGC-3'	touchdown[b]	1187	pJI3 (Morse *et al*., 1986)	Aminov *et al*., 2001
Ribo-2-RV		5'-TCIGMIGGIGTRCTIRCIGGRC-3'				
TetK-FW1	*tet* (K)	5'-TTATGGTGGTTGTAGCTAGAAA-3'	55	382	pAT102 (Gevers *et al*., 2003)	Gevers *et al*., 2003
TetK-RV1		5'-AAAGGGTTAGAAACTCTTGAAA-3'				
TetL-FW3	*tet* (L)	5'-GTMGTTGCGCGCTATATTCC-3'	55	717	pAT103 (Gevers *et al*., 2003)	Gevers *et al*., 2003
TetL-RV3		5'-TGAAMGRWAGCCCACCTAA-3'				
TetM-FW	*tet* (M)	5'-ACAGAAAGCTTATTATATAAC-3'	55	171	pJI3 (Morse *et al*., 1986)	Aminov *et al*., 2001
TetM-RV		5'-TGGCGTGTCTATGATGTTCAC-3'				
DI/TetM-RV	*tet* (M)		45	1513	pJI3 (Morse *et al*., 1986)	Clermont *et al*., 1997
TetO-FW1	*tet* (O)	5'-AATGAAGATTCCGACAATTT-3'	55	801	pAT121 (Gevers *et al*., 2003)	Sougakoff *et al*., 1987
TetO-RV1		5'-CTCATGCGTTGTAGTATTCCA-3'				
TetS-FW	*tet* (S)	5'-GAAAGCTTACTATACAGTAGC-3'	50	169	pVP2 (Perreten *et al*., 1997)	Aminov *et al*., 2001
TetS-RV		5'-AGGAGTATCTACAATATTTAC-3'				
TetT-FW	*tet* (T)	5'-AAGGTTTATTATATAAAAGTG-3'	55	169	Total DNA	Aminov *et al*., 2001
TetT-RV		5'-AGGTGTATCTATGATATTTAC-3'				
TetW-FW	*tet* (W)	5'-GAGAGCCTGCTATATGCCAGC-3'	64	168	pGEM-tetW (Barbosa *et al*., 1999)	Aminov *et al*., 2001
TetW-RV		5'-GGGCGTATCCACAATGTTAAC-3'				

[a] N = A, C , G and T; R = A and G; W = A and T; Y = C and T

[b] PCR conditions are described in Materials and Methods

Nucleotide sequence accession numbers

The *tet*(W) sequences determined in this study have been submitted to the EMBL database under the following accession numbers: *B. pseudocatenulatum* LMG 11593, AM181315; *B. pseudocatenulatum* LMG 10505^T, AM181316; *B. animalis* subsp. *lactis* LM 624, AM181317; *B. animalis* subsp. *lactis* LMG 18314, AM181318; *B. adolescentis* LMG 11579, AM181319.

Results

Agar overlay disc diffusion testing

The effect of the growth medium on the inhibition zone sizes of 12 antimicrobial agents determined with the agar overlay DD method was assessed for 10 *Bifidobacterium* strains. For this purpose, the MCA medium was compared with the LSM + cysteine medium. For all disc types tested, differences in inhibition zones between both media increased with the zone diameter thus indicating that the diffusion gradient of the antimicrobial agent is mostly affected by the medium composition at lower concentrations. Differences in inhibition zones between both media of more than 3 mm (40 of 120 strain-disc combinations) were mostly found for zone diameters > 20 mm (29 of 40 strain-disc combinations). In 72,4 % of these 29 strain-disc combinations a larger inhibition zone was found on LSM + cysteine medium which suggests that this medium exerts lower overall antagonistic effects compared to the MCA medium. Taken together with the fact that LSM + cysteine medium was able to sustain growth of all bifidobacterial strains so far tested (Klare *et al*., 2005), it was decided to use this formulation as standard medium in all subsequent DD and MIC assays.

In order to evaluate the reproducibility of the agar overlay DD method, reference strain LMG 10508^T was included in each series of antibiogram determinations. An overall mean standard deviation of ±1.9 mm with a maximum variation of 3 mm was obtained for all agents tested. For a subset of strains, mainly encompassing one strain/species, antibiotic susceptibility profiles were compared after 24h and 48h incubation. In most cases, diameters of inhibition zones measured after 48h coincided with those obtained after 24h of incubation (data not shown). Between both incubation times, an overall mean standard deviation of ±0.4 mm with a single maximum variation of 4 mm was obtained for all agents tested. The results of DD susceptibility testing of 100 *Bifidobacterium* strains to 12 antimicrobial compounds are summarized in **Table 2**. A unimodal distribution of large inhibition zones was observed for amoxicillin (≥25 mm), chloramphenicol (≥27 mm), erythromycin (≥28 mm), quinupristin-dalfopristin (≥25 mm) and rifampicin (≥21 mm). This type of distribution was noticed for all species tested, which implies that the overall susceptibility to these compounds probably is characteristic for the genus *Bifidobacterium*. Conversely, small unimodally distributed inhibition

Table 2. Inhibition zone diameters recorded for 100 *Bifidobacterium* strains in disc diffusion testing of 12 antimicrobial agents on LSM + cyteine medium

	Inhibition zone diameter range (mm)											
Species (number of strains)	**AMX10**	**GEN10**	**TET30**	**ERY15**	**CLI2**	**Q/D15**	**CIP5**	**RL100**	**TMP5**	**CHL30**	**RIF5**	**PB300**
B. adolescentis (6)	26-44	6-11*	10-41	34-38	16-36	32-43	20-23	6-35*	6-18*	31-41	29-39*	6-12*
B. angulatum (2)	38-43	6-9*	35-38	34-35	30-31	37-38	20-21	6	17-23*	35-37	28-29	6
B. animalis subsp. *animalis* (2)	29-39*	6-10*	28-36	30-38	13-16*	30-36	12-14*	6	35-44*	29-40	23-31*	6-10*
B. animalis subsp. *lactis* (44)	26-44	6-13*	11-37*	30-50	26-43	33-46	6-18*	6	21-46*	28-40	21-35*	6-16*
B. bifidum (8)	29-50	6-15	26-50	31-50	28-50	27-40	6-14*	6-29*	6-30*	29-50	23-40	6-22*
B. breve (7)	25-30	6-13*	28-34	28-33	18-30	29-35	9-12*	6	16-23*	27-33	26-32	6-10
B. catenulatum (2)	35-37	6-7*	31-32	31-32	15-18	30-33	18*	6-12*	16-20*	31-34	23*	6-8*
B. dentium (3)	33-38	6-9*	34-41	36-39	32-34	38-41	18-21	6	19-22*	37-40	27-33	6
B. gallicum (1)	31	8	40	39	31	35	17	6	6	42	37	10
B. longum biotype infantis (7)	31-50	12-17	27-50	32-50	25-50	34-50	12-16*	6-50*	9-50*	32-50	24-50	6-12*
B. longum biotype longum (11)	32-44	8-12*	33-48	34-47	31-43	38-50	6-14*	6-27*	6-22*	35-52	29-43	6-18 *
B. pseudocatenulatum (5)	32-42*	8-11*	14-35*	32-42*	28-40*	31-44*	19-21*	6-27*	11-19*	32-40*	22-30*	7-10*
B. scardovii (2)	27-31*	8-9*	30-33*	29-35*	21-25*	25-28*	14-15*	6	17*	32-35*	30-31*	7

* partial inhibition

AMX, amoxicillin; GEN, gentamicin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; Q/D, quinopristin-dalfopristin; CIP, ciprofloxacin; RL, sulphamethoxazole; TMP, trimethoprim; CHL, chloramphenicol; RIF, rifampicin, PB, polymyxin B

zone diameters were measured for gentamicin, sulphamethoxazole and polymyxin B, suggesting that bifidobacteria are intrinsically resistant to these agents. However, it should be noted that partial inhibition was occasionally noted for some of the tested species. In case of gentamicin, slightly larger inhibition zones were observed for strains belonging to *B. longum* biotype infantis compared to the other species tested (**Table 2**). A relatively broad distribution of inhibition zone diameters was noticed for tetracycline (10-50 mm) and trimethoprim (6-50 mm). For these two compounds, levels of resistance appeared to be strain-specific in particular species. In case of tetracycline, a large number of *B. animalis* subsp. *lactis* and *B. pseudocatenulatum* strains as well as one *B. adolescentis* strain displayed smaller inhibition zones compared to the other strains tested. In the trimethoprim DD assay, smaller inhibition zones were measured for *B. gallicum* and several representatives of *B. adolescentis*, *B. bifidum* and *B. longum* biotype longum. In this regard, it should be noted that the larger zones were mainly observed in cases of partial inhibition. Finally, bimodally distributed inhibition zone diameters were observed for clindamycin and ciprofloxacin. Strains displaying smaller inhibition zones for ciprofloxacin belonged to the species *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve* and *B. longum* biotype longum and can thus be considered as resistant to this agent. In case of clindamycin, smaller inhibition zones pointed to reduced susceptibility to this compound within *B. animalis* subsp. *animalis* and *B. catenulatum* and for some strains of *B. breve* and one *B. adolescentis* strain.

Determination of Minimal Inhibitory Concentrations (MIC)

MICs were determined for nine antimicrobial compounds. This selection included four agents for which bifidobacteria showed a broad or bimodal distribution of DD inhibition zone diameters, i.e. ciprofloxacin, trimethoprim, tetracycline and clindamycin, as well as two antibiotics to which bifidobacteria are presumed to be intrinsically resistant, i.e. polymyxin B and sulphamethoxazole. In addition, MICs were also determined for three compounds not included in the DD assays, i.e. minocycline, vancomycin and the therapeutic combination trimethoprim/sulphamethoxazole. For reproducibility testing, a control strain was included in every series of MIC determinations. A maximum deviation of one $\log_2$ dilution step was recorded for all antimicrobial agents tested. A selection of strains displaying a broad range of

inhibition zone diameters, usually encompassing two strains per species, was subjected to MIC determination. Based on the broad zone diameter distribution obtained for this agent (**Table 2**), an extended selection of strains was included for MIC measurements of tetracycline. In addition, strains that possessed a *tet*(W) gene as well as some *tet*(W)-negative strains were also subjected to MIC determination of minocycline. Because vancomycin was not included in the disc diffusion assay, 78 strains were subjected to MIC determination of this antimicrobial agent. MIC analysis of clindamycin was restricted to *B. animalis* subsp. *animalis* and subsp. *lactis* in order to substantiate the differences observed among inhibition zones (**Table 2**). MIC values could be classified in three categories (**Table 3**). In a first category, the tested bifidobacterial strains displayed high overall MIC values indicating intrinsic resistance of all members of the genus to the compound. This was the case for polymyxin B (MIC_{90}: >64 mg/L) and sulphamethoxazole (MIC_{90}: >1024 mg/L) and these findings thus confirm the results obtained with the DD assay. As could be predicted from the large zone diameter measured in DD testing, the type strain of *B. bifidum* displayed a much lower MIC of 1 mg/L for polymyxin B. In a second category, MIC values were more variable and broadly distributed within the strain set tested. In line with DD results, broad MIC distributions were obtained for trimethoprim (MIC range: ≤0.5 - 64 mg/L; MIC_{90}: 16 mg/L) and tetracycline (MIC range: ≤0.5 - 32 mg/L; MIC_{90}: 16 mg/L). In case of trimethoprim, most strains displayed an MIC of 8 mg/L although some strains of *B. adolescentis*, *B. longum* biotype infantis and *B. pseudocatenulatum* exhibited higher MIC values up to 64 mg/L. In agreement with DD data, higher MIC values of tetracycline were observed for *B. adolescentis*, *B. animalis* subsp. *lactis* and *B. pseudocatenulatum.* Overall, most strains (42,5%) displayed an MIC value ≤0.5 mg/L for this compound. MIC values of the second-generation tetracycline compound minocycline (MIC range: 1 - 32 mg/L; MIC_{90}: 16 mg/L) were usually distributed in a similar way as those of tetracycline. However, for all three *B. pseudocatenulatum* strains tested, considerably lower MIC values (1 - 2 mg/L) were obtained compared to those observed for tetracycline (32 mg/L) (**Table 4**). In contrast to the intrinsic sulphamethoxazole resistance observed in DD testing, MIC values of the combined therapeutic preparation trimethoprim/sulphamethoxazole (1/20) were broadly distributed and comparable to or lower than the MIC values observed for trimethoprim. Although to a lesser extent, a relatively broad

Table 3. MICs of *Bifidobacterium* strains to different antimicrobial agents determined by broth microdilution in LSM broth + cysteine

Species (no. of strains)	MIC range (mg/L)								
	Polymyxin B	Sulphamethoxazole	Ciprofloxacin	Trimethoprim	Tetracycline (no. of strains)*	Minocycline (no. of strains)*	Trimethoprim/ Sulphamethoxazole	Vancomycin (no. of strains)*	Clindamycin (no. of strains)*
B. adolescentis (2)	32	8, 512	1-2	2-64	<=0,5-32 (6)	32 (1)	4	<=0,125 (6)	ND
B. angulatum (2)	16, 32	64, >1024	1-8	<=0.5-1	<=0,5	ND	<=0,5-2	<=0,125	ND
B. animalis subsp. *animalis* (2)	>64	>512, >1024	4-8	<=0.5	1	2 (1)	<=0,5-8	<=0,125-0,5	0,5-1
B. animalis subsp. *lactis* (2)	>64	>1024	4	<=0.5-1	4-16 (10)	4-16 (10)	<=0,5	<=0,125-0,250 (24)	<=0,125 (10)
B. bifidum (2)	1, 64	64, >1024	16	4-8	<=0,5-4	4 (1)	2-4	<=0,125-1 (8)	ND
B. breve (2)	64	=>1024	8-16	2-8	<=0,5	ND	<=0,5-8	<=0,125-0,250 (7)	ND
B. catenulatum (2)	>64	64->1024	2	8	<=0,5	ND	2-4	<=0,125	ND
B. dentium (3)	32, 64	>1024	<=0.5-1	1-2	<=0,5	ND	1-2	<=0,125	ND
B. gallicum (1)	32	>512	2	<=0,5	<=0,5	4	<=0,5	<=0,125	ND
B. longum biotype infantis (2)	>64	64->512	4	4-16	1-2	1 (1)	2-16	<=0,125-0,250 (7)	ND
B. longum biotype longum (2)	32, >64	64->1024	4-16	8	<=0,5	ND	2-16	<=0,125-0,5 (9)	ND
B. pseudocatenulatum (2)	>64	64	1-4	16-32	1-32 (5)	1-2 (3)	1-2	<=0,125 (5)	ND
B. scardovii (2)	>64	>512->1024	2-4	2-8	<=0,5	ND	2	<=0,125-0,250	ND
MIC_{90} (all strains)	>64	>1024	16	16	16	16	8	0,25	0,5

ND: Not determined

*MIC values for tetracycline, minocycline, vancomycin and clindamycin were determined for an extended set of strains per species. Deviating numbers are indicated between brackets.

MIC distribution was also recorded for ciprofloxacin (MIC_{90}: 16 mg/L) for which the majority of the strains tested displayed an MIC of 4 mg/L. A third category was represented by overall low MIC values of clindamycin and vancomycin. The latter compound is known to diffuse poorly in agar media (Thomson *et al*., 1995) for which reason vancomycin resistance of bifidobacteria was only tested by means of the broth microdilution method. The highest MIC value of vancomycin (1 mg/L) was observed for some strains of the species *B. bifidum*, whereas the majority of the strains tested were found to be inhibited at even lower concentrations (MIC_{90}: 0.250 mg/L). These data indicate that most members of the genus *Bifidobacterium* are susceptible to this compound. As was also noticed from the DD results, MIC values of

Table 4. Phenotypic and genotypic characterization of tetracycline resistance for 29 *Bifidobacterium* strains

Species (number of strains)	MIC (mg/L)		*tet* gene
	Tetracycline	Minocycline	
B. adolescentis (n = 1)	32	32	*tet* (W)
B. pseudocatenulatum (n = 3)	32	1-2	*tet* (W)
B. animalis subsp. *lactis* (n = 3)	32	4-16	*tet* (W)
B. animalis subsp. *lactis* (n = 7)	16	4-16	*tet* (W)
B. animalis subsp. *lactis* (n = 3)	8	4-16	*tet* (W)
B. animalis subsp. *lactis* (n = 2)	4	4	*tet* (W)
B. bifidum (n = 2)	4	2-4	*tet* (W)
B. longum biotype infantis (n = 1)	2	1	NF
B. animalis subsp. *animalis* (n = 2)	1	2	NF
B. pseudocatenulatum (n = 2)	1	ND	NF
B. longum biotype infantis (n = 1)	1	ND	NF
B. bifidum (n = 1)	1	≤0.5	NF
B. breve (n = 2)	≤0.5	≤0.5	NF
B. scardovii (n = 2)	≤0.5	ND	NF
B. dentium (n = 2)	≤0.5	ND	NF
B. gallicum (n = 1)	≤0.5	4	NF
B. bifidum (n = 1)	≤0.5	ND	NF
B. adolescentis (n = 1)	≤0.5	ND	NF

ND: Not determined: NF: Not found

clindamycin for *B. animalis* subsp. *animalis* were found to be slightly higher (MIC range: 0.5 - 1 mg/L) than for *B. animalis* subsp. *lactis* (≤0.125 mg/L).

Genetic basis of tetracycline resistance

A subset of 29 strains, covering a broad tetracycline MIC range (≤0,5 - 32 mg/L), was subjected to PCR detection of tetracycline resistance genes (**Table 4**). In 15 strains displaying MICs in the range of 4 - 32 mg/L, the presence of the *tet*(W) gene conferring ribosomal protection (RP) against tetracycline was detected. These strains belonged to *B. adolescentis*, *B. pseudocatenulatum*, *B. animalis* subsp. *lactis* and *B. bifidum*. The identity of the *tet*(W) amplicons was confirmed by partial sequence analysis (positions 319 – 1263, i.e. 49% of the 1921 bp *tet*(W) gene of *Butyrivibrio fibrisolvens,* Acc. No. AJ427421). These analyses included three strains with comparable MIC values for both tetracycline and minocycline and two *B. pseudocatenulatum* strains for which lower minocycline MIC values (1 - 2 mg/L) were recorded compared to those observed for tetracycline (32 mg/L). At two positions, base substitutions resulting in a mutation at the protein level were detected in the partial *tet*(W) sequence. At amino acid positions 262 and 265 of the TetW protein of *Butyrivibrio fibrisolvens*, glycine and arginine were substituted in both *B. pseudocatenulatum* strains by aspartic acid and leucine, respectively.

None of the 29 strains tested were positive for the efflux genes *tet*(K) and *tet*(L).

In all 15 *tet*(W)-positive strains, the *tet*(W) gene was found to be present in a single copy on the chromosome, since no plasmids could be isolated. The presence of Tn*B1230* could not be demonstrated by PCR or by southern hybridisation in any of these strains. Likewise, attempts to transfer the *tet*(W) gene from *B. animalis* subsp. *lactis* LMG 11615 to *B. adolescentis* LMG 10734 by filter mating did not result in successful transconjugants (L. Masco, unpublished data).

Discussion

In contrast to clinically relevant bacteria for which resistance monitoring is indispensable (BSAC, Andrews and the BSAC Working Party on Susceptibility Testing, 2001; CLSI, 2005), no standard procedures or interpretive breakpoints have been established for antimicrobial susceptibility testing of bifidobacteria. In this regard, several test media have been used that meet the complex growth requirements of bifidobacteria, including tryptic soy broth supplemented with 0.2 % yeast extract and 0.06 % L-cystein-HCl (Lim *et al*., 1993), TPY medium (Charteris *et al*., 1998; Yazid *et al*., 2000; Matteuzzi *et al*., 1983) and Brucella agar supplemented with 5% laked sheep blood and vitamin K1 (Moubareck *et al*., 2005). However, the susceptibility test medium should not only sustain growth of the tested organisms but should also provide a non-interfering matrix exerting minimal antagonistic effects against a wide range of antimicrobial agents. Although the defined and universally applied test media Iso-Sensitest Agar (ISA) (BSAC) and Mueller-Hinton agar (NLSI) meet the latter requirement, it has been shown that they do not always support growth of any given LAB (lactic acid bacteria) food strain (Huys *et al*., 2002). Recently, the newly developed LSM + cysteine medium formulation was found to provide sufficient growth support of bifidobacterial reference strains (Klare *et al*., 2005). Furthermore, the use of this formulation in a microdilution method resulted in correct indications of known MICs for a set of international control strains. In an initial phase of the present study, the performance of the LSM + cysteine medium was compared to the undefined MCA medium which is routinely used to culture bifidobacteria for DD susceptibility testing of 10 *Bifidobacterium* reference strains. Especially at the lower concentrations of the gradients, it was found that inhibition zones gradually decreased on MCA compared to those recorded on LSM + cysteine agar. This observation substantiates the previous finding that the latter medium formulation is much more effective in minimizing antagonistic effects between antimicrobial agents and growth medium components (Klare *et al*., 2005).

Using the LSM + cysteine medium, the antibiogram of 100 *Bifidobacterium* strains belonging to 11 species and representing animal and human strains as well as isolates from probiotic products was recorded using the agar overlay DD method. A selection of these

strains were also included for MIC determination using a broth microdilution assay. Due to the lack of published cut-off values that allow separating strains with and without an acquired antimicrobial resistance mechanism in *Bifidobacterium*, susceptibility data were interpreted largely on the basis of histogram analyses. Depending on the relative position and the type of distribution (unimodal, bimodal or broad) of DD and/or MIC data in these histograms, strains were classified as resistant or susceptible. Ideally, 10 or more strains belonging to the same taxon need to be investigated in order to delineate epidemiological cut-off values at the species level. Because this condition was fulfilled for some but not all species in this study, interpretation of susceptibility data was mainly restricted to the genus level. In general, anaerobes such as bifidobacteria possess a natural resistance to aminoglycosides due to the lack of a cytochrome-mediated drug transport (Bryan and Kwan, 1970). Accordingly, overall resistance was observed to gentamicin, which confirms earlier findings (Matteuzzi *et al*., 1983; Lim *et al*., 1993; Charteris *et al*., 1998; Yazid *et al*., 2000; Moubareck *et al*., 2005). Likewise, our data also indicate that bifidobacteria are generally resistant to polymyxin B, a compound that is almost exclusively active against Gram-negatives (Hoeprich, 1970). Strains were generally resistant to sulphamethoxazole as a separate compound. However, the therapeutic combination trimethoprim/sulphamethoxazole was highly active against most bifidobacterial strains due to the synergetic inhibitory effect on the thymidine synthesis. This points to the fact that the reduced resistance towards the therapeutic combination trimethoprim/sulphamethoxazole is mainly due to the action of trimethoprim. All tested strains appeared to be uniformly susceptible to chloramphenicol, erythromycin, rifampicin and amoxicillin, which is in agreement with data of previous studies (Matteuzzi *et al*., 1983; Lim *et al*., 1993; Charteris *et al*., 1998; Yazid *et al*., 2000; Moubareck *et al*., 2005). The overall susceptibility to the β-lactam antimicrobial amoxicillin may be explained by the lack of β-lactamase activity in *Bifidobacterium* (Moubareck *et al*., 2005). Although not yet reported, quinupristin-dalfopristin was also found to be an active antimicrobial combination. Susceptibility to trimethoprim, ciprofloxacin, tetracycline and minocycline was variable and strain-specific. The range of MIC values of tetracyclines may be specific for some taxa (e.g. *B. pseudocatenulatum*), but clearly more strains need to be tested to substantiate this observation. Except for some *B. bifidum* strains, all tested *Bifidobacterium* strains were considered to be susceptible to vancomycin. This finding contradicts the conclusion of Charteris and co-workers (1998) stating that vancomycin

resistance is a general characteristic of bifidobacteria. Possibly, this discrepancy may be due to the limited reliability of the disc diffusion method used by the latter authors considering the fact that vancomycin is known to diffuse poorly in agar media (Thomson *et al*., 1995). Although our data suggest that bifidobacteria are susceptible to clindamycin, comparison of MIC data indicated reduced susceptibilities for some strains. In support of their recent taxonomic description (Masco *et al*., 2004), strains of *B. animalis* subsp. *animalis* and subsp. *lactis* included in this study could also be differentiated on the basis of quantitative differences in clindamycin MIC values.

The *tet*(W) gene is known to be responsible for acquired tetracycline resistance in several rumen anaerobes and in human *Bifidobacterium longum* strains (Scott *et al*., 2000). Recently, this gene was also detected in single strains of tetracycline-resistant *B. pseudocatenulatum* and *B. bifidum* (Moubareck *et al*., 2005). In the present study, *tet*(W) was found in 15 strains encompassing the species *B. pseudocatenulatum*, *B. bifidum, B. animalis* subsp. *lactis* and *B. adolescentis*. To our knowledge, this is the first report on the presence of *tet*(W) in the latter two *Bifidobacterium* species. All *tet*(W)-positive strains showed an MIC of tetracycline in the range of 4 – 32 mg/L, whereas all strains with lower MIC values contained none of the tested *tet* genes. These findings indicate that an MIC of ≤ 2 mg/L can be proposed as epidemiological cut-off value for defining tetracycline susceptibility in bifidobacteria, but more strains need to be analyzed to substantiate this. The observation that resistance towards tetracycline was not always joined by resistance to minocycline initiated partial *tet*(W) gene sequence analyses and revealed two amino acid substitutions. However, whether these substitutions are responsible for the difference in susceptibility remains to be investigated.

Previously, a sequence similarity of > 99,9% was reported between the *tet*(W) gene of a rumen isolate of *Butyrivibrio fibrisolvens* and human *B. longum* isolates, suggesting that the gene is potentially exchangeable between animals and humans (Scott *et al*., 2000). In *Butyrivibrio fibrisolvens*, the *tet*(W) gene is integrated in the conjugative transposon Tn*B1230* which is thought to be responsible for the environmental dissemination of *tet*(W) (Melville *et al*., 2004). In contrast, none of the 15 *tet*(W)-positive *Bifidobacterium* strains in this study were found to contain Tn*B1230* using both PCR-based and southern blotting detection. Similarly, Scott and co-workers (2000) were not able to identify this mobile element in human

B. longum isolates. This finding that a different genetic support exists for the *tet*(W) gene in bifidobacteria and merits further investigation. Previously, it has been shown that the *tet*(W) gene is transferable between genotypically diverse *Butyrivibrio fibrisolvens* strains (Scott *et al*., 1997). In this study, preliminary conjugation experiments between *B. animalis* subsp. *lactis* LMG 11615 and *B. adolescentis* LMG 10734 by filter mating did not result in successful transconjugants (L. Masco, unpublished data), but do not rule out the possibility that the gene is transferable using other recipients under different selective conditions.

Interest in the issue of antimicrobial resistance as a safety criterion for lactic acid bacteria used in probiotic applications is growing at a steady pass. In this context, interpretive reading of bifidobacterial resistance phenotypes has been significantly hampered by the lack of a validated method tested on a taxonomically diverse set of strains. The use of the recently developed LSM + cysteine medium formulation allowed us to discriminate between intrinsic and atypical resistance properties of bifidobacteria. Together with reduced susceptibilities to trimethoprim and/or ciprofloxacin in several strains, resistance to tetracyclines appears to occur in multiple *Bifidobacterium* species. In all cases, the tetracycline resistance phenotype was linked to the presence of an acquired non-plasmid located *tet*(W) gene. In follow-up studies, the LSM + cysteine medium needs to be tested using an extended strain panel (³ 10 strains per species) which will allow to define epidemiological cut-off values (http://www.srga.org/eucastwt/WT_EUCAST.htm) for all major *Bifidobacterium* species. This will not only lead to a more widespread acceptance of the method, but will also result in the definition of interpretive standards for use in the food industry and by regulatory agencies.

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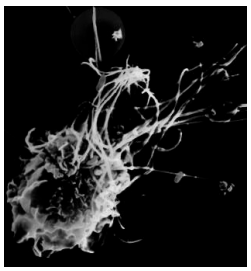
Chapter 6

Functionality assessment of potentially probiotic *Bifidobacterium* strains



6.1. *Bifidobacterium* strains induce *in vitro* cytokine production by human peripheral blood mononuclear cells in a strain-specific way.

Masco L., Pot B., Foligné B., Grangette C., Swings J. and Huys G. *Submitted to FEMS Immunology and Medical Microbiology*

Summary

Imbalance of the intestinal microflora, resulting from a reduction of 'protective' bacteria, is frequently associated with intestinal inflammation. Administration of probiotic strains has been suggested as a potential therapeutic approach for the prevention and treatment of inflammatory bowel disease (IBD). We investigated the immunomodulatory capacity of a taxonomically diverse set of 50 *Bifidobacterium* strains, including commercial probiotic product isolates as well as human commensal strains, with respect to their potential to induce the production of the cytokines IL-10, IL-12, TNF-α and IFN-γ by peripheral blood mononuclear cells isolated from healthy donors. The results of this *in vitro* analysis confirmed that cytokine stimulation profiles are strain-specific and revealed that bifidobacteria are potent inducers of the anti-inflammatory IL-10, while induction of IL-12, TNF-α and IFN-γ is low compared to a pro-inflammatory control strain. Given the key role of TNF-α in IBD pathogenesis and the fact that intestinal inflammation is associated with low IL-10 and high pro-inflammatory IL-12 and IFN-γlevels, our results suggest that administration of *Bifidobacterium* strains that promote high IL-10/IL-12 and IL-10/TNF-α ratios in combination with a low induction of IFN-γproduction may induce a shift towards a more anti-inflammatory state, resulting in alleviation of IBD symptoms. The *in vitro* approach used in this study allowed to screen a set of *Bifidobacterium* strains for their immunomodulatory potential in a high-throughput manner and enabled the selection of potential candidate strains for probiotic therapy of IBD.

Keywords: *Bifidobacterium*, IL-10, IL-12, TNF-α, IFN-γ, IBD, probiotics

Introduction

Most of the microorganisms that enter the human body are readily engulfed and destroyed by phagocytes, which provide an innate, antigen non-specific first line of defence against infection. Additional defence is provided by the acquired immune system to which mainly dendritic cells, macrophages and B cells contribute by acting as professional antigen-presenting cells (APC) proffering foreign antigens to naïve T cells. Macrophages in particular react to bacterial stimulation by secreting T-cell-activating cytokines and expressing membrane bound co-stimulatory molecules, which will bind to corresponding receptors on T cells. Local inflammatory responses are regulated by the secretion of both pro- and anti-inflammatory cytokines (Isolauri, 1999). Aberrant intestinal inflammatory responses can cause gastrointestinal diseases such as Crohn's disease and ulcerative colitis (UC). Balance control of pro- and anti-inflammatory cytokines has been suggested to alleviate intestinal inflammation, normalize gut mucosal dysfunction, and down-regulate hypersensitivity reactions (Isolauri *et al*., 2001).

Biological therapies including anti-inflammatory treatment with TNF monoclonal antibodies (mAbs), INF-γ mAbs or IL-12 mAbs have been shown to prevent the onset of colitis (Kojouharoff *et al*., 1997; Berg *et al*., 1996; Rennick *et al*., 1997). Likewise, the systemic delivery of the anti-inflammatory cytokine IL-10 (Van Deventer *et al*., 1997) as well as in situ synthesis by genetically engineered *Lactococcus lactis* bacteria in IL-10 knockout mice (Steidler *et al*., 2000) are promising biotherapeutic strategies in the treatment of inflammatory bowel disease (IBD).

Recent studies have focussed on the potential use of non-manipulated probiotic bacteria exhibiting natural anti-inflammatory properties to reduce inflammation (Venturi *et al*., 1999; Schultz *et al*., 2002; Ishikawa *et al*., 2003; Bibiloni *et al*., 2005; Peran *et al*., 2005). Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit to the host' (http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm). Members of the genus *Bifidobacterium* are frequently used as probiotics, primarily because they constitute a major part of the human and animal gastrointestinal tract and because they play an important role in the control of the intestinal microflora and in the maintenance of its normal state (Dunne *et al*., 1999). Recently, it was

shown that the administration of a *B. infantis* strain could alleviate symptoms in inflammatory bowel syndrome (IBS) patients. This effect was associated with a normalization of the ratio between the cytokines IL-10 and IL-12 towards a more anti-inflammatory state which was characterized by an enhanced IL-10 production (O'Mahony *et al*., 2005). The use of VSL#3, a probiotic preparation consisting of a mixture of eight lactic acid bacteria (LAB) including single strains of *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium infantis* was shown to inhibit mucosal TNF-α and IFN-γ production and has been found effective in treatment of UC (Bibiloni *et al*., 2005). Likewise, the preventive effect of a LAB-fermented milk, containing *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Lactobacillus acidophilus* on IBD in SAMP1/Yit mice was determined (Matsumoto *et al*., 2001). A lower release of TNF-α and IFN-γ and an increase of IL-10 from mesenteric lymph node cells was observed and associated with reduced histological injury and reduced ileal tissue weight.

The purpose of the present study was to identify pro- and anti-inflammatory characteristics of a taxonomically diverse set of *Bifidobacterium* strains, including probiotic product isolates, human commensal strains and strains implicated in clinical infections. The effect of bifidobacteria on the immunocompetent cell-derived production of the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines IL-12, IFN-γ and TNF-α was assessed *in vitro* using human peripheral blood mononuclear cells.

Materials and methods

Bacterial strains

A total of 50 *Bifidobacterium* strains were investigated in this study, including 22 type and reference strains obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm/lmg.htm) and 28 isolates obtained from various commercial probiotic products (Masco *et al*., 2005). The species identity of all strains was assessed or confirmed using BOX-PCR fingerprinting (Masco *et al*., 2003). The strain selection encompassed the following species: *B. adolescentis* (n = 2), *B. angulatum* (n = 2), *B. animalis* subsp. *lactis* (n = 21), *B. bifidum* (n = 5), *B. breve* (n = 3), *B. catenulatum* (n = 2), *B. dentium* (n = 1), *B. gallicum* (n = 1), *B. longum* biotype infantis (n = 3), *B. longum* biotype longum (n = 6), *B. pseudocatenulatum* (n = 2) and *B. scardovii* (n = 2). *Lactobacillus salivarius* Ls-33, kindly provided by Danisco (France), and *Lactococcus lactis* MG1363 (Gasson, 1983) were included as control strains, both of which have known effects on peripheral blood mononuclear cell (PBMC)-derived cytokine production (Foligné *et al*., submitted). All bifidobacterial strains were subcultured on modified Columbia agar [23 g special peptone (Oxoid), 1 g soluble starch, 5 g NaCl, 0.3 g L-cystein-HCl (Sigma), 5 g glucose and 15 g agar dissolved in 1 liter of distilled water (BCCM™/LMG, Medium 144)] at 37 °C under anaerobic conditions (84 % N_2, 8 % H_2, 8 % CO_2). Strain *L. salivarius* Ls-33 was subcultured at 37 °C in MRS broth (Difco), whereas strain *Lc. lactis* MG1363 was subcultured at 30 °C in M17 supplemented with 0.5 % glucose. Subsequently, all strains were grown overnight in appropriate broth medium. The bacteria were washed twice in phosphate-buffered saline (PBS, pH 7.2), and adjusted to McFarland 6, which corresponded to a final concentration of 10^9–10^{10} CFU ml^{-1}. The bacterial suspensions were stored at –80 °C in PBS solution containing 20% glycerol.

PBMC preparation

Fresh human blood was collected from five healthy individuals at the Centre Regional de Transmission Sanguine (CRTS) de Lille. Human PBMCs were isolated after dilution at a 1:2 ratio with PBS (Gibco BRL, supplemented with 1.32 mg l^{-1} $CaCl_2$ and 1 mg l^{-1} $MgCl_2$, i.e. supplemented PBS) on a Ficoll density gradient according to the instructions of the manufacturer (Amersham Biosciences). After centrifugation (400x g for 20 min. at 12 °C), the PBMCs were aspirated and washed 3 times in supplemented PBS (350x g for 10 min. at 12 °C). PBMCs were subsequently resuspended at a concentration of 2 x 10^6 cells ml^{-1} using RPMI 1640 medium (Gibco BRL) containing 10 % (w/v) heat-inactivated fetal calf serum (Gibco BRL), 1% (w/v) L-glutamine and 150 µg ml^{-1} gentamicin to prevent bacterial overgrowth.

Activation of mononuclear cells

PBMCs (2 x 10^6 cells ml^{-1}) were cultured in the presence of *Bifidobacterium* and control strains (2 x 10^7 CFU ml^{-1}) during 24 h at 37 °C in an atmosphere of air with 5 % CO_2. Control cultures only contained culture medium. After incubation, the culture supernatant was collected and stored at –20 °C until use.

Cytokine quantification

The levels of IL-10, IL12 (p70), TNF-α and IFN-γ were quantified by a specific enzyme-linked immunosorbent assay (ELISA) (Pharmingen, according to the manufacturer's recommendations). Briefly, maxisorp microtiter strip wells (Nunc™) were coated overnight at 4 °C with purified murine anti-human cytokine antibodies. After incubation, the wells were incubated with PBS containing 1 % BSA to block non-specific protein binding. A standard was prepared with known concentrations of recombinant cytokines, covering a detection range of 31.25 to 2000 pg ml^{-1} for TNF-α and IFN-γ, and 15.62 to 1000 pg ml^{-1} for IL-10 and IL-12. The standard series and appropriate dilutions of the samples (1/2 and 1/10 for quantification of IL-10 and IL-12; 1/10 and 1/50 for quantification of TNF-α and IFN-γ) were added to respective wells and incubated during 2 hours at room temperature. After washing, biotinylated antibodies (1 µg ml^{-1}) were added and incubated during 1 h at room temperature. Detection was performed after incubation with streptavidine-horseradish peroxidase conjugate (Jackson) and subsequent revelation using tetramethylbenzidine substrate. H_2SO_4 (2N) was added to stop the colorimetric reaction. The plates were read at 450 nm on an ELX808 Microplate Reader (Biotek Instrument, inc). Cytokine titers were determined using the KC4 for windows software (Biotek Instrument, inc).

Statistical analysis

For each cytokine, the response of human PBMCs to the individual strains was calculated from the responses of five independent donors to account for donor-to-donor variation and was expressed as means ± SEM. Statistical significance was evaluated by the Mann-Whitney U test. Differences were considered significant at a p value of <0.05.

Results

In the present study, human peripheral blood mononuclear cells (PBMC) isolated from five independent blood donors were cultured with live bifidobacteria, mostly represented by probiotic product isolates and human commensals. The results of their potential to induce PBMC-derived production of IL-10, IL-12, IFN-γ and TNF-α are shown in **Table 1**. Although a donor-related effect was observed in terms of absolute quantification, the ranking of strains based on cytokine profiles was highly comparable between the five donors (data not shown). Variation in induction of cytokine expression could hence be evaluated among strains, while pro- or anti-inflammatory tendencies were evaluated by comparison to control strains *L. salivarius* Ls-33 and *Lc. lactis* MG1363. The former strain is known to induce an overall anti-inflammatory cytokine profile, whereas *Lc. lactis* MG1363 stimulates the expression of pro-inflammatory cytokines (Foligné *et al*., submitted).

The bifidobacterial strains tested induced IL-10, IL-12, TNF-α and IFN-γ production in a strain-specific manner (**Table 1**). Because most species were represented by only a few strains, it was not possible to identify species-related effects on cytokine production. However, among the 21 *B. animalis* subsp. *lactis* strains tested, cytokine induction levels were as variable as the levels measured among strains of different species, which does not indicate a species-related effect. Of all strains tested, 46 % induced IL-10 levels above the expression level induced by the anti-inflammatory control strain *L. salivarius* Ls-33 (1306 ±774 pg ml^{-1}); only three strains (i.e. LM 311, LM 588 and LMG 21590) induced expression levels lower than the pro-inflammatory control strain *Lc. lactis* MG1363 (472 ±134 pg ml^{-1}). An overall low induction of IL-12 production was witnessed when PBMCs were cultured with bifidobacteria (50 ±0 to 692 ±325 pg ml^{-1}). For none of the tested strains, the expression level exceeded the one induced by *Lc. lactis* MG1363 (897 ±256 pg ml^{-1}). Together with *L. salivarius* Ls-33, 20 *Bifidobacterium* strains did not induce IL-12 production above a basal level of 50 pg ml^{-1}. None of the bifidobacteria induced IFN-γ expression levels above the ones induced by *Lc. lactis* MG1363 (73364 ±20641 pg ml^{-1}), while 58 % of the strains induced less IFN-γ than *L. salivarius* Ls-33 (17771 ±8134 pg ml^{-1}), of which 27,6 % was significantly lower. Noteworthy, even small amounts of IL-12 were able to induce IFN-γ

Table 1. Cytokine secretion by human PBMCs after exposure to *Bifidobacterium* strains[a]

Species	Strain No.	Origin	IL-10 (pg ml^{-1})	IFN-g (pg ml^{-1})	IL-12 (pg ml^{-1})	TNF-a (pg ml^{-1})
B. adolescentis	LMG 10502^{T}	Adult, intestine	1174 ± 322	21921 ± 11587	77 ± 17*	41727 ± 15198
	LMG 18897	Human, faeces	1039 ± 254	10491 ± 5872*	59 ± 9*	14957 ± 4012
B. angulatum	LMG 10503^{T}	Human, faeces	926 ± 199	3096 ± 1671*	54 ± 4*	19818 ± 16647
	LMG 11568	Sewage	931 ± 198	713 ± 474***	50 ± 0*	4171 ± 1232*
B. animalis subsp. *lactis*	LMG 18314	Yoghurt	1608 ± 299*	21773 ± 11017	72 ± 17*	35926 ± 16880
	LMG 11615	Infant, faeces	1292 ± 274*	33493 ± 12944	119 ± 37*	51177 ± 15138
	LM 2	Probiotic product	1618 ± 230*	5927 ± 3248*	50 ± 0*	29229 ± 9371
	LM 13	Probiotic product	1714 ± 572	4815 ± 2794*	50 ± 0*	15574 ± 5461
	LM 118	Probiotic product	1288 ± 181*	38620 ± 15185	125 ± 42*	46153 ± 14623
	LM 125	Probiotic product	1447± 334*	18061 ± 8260	98 ± 35*	30078 ± 10852
	LM 135	Probiotic product	1064 ± 234	40933 ± 19393	101 ± 32*	35296 ± 14822
	LM 165	Probiotic product	1700 ± 399*	9184 ± 4589*	51 ± 1*	37214 ± 16359
	LM 198	Probiotic product	1686 ± 430	1824 ± 1220*	50 ± 0*	7638 ± 2109*
	LM 216	Probiotic product	1911 ± 453*	12923 ± 6824*	50 ± 0*	34125 ± 9107
	LM 232	Probiotic product	1837 ± 304*	17140 ± 8972*	65 ± 9*	37593 ± 9847
	LM 241	Probiotic product	1909 ± 391*	19680 ± 10167	67 ± 17*	31891 ± 8234
	LM 271	Probiotic product	1272 ± 194*	33815 ± 15891	345 ± 123**	51475 ± 18517
	LM 350	Probiotic product	1713 ± 449*	15345 ± 9739	50 ± 0*	27516 ± 9154
	LM 371	Probiotic product	1224 ± 144*	41876 ± 16121	132 ± 40*	45789 ± 13905
	LM 391	Probiotic product	901 ± 191*	44556 ± 17780	173 ± 67***	38366 ± 11281
	LM 441	Probiotic product	1352 ± 335*	19981 ± 10773	64 ± 14*	21354 ± 4124
	LM 586	Probiotic product	1233 ± 198*	28793 ± 16414	107 ± 32*	52055 ± 21176
	LM 594	Probiotic product	1102 ± 118*	46889 ± 17821	142 ± 55*	37427 ± 11379
	LM 624	Probiotic product	1387 ± 494	3112 ± 2100*	50 ± 0*	15022 ± 8307
	LM 635	Probiotic product	1300 ± 236*	34517 ± 16272	82 ± 20*	44689 ± 16771
B. bifidum	LMG 11041^{T}	Breast-fed infant, faeces	890 ± 263	627 ± 389***	129 ± 70*	2881 ± 1118*
	LMG 13200	Not known	1613 ± 449*	14776 ± 13114	172 ± 109*	9130 ± 3856
	LM 311	Probiotic product	215 ± 70	50 ± 0***	50 ± 0*	170 ± 120***
	LM 381	Probiotic product	1072 ± 290	20610 ± 12347	150 ± 67*	26569 ± 11108
	LM 588	Probiotic product	372 ± 143	71 ± 19***	50 ± 0*	1506 ± 1194***
B. breve	LMG 13208^{T}	Infant, intestine	2144 ± 265*	782 ± 408***	50 ± 0*	21429 ± 8320
	LMG 10645	Not known	783 ± 302	15345 ± 8273	125 ± 72*	15692 ± 4934
	LM 646	Probiotic product	1218 ± 293	608 ± 393***	50 ± 0*	2182 ± 953***
B. catenulatum	LMG 11043^{T}	Adult, intestine or faeces	1709 ± 294*	20729 ± 6929*	70 ± 9*	45282 ± 14277
	LMG 18894	Sewage	1257± 199*	55498 ± 15137	238 ± 67*	38505 ± 10611
B. dentium	LMG 11045^{T}	Dental caries	1175 ± 272*	10487 ± 5444*	50 ± 0*	30728 ± 13659
B. gallicum	LMG 11596^{T}	Adult, intestine	745 ± 132	3115 ± 1806*	119 ± 69*	27113 ± 14616
B. longum biotype infantis	LMG 8811^{T}	Infant, intestine	982 ± 325	49762 ± 16546	692 ± 325	51933 ± 17890**
	LMG 18902	Infant, faeces	1877 ± 369*	25181 ± 12223	160 ± 76*	22683 ± 6235
	LM 418	Probiotic product	2075 ± 436*	5448 ± 3504*	50 ± 0*	19491 ± 6958
B. longum biotype longum	LMG 13197^{T}	Adult, intestine	484 ± 75	2772 ± 1631*	50 ± 0*	1954 ± 1570***
	LMG 18899	Adult, faeces	1341 ± 204*	3938 ± 1728*	50 ± 0*	19113 ± 10980
	LM 257	Probiotic product	1526 ± 400*	4740 ± 1892*	50 ± 0*	16006 ± 6097
	LM 614	Probiotic product	2118 ± 556*	2825 ± 1690*	50 ± 0*	12044 ± 3562
	LM 669	Probiotic product	1672 ± 227*	3047 ± 1517*	50 ± 0*	17602 ± 3514
	LM 676	Probiotic product	1409 ± 300*	9345 ± 3609*	72 ± 27*	18550 ± 6321
B. pseudocatenulatum	LMG 10505^{T}	Infant, faeces	1185 ± 208*	1094 ± 630***	50 ± 0*	6916 ± 2737*
	LMG 18904	Human, faeces	1424 ± 455	316 ± 114***	50 ± 0*	8019 ± 5569
B. scardovii	LMG 21589^{T}	50-year-old woman, blood	602 ± 133	24261 ± 17731	169 ± 61*	34385 ± 12692
	LMG 21590	44-year-old woman, hip	385 ± 161	21628 ± 14112	76 ± 24*	26396 ± 10292
Lactobacillus salivarius	Ls-33	Human isolate	1306 ± 774	17771 ± 8134*	50 ± 0*	14756 ± 7313
Lactococcus lactis	MG1363	Cheese starter	472 ± 134	73364 ± 20641**	897 ± 256**	26546 ± 8394
Non-stimulated control			50± 0	50± 0	50± 0	50± 0

[a] Results are expressed as means of five independent experiments ± SEM; values below the detection limit were appointed a basic value of 50 pg ml^{-1}

* significantly different from *Lactococcus lactis* MG1363 ($p < 0.05$)

** significantly different from *Lactobacillus salivarius* LS33 ($p < 0.05$)

*** significantly different from *Lactococcus lactis* MG1363 *and Lactobacillus salivarius* LS33 ($p < 0.05$)

production, which underlines its potent inducers function (Trinchieri, 2003). Finally, half of the strains tested induced TNF-α expression levels above the ones induced by *Lc. lactis* MG1363 (26546 ±8394 pg ml^{-1}), however none of these differences were significant. 22 % of the strains induced less TNF-α production than *L. salivarius* Ls-33 (14756 ±7313 pg ml^{-1}) of which 36,4% was significantly lower. For all cytokines quantified, *B. bifidum* LM 311 induced the lowest expression levels.

In order to evaluate the immunomodulatory properties of bifidobacteria, the IL-10 to IL-12 and IL-10 to TNF-α ratios were used as a marker to differentiate bifidobacteria with potential pro-inflammatory properties from those with anti-inflammatory properties (**Figure 1**). Of the 50 *Bifidobacterium* strains tested, 18 strains (i.e. 36 %) displayed IL-10/IL-12 ratios above the one calculated for *L. salivarius* Ls-33 (26.1 ±15.5), however, none were significantly different. None of the strains tested had lower IL-10/IL-12 ratios than *Lc. lactis* MG1363 (0.8 ±0.5) and only three strains (i.e. *B. bifidum* LM 588, *B. longum* biotype infantis LMG 8811^T, *B. scardovii* LMG 21589^T) were not significantly different from the *Lc. lactis* MG1363 control strain in this respect ($p > 0,05$). Thirty strains (i.e. 60 %) displayed IL-10/TNF-α ratios above the one calculated for *L. salivarius* Ls-33, however, none of these differences were significant ($p > 0.05$). None of the strains tested had lower IL-10/TNF-α ratios than *Lc. lactis* MG1363. Moreover, for 42 strains (i.e. 84 %) this value was significantly higher than the ratio of *Lc. lactis* MG1363 ($p < 0.05$). Except for some cases of low absolute values, ranking of strains based on the IL-10/TNF-α ratio corresponded to the ranking of strains based on the IL-10/IL-12 ratio.

Of all strains tested, *B. breve* LMG 13208^T, *B. longum* biotype longum LM 614 and biotype infantis LM 418, *B. animalis* subsp. *lactis* LM 13 and LM 198, exhibited the highest IL-10/IL-12 and IL-10/TNF-α ratios in association with high absolute values of IL-10 and low IFN-γ levels. Conversely, *B. animalis* subsp. *lactis* LM 391, *B. breve* LMG 10645, *B. scardovii* LMG 21589^T and LMG 21590 and *B. longum* biotype infantis LMG 8811^T were characterized by the lowest IL-10/IL-12 and IL-10/TNF-α ratios in association with low absolute values of IL-10 and high IFN-γ levels (**Figure 2**).

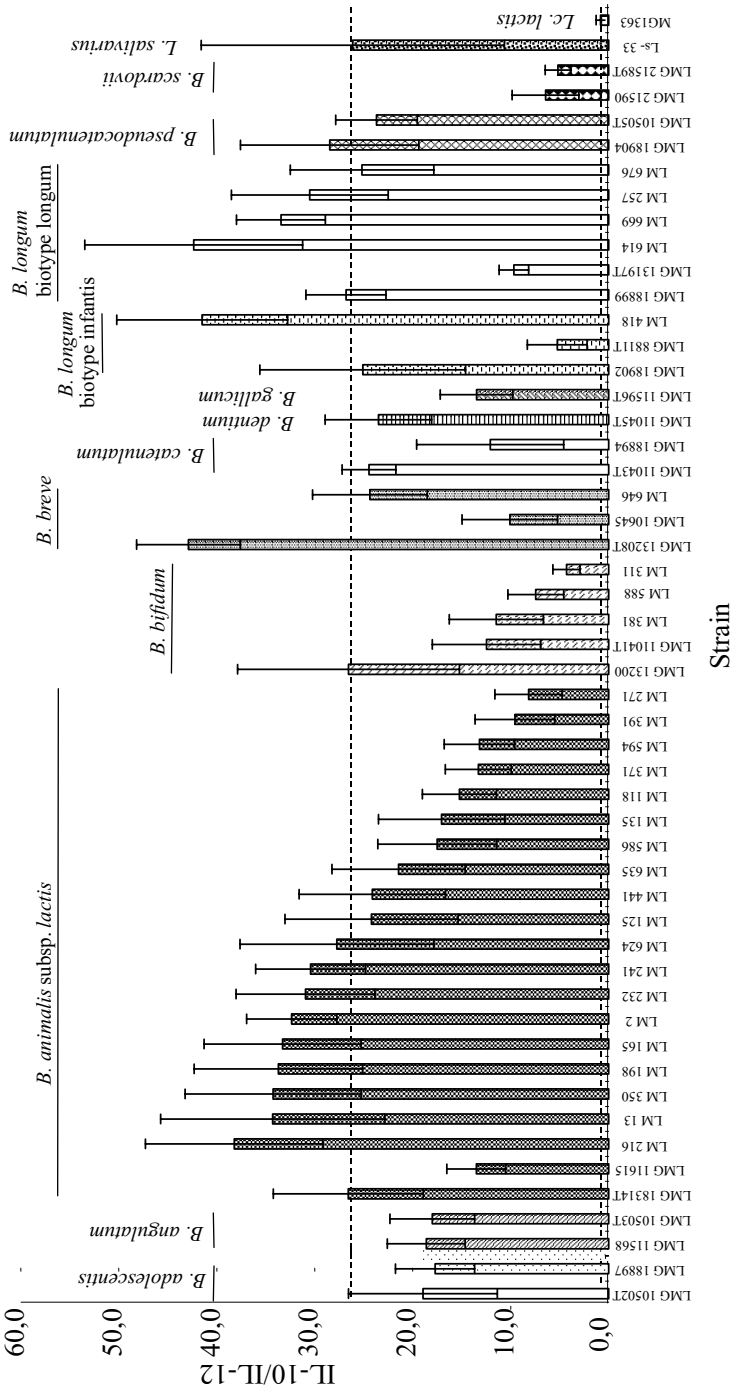


Figure 1. IL-10/IL-12 ratio, calculated for each strain and expressed as the mean ± standard error of five independent blood donors. Dashed lines correspond to the IL-10/IL-12 ratio of control strains *L. salivarius* Ls-33 and *Lc. lactis* MG1363 All strains were significantly different from *Lc. lactis* MG1363 ($p < 0.05$); only *Lc. lactis* MG1363 was significantly different from *L. salivarius* Ls-33 ($p < 0.05$).

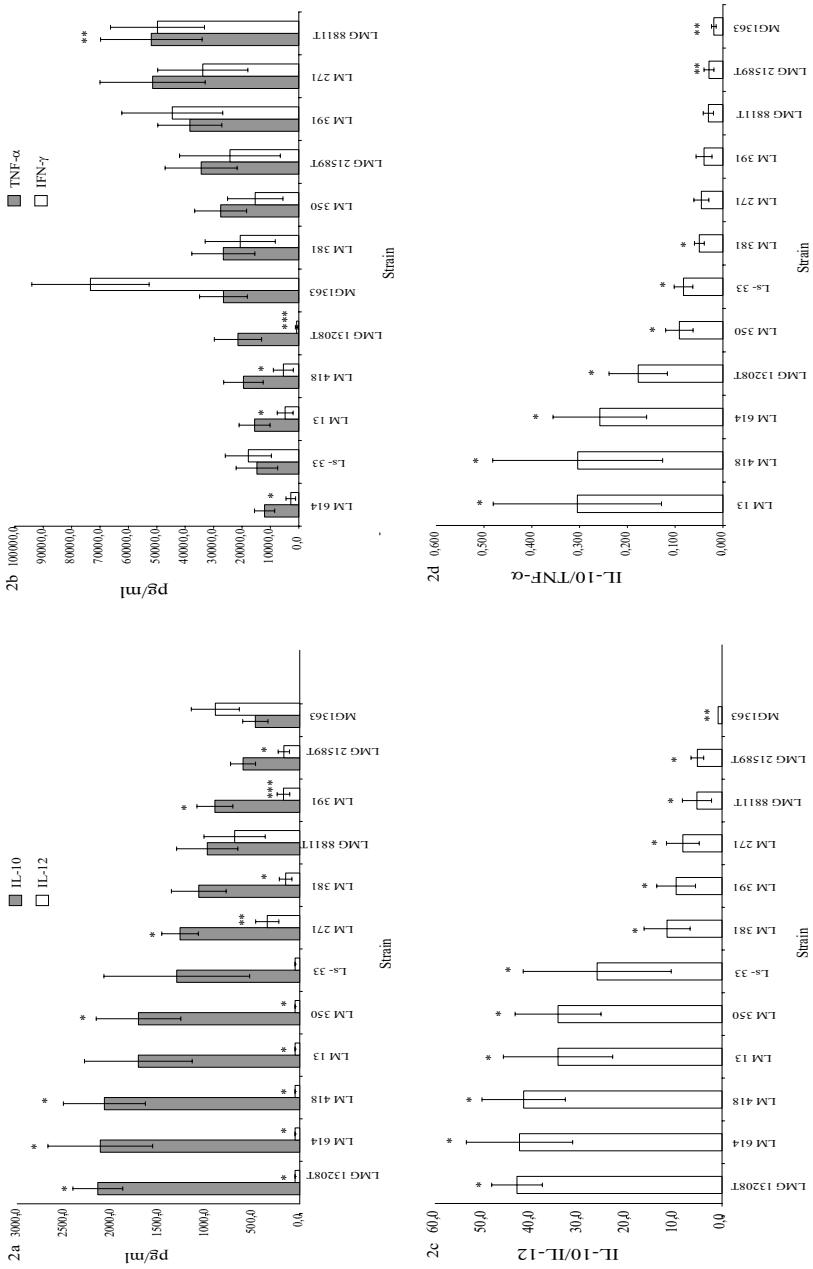


Figure 2. a) and b) Cytokine production by human PBMCs induced by / c) and d) IL-10/IL-12 and IL-10/TNF-α ratios of *B. animalis* subsp. *lactis* LM 13, LM 271, LM 391 and LM 350, *B. bifidum* LM 381, *B. breve* LMG 13208^{T}, *B. longum* biotype infantis LMG 8811T and LM 418, *B. longum* biotype longum LM 614, *B. scardovii* LMG 21589^{T}, *L. salivarius* Ls-33 and *Lc. lactis* MG1363. Bars represent the mean ± SEM. Significantly different ($p < 0.05$) from *Lc. lactis* MG1363 * and *L. salivarius* Ls-33** or both ***.

Discussion

The intestinal flora and its interaction with the host play an important role in maintaining the homeostasis of the immune system. Imbalance of the intestinal microflora, resulting from a reduced level of 'protective' bacteria, has been associated with intestinal inflammation (Fiocchi, 1998; Shanahan, 2000). In recent years, probiotic administration has been considered as a rational option in IBD therapy. Although the underlying mechanisms are not fully understood, some probiotics are believed to modulate the host defenses by influencing the intestinal immune system. In the present study, an *in vitro* comparison of a taxonomically diverse set of *Bifidobacterium* strains was performed in order to classify and select strains according to their indigenous pro- and anti-inflammatory properties for subsequent *in vivo* investigation.

All bifidobacterial strains tested induced the production of detectable amounts of the pro-inflammatory cytokines IFN-γ, TNF-α and of the anti-inflammatory cytokine IL-10 regardless of their respective species designation and in a strain-specific way. In contrast, very low amounts of the pro-inflammatory cytokine IL-12 were observed. These cytokine responses do not seem to be restricted to bifidobacteria, but have also been observed in other lactic acid bacteria (Mercenier *et al*., 2004).

In an attempt to classify *Bifidobacterium* strains tested on the basis of pro- and anti-inflammatory properties, the IL-10/IL-12 and IL-10/TNF-α ratios were determined and compared with the anti-inflammatory *L. salivarius* Ls-33 and pro-inflammatory *Lc. lactis* MG1363 control strains. The IL-10/IL-12 marker has proven to be useful to evaluate the *in vivo* effect of probiotic administration to irritable bowel syndrome (IBS) patients (O'Mahony *et al*., 2005). In the latter study, abnormal IL-10/IL-12 ratios resulting from low IL-10 and high IL-12 release by PBMCs in patients with IBS were normalized and accompanied by an alleviation of IBS symptoms after administration of a *B. infantis* strain. Likewise, the administration of the probiotic strain *Lactobacillus rhamnosus* GG, which reduces the TNF-α/IL-10 ratio, has been reported to exert intestinal anti-inflammatory effects both in human (Schultz *et al*., 2004) and in experimental intestinal inflammation (Dieleman *et al*., 2003). TNF-α plays a key role in the pathogenesis of IBD, as evidenced by the increased production of TNF-α in the intestinal mucosa from IBD patients (Reinecker *et al*., 1993; Reimund *et al*.,

1996), as well as by a number of clinical studies using anti-TNF-α mAb therapy (Rutgeerts *et al*., 2004). Furthermore, correlation between the *in vitro* characteristics of *Lactobacillus* strains determined using the PBMC model and their capability to modulate intestinal inflammation in a mouse model of chemically (TNBS) induced colitis has been established in previous studies (Mercenier *et al*., 2004; Foligné *et al*., in press; Foligné *et al*., submitted). Strains, that induce high IL-10 levels including control strain *L. salivarius* Ls-33, as well as strains like *Lc. lactis* MG1363 that induce substantially less IL-10 and higher TNF-α and IL-12 levels were introduced in the mouse colitis model. These experiments indicated that strains with a high IL-10/IL-12 ratio were able to reduce intestinal inflammation compared to low ratio strains that never improved the immunological status (Foligné *et al*., submitted). Furthermore, given the fact that IFN-γ levels are elevated in all genetic models of IBD and that monoclonal antibodies to IFN-γ have been successfully used in the treatment of Crohn's disease (Rutgeerts *et al*., 2002; Van Assche and Pearce, 2004), strains associated with low induction levels of IFN-γ are preferred for therapeutic application in IBD treatment. From our findings, it is to be expected that *Bifidobacterium* strains LMG 13208^{T} (*B. breve*), LM 614 (*B. longum* biotype longum), LM 418 (*B. longum* biotype infantis), LM 13 and LM 198 (*B. animalis* subsp. *lactis*), which display a higher IL-10/IL-12 and IL-10/TNF-α ratio than *L. salivarius* Ls-33 in addition to low induction of IFN-γ may reduce colitis in the *in vivo* TNBS model. By comparing data on cytokine responses obtained *in vitro* with reference strains having known *in vivo* probiotic effects, it is thus possible to obtain a rational indication of the probiotic potential of candidate strains. Furthermore, it may be possible to define *Bifidobacterium*-specific *in vitro* reference values, which can be used for the selection of candidate strains for subsequent *in vivo* testing.

It is clear from the present study that bifidobacteria induce the PBMC-derived cytokine production to a variable extent, as evidenced by the fact that the species *B. breve*, *B. animalis* subsp. *lactis* and *B. longum* biotype infantis harbour strains which elicit opposite effects as discussed above. This observation confirms earlier findings of strain-specificity of immunomodulation (Mercenier *et al*., 2004; Foligné *et al*., 2005). Despite the fact that the majority of the *B. animalis* subsp. *lactis* isolates as well as half of the *B. longum* biotype longum isolates have highly similar if not identical macrorestriction patterns in Pulsed Field Gel

Electrophoresis (PFGE) (data not shown), there was no correlation between cytokine induction profile and strain type. This finding confirms earlier indications that a high level of genomic relatedness among strains as determined by PFGE is not always sustained by phenotypic (and hence immunomodulatory) characteristics (Masco *et al*., 2005). This reinforces our opinion that, although PFGE is often considered as the gold standard of bacterial typing, caution is needed in the interpretation of such results during probiotic selection. Likewise, we were interested to see to what extent *Bifidobacterium* isolates from probiotic products would score better in cytokine profiling compared to the other strains included in the study. However, our results indicate that not all product isolates would be equally successful in IBD treatment. Conversely, the *in vitro* screening method enabled the identification of potential anti-inflammatory characteristics among the commensal bifidobacteria tested. However, *in vivo* analysis with a mouse colitis model needs to be performed to sustain these observations. In contrast to most other strains, the two *B. scardovii* strains isolated from clinical sites (Hoyles *et al*., 2002) were potent inducers of the pro-inflammatory cytokines tested and only weak inducers of IL-10. However, it is not clear whether this specific cytokine response is associated with the pathology of the strains.

In vivo experiments (animal models) and clinical trials can provide the ultimate evidence for the fact that specific probiotic strains may provide protective or therapeutic benefits in inflammatory bowel disease (IBD) (Fedorak and Madsen, 2004). However, because *in vivo* studies are time-consuming, laborious and for ethical reasons not adapted to perform large-scale strain screening assays, the *in vitro* approach presented in the current study provides a promising alternative to this end. Although the use of PBMCs for screening purposes does not take into account the complexity of the intestinal barrier, it involves the use of human immune cells that are easily collectable and allows high-throughput screening. The *in vitro* assay used in this study revealed that bifidobacteria have a pronounced anti-inflammatory potential, characterized by overall high IL-10 and low IL-12, TNF-α and IFN-γ induction levels compared to a pro-inflammatory control strain. This approach thus enables a more rational selection of promising therapeutic strains for further investigation *in vivo*.

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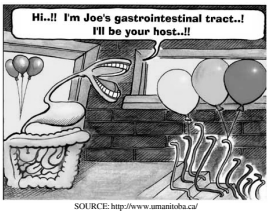

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6.2. *In vitro* assessment of the gastrointestinal transit tolerance of human reference strains and probiotic isolates of *Bifidobacterium*

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Summary

Next to health promoting effects, the functional aspect of probiotic strains also involves their capacity to reach the colon as viable metabolically active cells. The present study aimed to assess the potential of 24 probiotic product isolates and 42 human reference strains of *Bifidobacterium* to survive gastrointestinal transit under *in vitro* conditions. The survival capacity of exponential and stationary phase cultures upon exposure to gastric and small intestinal juices was determined using a recently developed microplate-based assay in combination with the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit. All 66 strains tested displayed a considerable loss in viability during exposure to an acidic pepsin containing solution (pH 2.0) ($p < 0.001$). Among the 10 taxa tested, cultures of *B. animalis* subsp. *lactis* appeared to be most capable to survive gastric transit. Although to a lesser extent, the presence of bile salts in the small intestinal tract also affected the viability of most of the strains tested. Except for three strains, all 66 strains were shown to express bile salt hydrolase activity using an agar-based assay. In contrast, the bifidobacterial strains used in this study appeared to possess a natural ability to survive the presence of pancreatin (pH 8.0). Although the effect was not significant, a slightly enhanced tolerance to gastrointestinal transit was observed when cells were in the stationary phase, especially when exposed to acid, compared to cells being in the exponential phase. Survival of the gastrointestinal tract appeared to be largely strain-dependent and hence implies that different strains will likely display a different behaviour in functionality. The assay used in this study allows an initial assessment of strains for use as probiotic cultures prior to selecting potential candidate strains for further investigation *in vivo*.

Keywords: Fluorescent dyes, microplate, *Bifidobacterium*, probiotics, gastrointestinal survival

Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf). A wide range of dairy-based and dried probiotic products for human consumption are currently available on the market (Stanton et al., 2001; Agrawal, 2005). Although cellular or culture components of dead probiotic bacteria are also thought to mediate beneficial effects in the host, it has been argued that most health benefits associated with probiotics are exerted by viable metabolically active microorganisms (Ouwehand and Salminen, 1998). Prior to inducing any effect, however, most living probiotic cultures are orally administered upon which they need to survive gastrointestinal (GI) transit in sufficient numbers.

During GI passage, cultures are required to tolerate the presence of pepsin and the low pH of the stomach, the protease-rich conditions of the duodenum and the anti-microbial activity of bile salts. Although the pH of the stomach may increase up to 6.0 or higher after food intake (Johnson, 1977), it generally ranges from 2.5 to 3.5 (Holzapfel et al., 1998). Fasting pH in the stomach may even be as low as 1.5 (Waterman and Small, 1998), which implies that survival in extreme acidic conditions is one of the first major physiological challenges faced by probiotic cultures upon oral administration. Following stomach passage, the small intestine is a second major barrier in the GI tract. Although the pH of the small intestine (i.e. 7.0-8.5) is more favourable towards bacterial survival, the presence of pancreatin and bile salts may have adverse effects.

Traditionally, the ability of a probiotic candidate to survive GI transit is assessed using conventional plating techniques that provide information on the number of viable and reproductive cells during incubation in simulated GI juices (Charteris et al., 1998, Huang and Adams, 2004, Mättö et al., 2004). In the present study, a recently developed technique based on the use of two fluorescent staining agents (Alakomi et al., 2005) was evaluated to assess the GI survival of probiotic cultures and human reference strains of *Bifidobacterium* under *in vitro* conditions. Together with several *Lactobacillus* species, bifidobacteria are amongst the most commonly used bacterial organisms in commercial probiotic products. Using

a microplate-based assay, the relative degree of tolerance towards gastric and pancreatic juices and the ability to survive in presence of bile salts was assessed by differentiation between viable and dead cells. In addition, an agar-based culture method was applied to determine the potential of *Bifidobacterium* strains to deconjugate bile salts.

Materials and Methods

Bacterial Strains

A total of 66 *Bifidobacterium* strains were investigated in this study including 42 type and reference strains obtained from the BCCM™/LMG Bacteria Collection, Ghent University, Belgium (http://bccm.belspo.be/index.php) and 24 isolates obtained from 23 commercial probiotic products (Masco et al., 2005). The species identity of all strains was previously checked by BOX-PCR fingerprinting (Masco et al., 2005). The selection encompassed the following *Bifidobacterium* (sub)species: *B. adolescentis* (n = 6), *B. angulatum* (n = 2), *B. animalis* subsp. *lactis* (n = 19), *B. bifidum* (n = 8), *B. breve* (n = 7), *B. catenulatum* (n = 2), *B. gallicum* (n = 1), *B. longum* biotype infantis (n = 7), *B. longum* biotype longum (n = 9) and *B. pseudocatenulatum* (n = 5). All strains were subcultured in MRS broth (288130, BD, Le Pont de Claix, France) supplemented with 0.5 g l^{-1} L-cystein-HCl (C-6852, Sigma, Bornem, Belgium) (MRS-cystein broth) at 37 °C under anaerobic conditions (84 % N_2, 8 % H_2, 8 % CO_2). Subsequently, overnight subcultures were grown in MRS-cystein broth until they reached the early exponential or the stationary growth phase, respectively.

Microplate assays

Survival rates in gastrointestinal juices (i.e. gastric, pancreatic and bile salt solutions) were assessed using a microplate-based fluorochrome assay in combination with the LIVE/DEAD® *Bac*Light™ Bacterial Viability (L/D) kit (L7012, Molecular Probes Inc., Leiden, The Netherlands) as previously described (Alakomi et al. 2005). The L/D kit combines the nucleic acid dyes propidium iodide (PI), a red-coloured agent that is excluded from intact cells and SYTO9, a green-coloured agent that is membrane-permeant and stains both viable and non-viable cells. Because PI has a higher affinity for DNA than SYTO9, it is able to displace SYTO9 from the DNA. Hence, intact viable cells will stain fluorescent green, whereas dead cells will colour red.

Exponential and stationary phase cells were harvested by centrifugation, washed with 0.85 % (w/v) NaCl and incubated with (challenged culture) or without (control culture) each of the gastrointestinal juices. Gastric juice [0.3 % (w/v) pepsine (P-7000, Sigma, Bornem, Belgium), 0.5 % (w/v) NaCl, pH 2.0, adjusted with HCl] or pancreatic juice [0.1 % (w/v) pancreatin (P-1500, Sigma), 0.5 % (w/v) NaCl, pH 8.0, adjusted with NaOH] was added to the harvested cells and aliquots were taken after 1, 90 and 180 min. of incubation under microaerobic (6 % O_2) or anaerobic conditions, respectively. Cells were treated with bile salt solution [0.3 % (w/v) bovine bile (B3883, Sigma), 0.5 % (w/v) NaCl, pH 8.0, adjusted with NaOH] during 60 min. in anaerobic conditions. After incubation, cells were harvested by centrifugation, washed and resuspended in 0.85 % (w/v) NaCl solution. Of each bacterial cell suspension, 100 µl was pipetted in triplicate into separate wells of a white 96-well fluorescence microplate (C8 white maxisorp 437591, Nunc™, Roskilde, Denmark). Staining solutions of PI and SYTO9 were prepared according to the

manufacturer's instructions. Aliquots of 100 µl staining solution were added to each well and mixed. Subsequently, plates were incubated in the dark at room temperature for 15 min. Fluorescence measurements were performed using a fluorescence microplate reader (HTS 7000 Bio Assay Reader, Perkin-Elmer, Monza, Italy). Intensities of green (535 nm) and red (635 nm) emission were recorded after excitation at 485 nm. Following fluorescence background substraction, the mean ratio of green to red fluorescence emission ($Ratio_{G/R}$), which is proportional to the relative numbers of live bacteria and hence the survival rate, was calculated from three measurements. For every *in vitro* test, *B. animalis* subsp. *lactis* LMG 18314^T was included for reproducibility assessment.

Bile salt hydrolase (BSH) assay

Strains were tested for taurodeoxycholic acid (TDCA) hydrolase activity using MRS-cystein agar plates to which 0.3 % TDCA sodium salt (86345, Fluka-Biochemica, Buchs, Switzerland) was added (MRS-TDCA). Overnight grown MRS-cystein broth cultures were inoculated on MRS-TDCA and incubated for 24 h at 37 °C under anaerobic conditions. Strains were scored positive for BSH activity when a white precipitate of deoxycholic acid beneath and around the colonies was observed. Growth performance was compared with cultures grown on MRS-cystein agar.

Statistical analysis

Statistical analysis comprised significance testing of the difference between means of trendline slopes calculated from the logarithmic (% $ratio_{G/R}$) values using the nonparametric Kruskal-Wallis H and Mann-Whitney U test.

Results

Despite some exceptions, there was a general tendency indicating that gastrointestinal survival under *in vitro* conditions as determined from $Ratio_{G/R}$ values was strain-specific among the selected *Bifidobacterium* strain set. The overall mean coefficient of variation of triplicate measurements of the $Ratio_{G/R}$ was ± 0.94% with a maximum variation of 3.63%. Although most strains performed slightly better in presence of gastric juice when being in the stationary phase (Mean $slope_{STAT}$: -0.00302 ±0.00020) compared to exponential phase cells (Mean $slope_{EXP}$: -0.00356 ±0.00016) ($p > 0.05$), all strains tested showed significant loss in viability after incubation for 180 min ($p < 0.001$) (Table 1). Of all strains tested, reference strains and probiotic isolates of *B. animalis* subsp. *lactis* displayed the highest survival rates during simulated gastric transit compared to the other taxa ($p < 0.05$). Only single strains of *B. adolescentis* (LMG 10502^T), *B. angulatum* (LMG 10503^T), *B. bifidum* (LM 588), *B. breve* (LM 646), *B. catenulatum* (LMG 11043^T), *B. longum* biotype longum (LMG 13196) and *B. pseudocatenulatum* (LMG 10505^T) exhibited survival rates comparable to those observed for the *B. animalis* subsp. *lactis* strains. Remarkably, probiotic product isolates of *B. bifidum* and *B. breve* LM 646, showed higher survival rates compared to the *B. bifidum* and *B. breve* reference strains ($p < 0.05$) (Figure 1), respectively.

A subset of 30 strains, encompassing the 10 *Bifidobacterium* taxa that exhibited variable survival rates during simulated gastric transit, was selected for *in vitro* analysis of the small intestinal transit (Tables 2-3). For most strains tested, no loss in viability was witnessed after 180 min of incubation in presence of pancreatin when being in the stationary phase compared to exponential phase cells that were more susceptible ($p < 0.05$). Exponential phase cells belonging to *B. angulatum*, *B. animalis* subsp. *lactis* and *B. catenulatum* as well as *B. bifidum* probiotic product isolates and *B. breve* reference strains showed a significant decrease in viability ($p < 0.05$). As was the case for gastric transit, the survival capacity in the presence of pancreatin proved to be largely strain dependent. However, cultures of *B. animalis* subsp. *lactis* now grouped among the least pancreatin tolerant strains. Differences in survival rates were noted between probiotic product isolates and reference strains of the same species, the latter showing higher survival rates ($p < 0.05$).

During testing of conjugated bile salt tolerance, a general slight decrease in viability was witnessed after 60 minutes of incubation (Mean $slope_{EXP}$: -0.0112 ±0.0065; Mean $slope_{STAT}$: -0.0110 ±0.0071) ($p < 0.05$) (Table 3). Of the 30 strains tested, only *B. longum* biotype infantis strains LMG 18902 (Mean $slope_{STAT}$: 0.0007 ±0.0004) and LM 418 (Mean $slope_{STAT}$: 0.0060 ±0.0001) and *B. longum* biotype longum LM 257 (Mean $slope_{EXP}$: 0.0016 ±0.0001) were able to retain their metabolically active state during this incubation period. Within the species *B. bifidum*, human reference strains performed better than the probiotic product isolates, while the opposite was the case for the *B. breve* strains ($p < 0.05$). For the other species tested, no pronounced differences in $Ratio_{G/R}$ were witnessed between probiotic product isolates and reference strains at a level of $p = 0.05$. Likewise, no significant differences were noted between stationary phase cells and exponential phase cells.

Of the 66 strains tested, only three strains (*B. gallicum* LMG 11596^T and *B. longum* biotype infantis LMG 8811^T and LMG 11588) did not express TDCA hydrolase activity in the BSH assay. Noteworthy, a slight reduction in growth performance was witnessed in the presence of TDCA compared to growth on the MRS control plates.

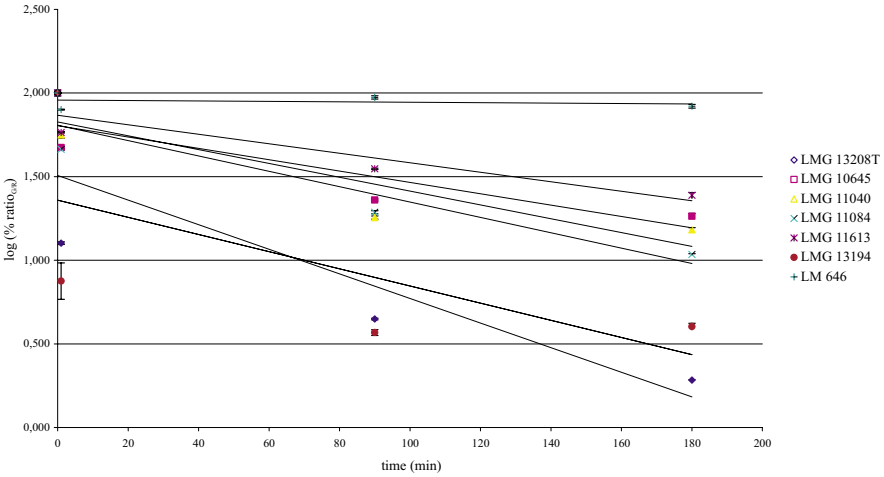

Figure 1. Graph illustrating the survival behaviour of probiotic product isolate *B. breve* LM 646 compared to *B. breve* reference strains (stationary phase cells) over a 180 min incubation period. Trendlines were calculated for each strain based on the log (% $ratio_{G/R}$) values which are expressed as the mean ± standard deviation (bars) of three measurements. Comparison and statistical analysis were based on the slopes of these trendlines [slope range for *B. breve* reference strains: -0.0028 – -0.0074 and *B. breve* LM 646: -0.0001].

Table 1. Effect of simulated gastric juice on the viability of 66 *Bifidobacterium* strains as calculated from the trendline slopes[a]

Species (no. of strains)		Exponential growth phase Mean slope ± SD	Stationary growth phase Mean slope ± SD
B. adolescentis (6)		-0,0033 ± 0,0015	-0,0054 ± 0,0025
B. angulatum (2)		-0,0028 ± 0,0022	-0,0027 ± 0,0022
B. animalis subsp. *lactis*	Reference strains (4)	-0,0016 ± 0,0012	-0,0007 ± 0,0006
	Probiotic isolates (15)	-0,0009 ± 0,0015	-0,0012 ± 0,0028
B. bifidum	Reference strains (5)	-0,0046 ± 0,0013	-0,0055 ± 0,0033
	Probiotic isolates (3)	-0,0026 ± 0,0031	-0,0019 ± 0,0014
B. breve	Reference strains (6)	-0,0054 ± 0,0014	-0,0047 ± 0,0015
	Probiotic isolates (1)	-0,0008 ± 0,0001	-0,0001 ± 0,0001
B. catenulatum (2)		-0,0035 ± 0,0023	-0,0013 ± 0,0026
B. gallicum (1)		-0,0063 ± 0,0000	-0,0051 ± 0,0002
B. longum biotype infantis	Reference strains (6)	-0,0044 ± 0,0019	-0,0044 ± 0,0021
	Probiotic isolates (1)	-0,0050 ± 0,0000	-0,0042 ± 0,0000
B. longum biotype longum	Reference strains (5)	-0,0039 ± 0,0016	-0,0016 ± 0,0019
	Probiotic isolates (4)	-0,0047 ± 0,0006	-0,0024 ± 0,0011
B. pseudocatenulatum (5)		-0,0036 ± 0,0010	-0,0040 ± 0,0021
	Total (66)	-0,00356 ± 0,00162	-0,00302 ± 0,00183
	Reference strains (42)	-0,00394 ± 0,00134	-0,00355 ± 0,00181
	Probiotic isolates (24)	-0,00281 ± 0,00201	-0,00198 ± 0,00150

[a] Trendline slopes are expressed as the mean value of the measurements of the tested strains.

Individual trendlines were calculated based on triplicate measurements of the log (% $ratio_{G/R}$) values at times 0, 1, 90 and 180 min.

Table 2. Effect of simulated pancreatic juice on the viability of 30 *Bifidobacterium* strains as calculated from the trendline slopes[a]

Species (no. of strains)		Exponential growth phase Mean slope ± SD	Stationary growth phase Mean slope ± SD
B. adolescentis (3)		-0,000078 ± 0,00102	0,00066 ± 0,00061
B. angulatum (2)		-0,000083 ± 0,00008	-0,00027 ± 0,00019
B. animalis subsp. *lactis*	Reference strains (2)	-0,00057 ± 0,00045	0,00098 ± 0,00019
	Probiotic isolates (3)	-0,00111 ± 0,00045	-0,00096 ± 0,00085
B. bifidum	Reference strains (1)	0,00170 ± 0,00035	0,00110 ± 0,00000
	Probiotic isolates (2)	-0,00085 ± 0,00035	0,00082 ± 0,00032
B. breve	Reference strains (2)	-0,00035 ± 0,00014	0,00070 ± 0,00143
	Probiotic isolates (1)	0,00147 ± 0,00023	0,00003 ± 0,00006
B. catenulatum (2)		-0,00050 ± 0,00026	0,00033 ± 0,00077
B. gallicum (1)		0,00060 ± 0,00000	0,00067 ± 0,00006
B. longum biotype infantis	Reference strains (3)	0,00083 ± 0,00080	0,00080 ± 0,00082
	Probiotic isolates (1)	-0,00067 ± 0,00029	0,00033 ± 0,00012
B. longum biotype longum	Reference strains (2)	0,00058 ± 0,00058	0,00008 ± 0,00079
	Probiotic isolates (2)	-0,00023 ± 0,00052	-0,00053 ± 0,00015
B. pseudocatenulatum (3)		-0,00014 ± 0,00026	0,00068 ± 0,00082
	Total (30)	0,00004 ± 0,00083	0,00036 ± 0,00059
	Reference strains (21)	0,00020 ± 0,00072	0,00057 ± 0,00041
	Probiotic isolates (9)	-0,00028 ± 0,00103	-0,00006 ± 0,00070

[a] Trendline slopes are expressed as the mean value of the measurements of the tested strains.

Individual trendlines were calculated based on triplicate measurements of the log (% $ratio_{G/R}$) values at times 0, 1, 90 and 180 min.

Table 3. Effect of conjugated bile salt on the viability of 30 *Bifidobacterium* strains as calculated from the trendline slopes[a]

		Exponential growth phase	Stationary growth phase
Species (no. of strains)		**Mean slope ± SD**	**Mean slope ± SD**
B. adolescentis (3)		-0,0099 ± 0,0086	-0,0095 ± 0,0049
B. angulatum (2)		-0,0093 ± 0,0073	-0,0210 ± 0,0011
B. animalis subsp. *lactis*	Reference strains (2)	-0,0120 ± 0,0004	-0,0151 ± 0,0064
	Probiotic isolates (3)	-0,0206 ± 0,0011	-0,0128 ± 0,0039
B. bifidum	Reference strains (1)	-0,0111 ± 0,0002	-0,0068 ± 0,0001
	Probiotic isolates (2)	-0,0167 ± 0,0042	-0,0073 ± 0,0067
B. breve	Reference strains (2)	-0,0154 ± 0,0018	-0,0083 ± 0,0007
	Probiotic isolates (1)	-0,0018 ± 0,0001	-0,0057 ± 0,0001
B. catenulatum (2)		-0,0174 ± 0,0017	-0,0180 ± 0,0069
B. gallicum (1)		-0,0238 ± 0,0003	-0,0222 ± 0,0004
B. longum biotype infantis	Reference strains (3)	-0,0033 ± 0,0022	-0,0068 ± 0,0057
	Probiotic isolates (1)	-0,0118 ± 0,0001	0,0060 ± 0,0001
B. longum biotype longum	Reference strains (2)	-0,0054 ± 0,0035	-0,0081 ± 0,0057
	Probiotic isolates (2)	-0,0052 ± 0,0075	-0,0150 ± 0,0018
B. pseudocatenulatum (3)		-0,0049 ± 0,0075	-0,0150 ± 0,0053
	Total (30)	-0,0112 ± 0,0065	-0,0110 ± 0,0071
	Reference strains (21)	-0,0113 ± 0,0063	-0,0131 ± 0,0059
	Probiotic isolates (9)	-0,0112 ± 0,0078	-0,0070 ± 0,0082

[a] Trendline slopes are expressed as the mean value of the measurements of the tested strains.

Individual trendlines were calculated based on triplicate measurements of the log (% $ratio_{G/R}$) values at times 0 and 60 min.

Discussion

Together with safety and technological aspects, functionality screening plays a key role in the selection of potential probiotic strains for human use. Next to health promoting characteristics, the functional aspect of probiotic strains also involves their capacity to reach the colon in a metabolically active state. In the present study, a number of *in vitro* tests were used to screen a large number of *Bifidobacterium* strains of intestinal and food origin for their ability to survive in the presence of pepsin, pancreatin and bile salts. Survival rates were assessed using a recently described microplate scale fluorochrome assay (Alakomi et al., 2005) following incubation in each of the gastrointestinal juices. This approach proved to be highly reproducible and provides a rapid alternative to laborious plate count techniques. As a general tendency, survival rates of the bifidobacterial strains tested proved to be largely strain-dependent. As previously reported for bifidobacteria of human origin, most of the strains were susceptible to low pH and bile salts, while an apparently intrinsic ability to survive the presence of pancreatin was witnessed (Charteris et al., 1998).

Of all strains tested, representatives of *B. animalis* subsp. *lactis* appeared to be most capable to survive gastric transit, which is probably due to their enhanced acid tolerance compared to other *Bifidobacterium* species (Matsumoto et al., 2004; Mättö et al., 2004). When exposed to acidic conditions, bacteria try to maintain a pH homeostasis by discharging H^+ from the cell by H^+-ATPase (Booth, 1985). It has previously been shown that upon incubation under acidic conditions, the H^+-ATPase activity in *B. animalis* subsp. *lactis* increases whereas that of other, non acid-tolerant bifidobacteria, such as *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. longum* biotype infantis and longum and *B. pseudocatenulatum*, diminishes and results in a general decrease or loss of viability (Matsumoto et al., 2004). These results suggest that many *B. animalis* subsp. *lactis* cultures incorporated in probiotic food products are sufficiently acid tolerant to reach the intestinal tract in a metabolically active state after oral administration. For probiotic strains with limited acid tolerance, gastric passage can be enhanced by the presence of milk proteins due to a buffering or protective effect, suggesting that milk-based products constitute an important carrier of probiotic strains (Charteris et al., 1998). Other studies have demonstrated that probiotic cultures can be significantly

protected by the addition of (cryo)protectants during spray- and freeze-drying or via encapsulation in milk proteins and complex (prebiotic) carbohydrates (Ross et al., 2005). Furthermore, the up-regulation of genes involved in stress responses has been shown to enhance acid tolerance of probiotic bacteria (Kullen and Klaenhammer, 1999). To some extent, this type of adaptation to acidic stress conditions might explain why the relative decrease in viability after 180 minutes of incubation was lower than after 1 minute for all strains tested in our study. Finally, also food-grade genetic manipulation has been used to improve probiotic performance (Desmond et al., 2004), which leaves the option to further exploit promising cultures that are sensitive to gastric transit.

Despite considerable loss of viability during simulated gastric transit, most of the bifidobacterial strains used in this study appeared to possess a natural ability to survive the presence of pancreatin. The survival of lactobacilli (Charteris et al., 1998) and dairy propionibacteria (Huang and Adams, 2004) also seems unaffected when incubated with a simulated pancreatin containing solution. Consequently, the presence of pancreatin in the small intestine does not appear to confer an insuperable barrier for probiotic cultures. In contrast, our results indicate that the presence of bile salts slightly reduces the viability of most of the strains tested. After synthesis from cholesterol and conjugation to either glycine or taurine in the liver, conjugated bile salts are secreted into the small intestine and undergo extensive chemical modifications upon arrival in the colon due to microbial activity. Bile salt hydrolase (BSH) catalyses the hydrolysis of conjugated bile salts into the bile salt and the amino acid residue. Although the functions of this enzyme and the physiological impact on its host are far from understood, conjugated bile salt hydrolysis is a commonly observed phenomenon among gastrointestinal bacteria, including the genera *Bacteroides*, *Bifidobacterium, Clostridium*, *Enterococcus*, *Fusobacterium, Lactobacillus,* and *Peptostreptococcus* (Aries and Hill, 1970; Hylemon, 1985; Chateau et al., 1994). Deconjugation of bile salts is an important metabolic reaction in the bile salt metabolism of mammals and has been associated with a reduction of serum cholesterol (Anderson and Gilliland, 1999; Pereira and Gibson, 2002). On the other hand, excessive bile salt deconjugation can also exert pathological effects. Deconjugated bile salts are thought to be involved in the formation of gallstones (Thomas et al., 2000) and the development of colorectal cancer (Singh et al., 1997). In line with this

functional paradox, it has been suggested that the release of deconjugated bile salts seems to have a higher anti-microbial effect than the unmodified bile salts (Grill et al., 1995; 2000). Although most bifidobacterial strains tested in the present study exhibited BSH activity, we observed a slight overall sensitivity towards bile salts, which confirms earlier findings (Kociubinski et al., 1999). Since BSH activity is expressed constitutively within the genus *Bifidobacterium* (Grill et al., 1995; 2000), the absence of BSH activity in two *B. longum* biotype infantis strains and in the type strain of *B. gallicum* might indicate absence, inactivation or mutation of the BSH gene. Although interest has been shown to use strains that produce BSH to lower serum cholesterol levels (De Smet et al., 1994), the lack of BSH activity is one of the criteria currently used in the selection of probiotic candidates. However, given the wide distribution and high activity of BSH in bifidobacteria compared to other probiotic groups (Grill et al., 1995, 2000; Tanaka et al., 1999), the lack of BSH as a selection criterion seems controversial and might need revision.

Stress responses of bacterial cultures generally vary with the growth phase. Bacterial cells that enter the stationary phase tend to develop a general stress resistance and are thus more resistant to various types of stress factors (van de Guchte et al., 2002) including the ones encountered during gastrointestinal transit. When exposed *in vitro* to gastro-intestinal juices, the *Bifidobacterium* strains tested in our study exhibited a slightly more tolerant profile during the stationary phase than during the exponential phase, however, these differences were not significant.

In conclusion, the results obtained during this study indicate that tolerance towards bile salts is potentially more important during probiotic selection compared to gastric and pancreatic tolerance. With the development of new delivery systems and the use of specific foods, acid-sensitive strains can be buffered through the stomach. In addition, bifidobacteria seem to possess a natural tolerance towards pancreatin. Consequently, the potential of a probiotic strain to survive passage through the gastrointestinal tract after ingestion may largely depend on its ability to resist the antimicrobial action of bile salts. Furthermore, the strain-dependent tendency observed for transit survival implies that different strains will likely display a different behaviour in functionality. For this reason, preliminary characterization of strains for

use as probiotic cultures through *in vitro* screening is of great value in selecting functional candidate strains for further *in vivo* studies.

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Part 4
Conclusions and perspectives

Probiotic membership application form

NAME: ***Bifidobacterium bifidum*** **Strain X**

ORIGIN: **Human intestine**

FUNCTION: **Prevention and alleviation of symptoms associated with Inflammatory Bowel Disease (IBD)**

SPECIAL FEATURES:
The applicant is able to survive passage through the gastro-intestinal tract and declares to be free of acquired antibiotic resistance genes.

Signature,

Bifidobacterium bifidum Strain X

Conclusions and perspectives

In order to successfully commercialise probiotic products, it is important to correctly **identify** the incorporated strains and to document their **safety** and **functionality** properties. The research performed in this Ph.D. study has delivered a set of optimised and/or new methodologies to analyse these key aspects for potentially probiotic bifidobacteria. Provided that they are subjected to ***in vivo* validation**, these **standardized *in vitro* protocols** can be used in the polyphasic screening of new candidate cultures. Only by doing so, *Bifidobacterium*-specific cut-off values can be defined, which will allow a reliable *in vitro* comparison and subsequent selection of promising strains for probiotic use.

The past few years have witnessed the development and evaluation of a broad range of techniques for the identification of bifidobacteria (Ventura *et al*., 2004; Ward and Roy, 2005). Although the identification of most *Bifidobacterium* taxa is relatively straightforward, some techniques fail to differentiate closely related species. In this study a database based on **BOX-PCR fingerprints** from well-characterized type and reference strains was constructed for the identification of unknown bifidobacteria. This technique was very suitable for unambiguous discrimination of all validly described (sub)species within the genus *Bifidobacterium*, and to some extend also allowed strain differentiation. As a result, only those isolates that display highly similar if not identical BOX-PCR banding patterns may need further typing. Despite the fact that this technique does not provide phylogenetic information, it has contributed to an enhanced understanding of the classification of species with a controversial taxonomic position. For instance, BOX-PCR clearly discriminates among the recently proposed biotypes longum, infantis and suis in the species *B. longum* (Sakata *et al*., 2002). Likewise, the results obtained with BOX-PCR fingerprinting formed the onset for further investigation of the **affiliation of *B. lactis* to the closely related species *B. animalis*** (Scardovi and Trovatelli, 1974), a matter that had been much debated since its original description (Meile *et al*., 1997). Triggered by recent proposals to elucidate the taxonomic

standing of *B. lactis* within the genus *Bifidobacterium* and given its importance in the probiotic industry, a polyphasic study was carried out to clarify its taxonomic position. New DNA-DNA hybridisation data and phenotypic results formed the taxonomic basis to unify *B. animalis* and *B. lactis*. Results of protein profiling, genotypic analyses and growth evaluation in milk, on the other hand, indicated that both former species constituted different taxa at the subspecies level. These findings resulted in the proposal to create two subspecies in *B. animalis*, namely *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*. Despite this reclassification, representatives of the latter subspecies continue to be referred to as *B. lactis*. Because the species epithet 'animalis' suggests that strains in probiotic products are from animal origin, the use of the correct taxonomic name may not appeal to consumer and/or producer from the commercial point of view.

The research performed in this Ph.D. work also included a post-production surveillance study of worldwide collected commercial probiotic products claiming to contain bifidobacteria. Both qualitative and quantitative microbial analysis was performed using culture-dependent and culture-independent methods. **Qualitative microbial analysis** revealed that a rather high percentage of probiotic products were incorrectly or inadequately labeled with respect to the identity of the incorporated strains and that a substantial number of dried products inadmissibly lack the presence of any viable microorganisms, which must raise questions about their postulated probiotic effects. In all product types tested, *B. animalis* subsp. *lactis* was most frequently found, which confirms the industrial importance of strains assigned to this subspecies. The use of conventional isolation and subsequent identification of the implemented bifidobacterial strains in combination with Denaturing Gradient Gel Electrophoresis (DGGE) proved to be successful in the characterization of the taxonomic content of *Bifidobacterium*-claiming probiotic products. However, while the complementary use of these strategies provided reliable qualitative data, both approaches struggled with their limitations in terms of quantification. This is partly due to the lack of suitable media for the selective isolation of bifidobacteria from probiotic products, which compromises the reliability of many enumeration procedures.

Triggered by these shortcomings, real-time PCR based on the non-specific SYBR Green I chemistry was evaluated for the culture-independent quantification of bifidobacteria in probiotic products. Although preliminary research performed during this Ph.D. has clearly demonstrated the potential of real-time PCR in **quantitative microbial analysis**, the accuracy and sensitivity of the method has to be further evaluated. Such an evaluation should include further optimization of the sample processing and DNA extraction protocol, which are essential in the interpretation of real-time PCR data. Likewise, a reliable estimate of the detection limit needs to be obtained e.g. based on spiking experiments. Additionally, implementation of Reverse Transcriptase real-time PCR will allow differentiation between live and dead bifidobacteria. Future developments including the use of species-specific or even strain-specific primers and probes will certainly broaden the potential of real-time PCR and make it applicable for the complete microbial analysis of probiotic products. However, when there is little or no information on the bacterial content of a probiotic sample, a separate probe or primer is needed for each possible probiotic species, resulting in a significant increase in cost and workload. Alternatively, the combined use of real-time PCR based on the SYBR Green I chemistry and *Bifidobacterium*-specific primers and DGGE analysis may also allow accurate quantitative and qualitative analysis of probiotic products.

Culture-dependent analysis is still indispensable in the investigation of safety and functionality related properties of individual probiotic candidates. The first step in this respect is the **identification of a probiotic organism at the strain level,** which is of paramount importance when attributing specific features to a specific strain. Results obtained from Pulsed-Field Gel Electrophoresis (PFGE) typing performed during this Ph.D. study clearly indicated a relatively high degree of genomic homogeneity among the *Bifidobacterium* strains currently used in the probiotic industry, which is in contrast with the high diversity of *Bifidobacterium* products available on the market. Although PFGE is considered as the 'gold standard' for strain typing, phenotypic data obtained during this study occasionally revealed subtle differences among certain strains, which were not reflected by PFGE data. These findings indicate that probiotic cultures with indistinguishable PFGE profiles can still show phenotypic variation.

Recent years have witnessed a growing concern about the risk related to the potential transfer of **antimicrobial resistances** from probiotic strains to intestinal pathogens, especially after reports on the presence of antibiotic resistance genes and transfer of plasmids and transposons in *Enterococcus* and *Lactobacillus* species (Teuber *et al*., 1999). Due to the fact that bifidobacteria are considered as safe organisms, antimicrobial susceptibility of *Bifidobacterium* strains has rarely been investigated. Furthermore, interpretive reading of bifidobacterial resistance phenotypes has been significantly hampered by the lack of a validated method tested on a taxonomically diverse set of strains. The use of the recently developed LSM + cysteine medium formulation (Klare *et al*., 2005) allowed us to discriminate between intrinsic and atypical resistance properties of bifidobacteria. Although the presence of non-transmissible antibiotic resistances does not usually confer a safety concern and even might be useful in the treatment of antibiotic associated diarrhoea (Charteris *et al*., 1998), the finding of atypical antimicrobial resistances in some *Bifidobacterium* strains urges for a continuous vigilance in the selection of strains for probiotic use. In this Ph.D. work, atypical **tetracycline** resistance could be linked to the presence of an acquired *tet*(W) gene. Although this implies the presence of a mobile genetic support, the *tet*(W) gene was not associated with a plasmid nor with transposon Tn*B1230* (Melville *et al*., 2004). Further investigation, including up- and downstream sequencing, as well as extended conjugation experiments should enhance our understanding on the dissemination of the *tet*(W) gene. The fact that several probiotic product isolates, including *B. animalis* subsp. *lactis* and *B. bifidum* strains were shown to possess an acquired *tet*(W) gene stresses the need for a minimal safety evaluation during the selection of *Bifidobacterium* strains for probiotic use.

An increasing number of clinical and experimental studies demonstrate that the resident microbiota may steer the inflammatory responses in allergic and inflammatory bowel diseases (Guarner and Malagelada, 2003). If this concept proves right, unbalanced indigenous microbiota may be modulated by the administration of probiotics. An important part of the beneficial effects of probiotics are related to their **immunomodulatory effects** including immune-enhancing as well as anti-inflammatory activities. In this Ph.D. work, the potential of selected *Bifidobacterium* strains to induce cytokine production by human peripheral blood

mononuclear cells was investigated. For this purpose, an existing *in vitro* method was further validated for high-throughput screening and subsequent selection of promising candidate strains for possible biotherapeutic applications. Although the objective of the screening was focussed on selecting strains for future application in IBD treatment, this technique also allows a more rational selection of promising strains for probiotic therapy of other immune disorders such as allergy.

Although cellular or culture components of dead probiotic bacteria are also thought to mediate beneficial effects in the host, it has been argued that most health benefits associated with probiotics are exerted by viable metabolically active microorganisms (Ouwehand and Salminen, 1998). Prior to inducing any effect, however, most living probiotic cultures are orally administered upon which they need to **survive gastrointestinal (GI) transit** in sufficient numbers. In this Ph.D. study, a recently developed *in vitro* technique (Alakomi *et al*., 2005) combining the use of fluorescent stains, which allow the differentiation between viable and dead bacterial cells, with detection of fluorescence using a microplate reader was optimised and used to screen human and probiotic *Bifidobacterium* isolates for their potential to survive gastric and small intestinal transit. Although these functional properties appeared to be largely strain-dependent, most of the strains were found to be susceptible to low pH and bile salts, while an apparently intrinsic ability to survive the presence of pancreatin was witnessed. With the development of new delivery systems and the use of specific foods, acid-sensitive strains can be buffered through the stomach. Consequently, the potential of a probiotic strain to survive passage through the gastrointestinal tract after ingestion may largely depend on its ability to resist the antimicrobial action of bile salts and hence might be more important during probiotic selection compared to gastric and pancreatic tolerance.

Except for three strains, all *Bifidobacterium* strains tested were shown to express bile salt hydrolase (BSH) activity using an agar-based assay. Although interest has been shown to use strains that produce BSH to lower serum cholesterol levels (De Smet *et al*., 1994), the lack of BSH activity is one of the criteria currently used in the selection of probiotic candidates. However, given the wide distribution and high activity of BSH in bifidobacteria compared to

other probiotic groups (Grill *et al*., 1995, 2000; Tanaka *et al*., 1999), the lack of BSH as a selection criterion seems controversial and might need revision.

It is clear from the results obtained in this study that several important probiotic properties are **strain-specific**, which again highlights the need for accurate strain identification and for characterization of probiotic attributes on an individual strain basis rather than designating an entire species as probiotic. Of all *Bifidobacterium* taxa that have been associated with probiotic use (Holzapfel *et al*., 1998), *B. adolescentis* was never found in any of the commercial probiotic products analyzed. On the other hand, the scientific data gathered in this study indicates that we might need to broaden our perspectives and consider the admission of strains belonging to the species *B. angulatum*, *B. catenulatum*, *B. gallicum* and *B. pseudocatenulatum* to the probiotic consortium. Several promising strains of these taxa definitely merit further investigation. Conversely, given the involvement of *B. dentium* in cariogenic processes and the clinical source of isolation of *B. scardovii*, it is evident that strains belonging to these species should not be considered for use as probiotics.

In the light of the current genomics era, it is beyond doubt that future large-scale comparative genomics studies will contribute to a better understanding of the evolutionary relationships, the ability of horizontal gene transfer, and the unravelling of mechanisms linked to probiotic effects. These new developments will also form the platform for microarray and proteomic technologies for real-time analysis of RNA and protein expression by probiotic *Bifidobacterium* strains. Investigation of probiotic organisms with these new and potentially powerful tools will facilitate the selection of bacteria as therapeutic agents.

Several of the methods described in this Ph.D. thesis may contribute to a better characterization of candidate probiotic strains with respect to their identity, safety and functionality (**Figure 1**).

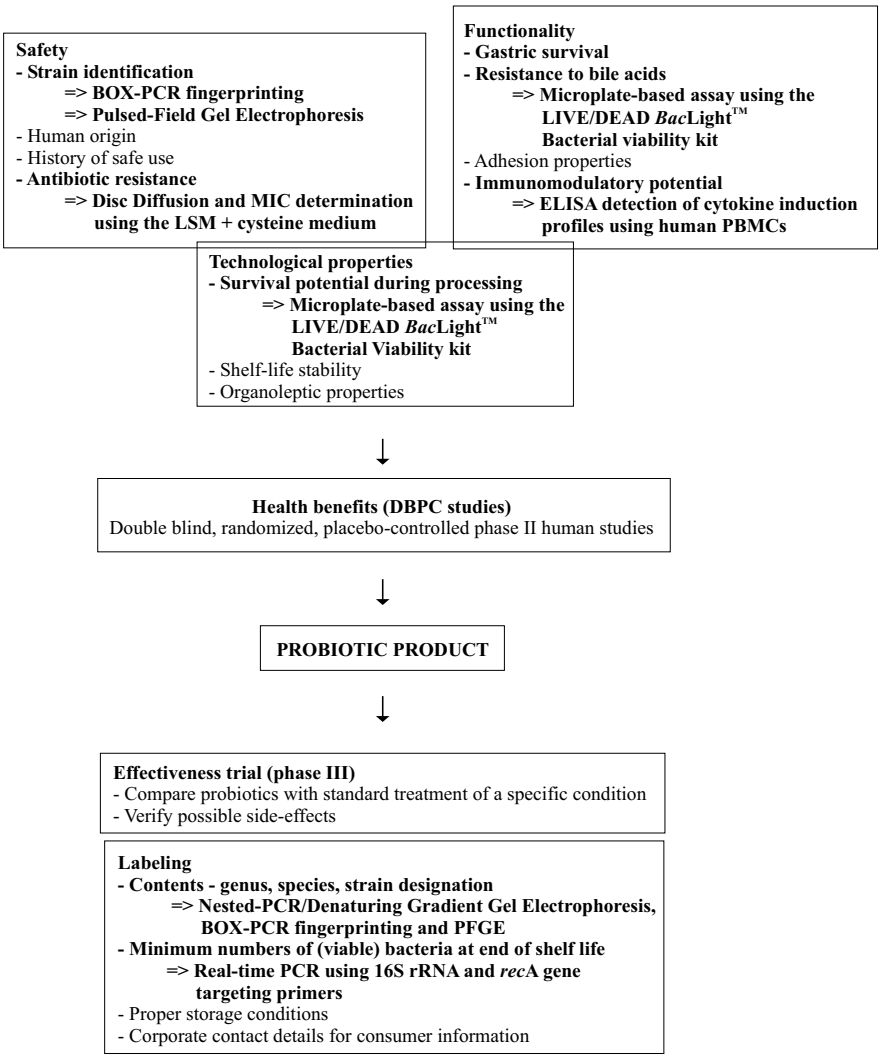

Figure 1. Positioning of the methods described in this Ph.D. study in the general scheme of proposed guidelines for the selection of potential probiotic strains for human use (based on Reid *et al*., 2002 and Reid, 2005).

Although the research performed in this Ph.D. study did not aim at putting the current definition of a 'probiotic' to the test, some considerations might set the scene for further discussion. In contrast to the growing concern about the safety of human probiotics and growing efforts to provide a qualified generic approval system to their safety assessment (von Wright, 2005), this issue is not reflected in the definition. Although there is no question that probiotics intended for human use should be safe, the appendage of "a live '**QPS status**' microorganism..." would ascertain that the organism in question has been subjected to an individually relevant safety review, as recommended by the EFSA. In addition to the importance of careful strain selection evidenced in this Ph.D. work, there is also the emerging need for a more profound description of the term '**host**' to which carefully selected strains are administered. A possible way forward would be to make a primary distinction between organisms applied to **maintain the healthy status of a healthy individual**, in which case the current definition might subsist, and those applied as **biotherapeutics to individuals suffering from a specific clinical disorder**. In the latter case, probiotic candidates may need to be subjected to profound analyses currently applicable to standard drug therapy. These studies would eventually lead to specifications of the health benefit as well as the route of administration, the active compound and hence whether or not viability is required, and the dosage needed to achieve this benefit. These are merely some reflections on a definition that attempts to harmonize the knowledge on a circumstantially complex, though increasingly important matter.

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Summary

In order to introduce probiotic products on the market on a scientific basis, it is important that the organisms incorporated in such products are **correctly identified** and subjected to profound analyses documenting their **safety** and **functionality** before conducting clinical trials and entering a marketing strategy. Only in this way, successful probiotic products can be delivered with long-term marketing potential. The **goal of this Ph.D. work** was to **evaluate and optimise new and existing methodologies to examine the microbial aspects of probiotic product quality control** and **to provide scientific documentation related to safety and functionality** on commercially applied strains as well as on human reference strains of the genus ***Bifidobacterium***. Essentially, a post-production surveillance study covering both **qualitative and quantitative microbial analysis** of worldwide collected commercial probiotic products claiming to contain bifidobacteria was performed using **culture-dependent and culture-independent methods**. Based on the results obtained with these methods, a subset of probiotic product isolates was selected which was supplemented with human reference strains of *Bifidobacterium* to assess the presence of atypical **antibiotic resistances**, to investigate their **immunomodulatory properties** and to determine their potential to survive **gastrointestinal transit**.

The aim of the first part of this study was to evaluate the use of repetitive DNA element PCR fingerprinting (rep-PCR) for the taxonomic discrimination among the majority of validly described species within the genus *Bifidobacterium*. After comparing several primer sets targeting the repetitive DNA elements BOX, ERIC, $(GTG)_5$ and REP, the BOXA1R primer was found to be the most optimal choice for the establishment of a taxonomic framework of *Bifidobacterium* type and reference strains. The **BOX-PCR fingerprinting** technique is a rapid, easy-to-perform and reproducible tool for the **unambiguous identification** of a wide range of bifidobacteria at the species, subspecies and potentially up to the strain level (see Chapter 3.1.). The results obtained with BOX-PCR fingerprinting formed the onset for a polyphasic study of the **taxonomic affiliation between *B. lactis*** (Meile *et al*., 1997) **and the closely related species *B. animalis*** (Scardovi and Trovatelli, 1974). DNA-DNA

hybridisation data and phenotypic results reinforced previous suggestions to consider *B. lactis* as a later synonym of *B. animalis*. Despite their unification at the species level, however, results of protein profiling, genotypic analyses and growth evaluation in milk indicated that the two former species clearly belonged to different subtaxa. Therefore, we proposed to create **two subspecies in *B. animalis*, namely *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis*** (see Chapter 3.2.). As a consequence, probiotic products previously shown to contain *B. lactis* need to be relabelled with the new taxon name *B. animalis* subsp. *lactis*.

In a second part, a set of 58 commercially available, worldwide collected probiotic products claiming to contain bifidobacteria including 22 yoghurts, 5 dairy fruit drinks, 28 food supplements and 3 pharmaceutical preparations, were subjected to **qualitative microbial analysis by means of culture-dependent as well as culture-independent methods** (see Chapter 4.1.). Using two selective culture media, a total of 434 confirmed bifidobacterial isolates were recovered of which 154 isolates, mainly corresponding to three isolates/product/culture medium, were identified to the species level by BOX-PCR fingerprinting. Members of *Bifidobacterium animalis* subsp. *lactis* were most frequently found, although also isolates belonging to *Bifidobacterium longum* biotypes longum and infantis, *Bifidobacterium bifidum* and *Bifidobacterium breve* were recovered. In parallel, all products were also subjected to **culture-independent analysis** which involved a nested-PCR step on total bacterial DNA extracted directly from the product, followed by separation of the amplicons by Denaturing Gradient Gel Electrophoresis (DGGE) and subsequent species identification by band position analysis and database comparison. By conventional cultivation, 70.7 % of the products analysed were found to contain culturable bifidobacteria whereas by culture-independent DGGE analysis, members of the genus *Bifidobacterium* could be detected in 96.5 % of the analysed products. Qualitative microbial analysis revealed that a rather high percentage of probiotic products were incorrectly or inadequately labeled with respect to the identity of the incorporated strains and that a substantial number of dried products (51,6 %) inadmissibly lack the presence of any viable microorganisms, which raised questions about their postulated probiotic effects. From our data, it can be concluded that conventional isolation and subsequent identification of the implemented bifidobacterial strains in combination with DGGE is a successful integrated

strategy in the characterization of the taxonomic content of *Bifidobacterium*-claiming probiotic products. However, while the complementary use of these strategies provided reliable qualitative data, both approaches struggled with their limitations in terms of quantification. This is partly due to the lack of suitable media for the selective isolation of bifidobacteria from probiotic products, which compromises the reliability of many enumeration procedures. Therefore, to complete microbial analysis, real-time PCR targeting the multicopy 16S rRNA gene and the single copy *recA* gene was evaluated for the culture-independent quantification of bifidobacteria in 29 probiotic products. Both assays relied on the use of genus-specific primers and non-specific SYBR Green I detection. Based on the preliminary results obtained in this study and the common assumption that products need to contain a minimum of 10^6 CFU/ml of probiotic bacteria to exert any health effect, only 10 products (35 %) in the study fulfilled this criterion for bifidobacteria. Independent from the gene target used for real-time PCR quantification, a very broad distribution of bifidobacterial concentrations ranging from 0 – 10^8 CFU g^{-1} or ml^{-1} was observed among the products tested. The preliminary research performed in this study clearly demonstrates the potential of real-time PCR as alternative for the culture-based approach in **quantitative microbial analysis** of probiotic products (see Chapter 4.2.).

Genotypic characterization of a number of bifidobacterial isolates at the strain level by means of Pulsed-Field Gel Electrophoresis (PFGE) revealed a relatively high degree of genomic homogeneity among the *Bifidobacterium* strains currently used in the probiotic industry (see Chapter 4.1.). Furthermore, this approach allowed to compose a set of well-typed probiotic product isolates encompassing the (sub)species *B. animalis* subsp. *lactis*, *B. longum* biotypes longum and infantis, *B. bifidum* and *B. breve*. Supplemented with a set of unique type- and reference strains encompassing all human *Bifidobacterium* (sub)species, this collection of strains was subsequently characterised with respect to the presence of atypical antimicrobial resistances, immunomodulatory properties and gastrointestinal transit tolerance.

The susceptibility of 100 strains encompassing 11 bifidobacterial species to 15 antimicrobial agents was tested by the agar overlay disc diffusion and broth microdilution method using the Lactic acid bacteria Susceptibility test Medium (LSM) supplemented with

cysteine. Based on the distribution of inhibition zone diameters and MIC values, all strains tested were susceptible to amoxicillin, chloramphenicol, erythromycin, quinupristin-dalfopristin, rifampicin and vancomycin. Our data also indicated that bifidobacteria are intrinsically resistant to gentamicin, sulphamethoxazole and polymyxin B. Susceptibility to trimethoprim, trimethoprim/sulphamethoxazole, ciprofloxacin, clindamycin, tetracycline and minocycline was variable. The genotypic basis of atypical tetracycline resistance was further characterized using PCR, Southern blotting and partial sequencing. The *tet*(W) gene was found to be responsible for tetracycline resistance in 15 strains including 7 probiotic isolates belonging to the taxa *B. animalis* subsp. *lactis* and *B. bifidum*. This gene was present in a single copy on the chromosome and did not appear to be associated with the conjugative transposon Tn*B1230* previously found in *tet*(W)-containing *Butyrivibrio fibrisolvens*. The use of the LSM + cysteine medium allowed us to discriminate between intrinsic and atypical resistance properties of bifidobacteria, and sets the scene for future definition of epidemiological cut-off values for all important *Bifidobacterium* species. Although the presence of specific antibiotic resistance traits among probiotic strains may be desirable in certain applications, the detection of an acquired *tet*(W) gene in several probiotic product isolates stresses the need for a minimal safety evaluation during the selection of *Bifidobacterium* strains for probiotic use (see Chapter 5.1.).

Subsequently, we investigated the immunomodulatory capacity of a subset of 50 *Bifidobacterium* strains with respect to their potential to induce the production of the cytokines IL-10, IL-12, TNF–α and IFN-γ by peripheral blood mononuclear cells isolated from healthy donors. The results of this *in vitro* analysis confirmed that cytokine stimulation profiles are strain-specific and revealed that bifidobacteria are potent inducers of the anti-inflammatory IL-10, while induction of IL-12, TNF–α and IFN-γ were low compared to a pro-inflammatory control strain. Given the key role of TNF–α in inflammatory bowel disease (IBD) pathogenesis and the fact that intestinal inflammation is associated with low IL-10 and high pro-inflammatory IL-12 and IFN-γ levels, our results suggest that administration of *Bifidobacterium* strains that promote high IL-10/IL-12 and IL-10/TNF–α ratios in combination with a low induction

of IFN-γ production may induce a shift towards a more anti-inflammatory state, resulting in alleviation of IBD symptoms (see Chapter 6.1.).

In addition to these immunomodulating properties, which constitute a part of the underlying mechanisms of health-promoting effects, the ability of probiotic bacteria to survive passage through the GI-tract in order to reach the large intestine is an important issue of basic functionality. A recently developed *in vitro* technique combining the use of fluorescent stains, which allow the differentiation between viable and dead bacterial cells, with detection of fluorescence using a microplate reader was optimised and used to screen human and probiotic *Bifidobacterium* isolates for their degree of resistance against gastric and pancreatic juices and their ability to survive in presence of bile salts. Although these properties were highly strain-dependent, most strains were susceptible to low pH and bile salts, while an apparently intrinsic resistance was witnessed against pancreatin. Furthermore, almost all strains possessed bile salt hydrolase activity (see Chapter 6.2.).

The development of a successful *Bifidobacterium*-containing probiotic product includes many aspects of safety, functionality as well as technological and labelling issues. Overall, our results demonstrate the need for a profound microbial analysis of probiotic products, in which a combined approach of culture-dependent and culture-independent methods has proven its high competence. Furthermore, the *in vitro* approaches used in this study provide high-throughput means for the correct identification and screening of *Bifidobacterium* strains for the presence of atypical antibiotic resistances, gastrointestinal transit survival capacity and immunomodulatory properties. In this way basic information documenting safety and functionality is generated which permits a wilfully selection of potential candidate strains for further investigation *in vivo*. The results obtained in the course of this work indicate that besides the strains already implicated in functional food production, also other *Bifidobacterium* strains of human origin may function as potential alternative sources for future probiotic development.

Samenvatting

Om een wetenschappelijk verantwoord probiotisch product op de markt te introduceren is het van uitermate groot belang dat de gebruikte probiotische stammen **correct geïdentificeerd** zijn en onderworpen worden aan grondig onderzoek naar hun **veiligheid** en **functionaliteit** vooraleer klinische studies worden aangevat en voor eigenlijke commercialisering van het product. Enkel op die manier kunnen probiotische producten met lange-termijn perspectieven succesvol geïntroduceerd worden. **Dit doctoraat had tot doel nieuwe en bestaande technieken te evalueren en optimaliseren voor microbiële kwaliteitscontrole van probiotische producten evenals voor het verschaffen van wetenschappelijke informatie gerelateerd aan de veiligheid en functionaliteit van commercieel gebruikte en humane referentie stammen van het genus *Bifidobacterium*.** Dit omvatte de **kwalitatieve en kwantitatieve microbiële analyse** van wereldwijd verzamelde probiotische producten die beweerden bifidobacteriën te bevatten, gebruik makende van zowel **kweekahankelijke als kweekonafhankelijke methoden**. Op basis van deze resultaten werd een subset probiotische product isolaten geselecteerd die samen met humane referentie stammen van het genus *Bifidobacterium* onderzocht werden naar de aanwezigheid van atypische **antibiotica resistenties**, naar hun **immuunmodulerende eigenschappen** en hun capaciteit om **transit doorheen het gastrointestinaal stelsel te overleven**.

Een eerste deel van de studie omvatte de evaluatie van repetitief DNA element PCR fingerprinting (rep-PCR) voor de taxonomische discriminatie van de overgrote meerderheid van valide beschreven species binnen het genus *Bifidobacterium*. Na vergelijking van verschillende primer sets die respectievelijk de repetitieve elementen BOX, ERIC, $(GTG)_5$ en REP herkennen, werd de BOXA1R primer gekozen voor de constructie van een taxonomisch kader van *Bifidobacterium* type en referentie stammen. De **BOX-PCR fingerprinting** techniek is een snelle, gemakkelijk uit te voeren en reproduceerbare methode die toelaat een brede variëteit aan bifidobacteriën op een **éénduidige** manier te **identificeren** tot op species, subspecies en soms zelfs tot op stamniveau (zie Hoofdstuk 3.1.). De resultaten

bekomen met BOX-PCR fingerprinting gaven vervolgens aanleiding tot het aanvatten van een polyfasische studie die de **taxonomische verwantschap tussen *B. lactis*** (Meile *et al*., 1997) **en het nauwverwante species *B. animalis*** (Scardovi and Trovatelli, 1974) diende op te helderen. DNA-DNA hybridisatie gegevens en fenotypische data bevestigden eerdere suggesties om *B. lactis* te beschouwen als een recenter synonym van *B. animalis*. Niettegenstaande, ondanks hun vereniging op species niveau, bewezen de resultaten bekomen met eiwitprofilering, genotypische analyzes en groeitesten in melk dat de twee origineel beschreven species duidelijk behoren tot verschillende subtaxa. Bijgevolg stelden we voor **twee subspecies te creëren binnen *B. animalis*, namelijk *B. animalis* subsp. *animalis* en *B. animalis* subsp. *lactis*** (zie Hoofdstuk 3.2.). Zodoende zouden probiotische producten waarvan eerder werd aangetoond dat ze *B. lactis* bevatten opnieuw geëtiketteerd moeten worden met de nieuwe taxon naam *B. animalis* subsp. *lactis*.

In een tweede deel werd een set van 58 wereldwijd commercieel verkrijgbare probiotische producten die claimden bifidobacteriën te bevatten, verzameld. Deze selectie omvatte 22 yoghurts, 5 zuivel fruitdranken, 28 voedingssupplementen en 3 farmaceutische preparaten die onderworpen werden aan **kwalitatieve microbiële analyze met behulp van zowel een cultuur-afhankelijke als cultuur-onafhankelijke methode** (see Chapter 4.1.). In totaal werden 434 bifidobacteriën geïsoleerd op twee selectieve media. Hiervan werden drie isolaten/product/medium, in totaal 154 isolaten, geïdentificeerd tot op speciesniveau met behulp van BOX-PCR fingerprinting. Representatieven van het species *Bifidobacterium animalis* subsp. *lactis* werden het meeste teruggevonden, maar ook isolaten behorende tot *Bifidobacterium longum* biotypes longum en infantis, *Bifidobacterium bifidum* en *Bifidobacterium breve* werden teruggevonden. In parallel werden alle producten ook onderworpen aan een **cultuur-onafhankelijke analyse**. Dit omvatte een nested-PCR stap op totaal bacterieel DNA geëxtraheerd rechtstreeks uit het product, gevolgd door scheiding van de amplicons door Denaturing Gradient Gel Electrophoresis (DGGE) en vergelijking van de bandposities met deze van een voordien opgebouwde identificatiedatabank van *Bifidobacterium* referentiestammen. Na analyse gebruik makend van de traditionele kweekmethode bleek 70.7 % van de producten levensvatbare bifidobacteriën te bevatten

terwijl met kweek-onafhankelijke DGGE analyse het genus *Bifidobacterium* in 96.5 % van de geanalyseerde producten kon worden gedetecteerd. Kwalitatieve microbiële analyze onthulde voor een relatief hoog percentage aan probiotische producten een foutieve of onvolledige vermelding van de identiteit van de stammen aanwezig in het product. Tevens werd aangetoond dat 51.6 % van de gedroogde producten geen levende microorganismen bevatten, wat hun geclaimde probiotische effecten in twijfel doet trekken. Uit onze resultaten blijkt dat conventionele isolatie en daaropvolgende identificatie van de gebruikte *Bifidobacterium* stammen in combinatie met DGGE een succesvolle strategie is voor de analyze van de taxonomische inhoud van *Bifidobacterium*-claimende probiotische producten. Niettegenstaande de combinatie van deze strategieën betrouwbare kwalitatieve data opleverde, bleken beide methoden beperkt in hun mogelijkheid om kwantificatieve data te genereren. Dit is deels te wijten aan het gebrek aan geschikte media voor de selectieve isolatie van bifidobacteriën uit probiotische producten waardoor betrouwbare plaattellingen niet mogelijk zijn. Bijgevolg werd real-time PCR gericht tegen het multicopy 16S rRNA gen en het single copy *recA* gen geëvalueerd voor de cultuur-onafhankelijke kwantificatie van bifidobacteriën in 29 probiotische producten teneinde deze producten volledig te kunnen onderwerpen aan microbiële analyze. Beide methoden berustten op het gebruik van genus-specifieke primers en niet-specifieke SYBR Green I detectie. Op basis van de resultaten bekomen in deze studie en de algemene veronderstelling dat producten minstens 10^6 probiotische bacteriën per ml dienen te bevatten om een gezondheidseffect teweeg te brengen, bleken slechts 10 producten (35 %) te voldoen aan dit criterium. In de geteste producten werden, onafhankelijk van het target gen, een zeer brede spreiding aan bifidobacteriële concentraties vastgesteld gaande van 0 – 10^8 CFU g^{-1} of ml^{-1}. De preliminaire resultaten bekomen in deze studie bewijzen zeker het potentieel van real-time PCR als alternatief voor de kweek-gebaseerde aanpak in de **kwantitatieve microbiële analyze** van probiotische producten (zie Hoofdstuk 4.2.).

Genotypische stamtypering van een aantal *Bifidobacterium* isolaten met behulp van Pulsed-Field Gel Electrophoresis (PFGE) toonde aan dat de *Bifidobacterium* stammen die momenteel gebruikt worden in de probiotische industrie een relatief hoge graad van genomische gelijkenissen vertonen (zie Hoofdstuk 4.1.). Deze methode liet tevens toe een verzameling

van correct-getypeerde probiotische product isolaten samen te stellen behorende tot de taxa *B. animalis* subsp. *lactis*, *B. longum* biotypes longum en infantis, *B. bifidum* en *B. breve*. Deze stammencollectie werd samen met een set unieke type- en referentie stammen die alle humane *Bifidobacterium* (sub)species vertegenwoordigen vervolgens onderzocht op de aanwezigheid van atypische antimicrobiële resistenties, hun immuunmodulerende eigenschappen en gastrointestinale transit tolerantie.

De gevoeligheid van 100 stammen behorende tot 11 bifidobacteriële species voor 15 antimicrobiële agentia werd getest door middel van de agar overlay disk diffusie en broth microdilutie methode gebruik makende van het Lactic acid bacteria Susceptibility test Medium (LSM) gesupplementeerd met cysteine. Op basis van de spreiding van de inhibitie zone diameters en MIC waarden bleken alle geteste stammen gevoelig te zijn voor amoxicilline, chloramphenicol, erythromycine, quinupristin-dalfopristin, rifampicine en vancomycine. Uit onze data blijkt tevens dat bifidobacteriën een inherente resistentie vertonen voor gentamicine, sulphamethoxazole en polymyxin B. Gevoeligheid voor trimethoprim, trimethoprim/ sulphamethoxazole, ciprofloxacine, clindamycine, tetracycline en minocycline was variabel. De genotypische achtergrond van de atypische tetracycline resistentie werd verder onderzocht met behulp van PCR, Southern blotting en partiële sequenering. Het *tet*(W) gen bleek verantwoordelijk te zijn voor de tetracycline resistentie die werd aangetoond in 15 stammen waarvan 7 probiotische isolaten behorendende tot de taxa *B. animalis* subsp. *lactis* en *B. bifidum*. Dit gen was aanwezig in één enkele copij op het chromosoom en bleek niet geassocieerd te zijn met het conjugatieve transposon Tn*B1230* eerder gevonden in *tet*(W)-positieve *Butyrivibrio fibrisolvens* isolaten. Het gebruik van het LSM + cysteine medium liet ons toe onderscheid te maken tussen intrinsieke en atypische resistenties binnen het genus *Bifidobacterium* en vormt de basis voor het definiëren van epidemiologische grenswaarden voor alle belangrijke *Bifidobacterium* species. Niettegenstaande de aanwezigheid van specifieke antibioticum resistenties bij probiotische stammen voordelig kan zijn in een aantal toepassingen, benadrukt het vinden van een verworven *tet*(W) gen in verschillende probiotische product isolaten de nood aan een minimale veiligheidsevaluatie in de selectie van *Bifidobacterium* stammen voor probiotisch gebruik (zie Hoofdstuk 5.1.).

Vervolgens onderzochten we de immuunmodulerende eigenschappen van een selectie van 50 *Bifidobacterium* stammen. Hun capaciteit om de productie van de cytokines IL-10, IL-12, TNF-α en IFN-γ door peripheral blood mononuclear cells geïsoleerd uit gezonde donoren te induceren werd nagegaan. De resultaten bekomen met deze *in vitro* methode bevestigden dat cytokine inductie profielen stam-specifiek zijn en dat bifidobacteriën sterke induceerders zijn van het anti-inflammatoire IL-10, terwijl de inductie van IL-12, TNF-α en IFN-γ beperkt bleek in vergelijking met een pro-inflammatoire controle stam. Gezien de belangrijke rol van TNF-α bij intestinale ontsteking welke tevens geassocieerd is met lage IL-10 en hoge pro-inflammatoire IL-12 en IFN-γ niveaus, suggereren onze resultaten dat *Bifidobacterium* stammen die hoge IL-10/IL-12 en IL-10/TNF-α ratios teweeg brengen in combinatie met lage IFN-γ inductiewaarden mogelijks een positief effect zullen teweegbrengen wanneer zij worden toegediend aan patiënten die lijden aan chronische intestinale ontsteking (zie Hoofdstuk 6.1.).

Naast deze immuunmodulerende eigenschappen die de basis vormen van verschillende gezondheidsbevorderende effecten, maakt de mogelijkheid van probiotische stammen om de transit naar de dikke darm te overleven een belangrijk onderdeel van hun functionaliteit. Een recent ontwikkelde *in vitro* techniek die het gebruik van fluorescentie kleurstoffen, welke onderscheid maken tussen levende en dode bacteriële cellen, combineert met detectie van die fluorescentie op microplaat schaal werd geoptimaliseerd en vervolgens gebruikt voor het nagaan van de tolerantie van humane en probiotische *Bifidobacterium* isolaten tegen maag- en pancreassap en hun mogelijkheid te overleven in aanwezigheid van galzouten. Alhoewel deze eigenschappen sterk stam-afhankelijk zijn, bleken de meeste stammen toch gevoelig te zijn voor een lage pH en galzouten, terwijl een inherente resistentie werd vastgesteld voor pancreatine. Bijna alle stammen vertoonden galzout hydrolase activiteit (zie Hoofdstuk 6.2.).

De succesvolle commercialisering van een *Bifidobacterium*-bevattend probiotisch product vereist grondig voorafgaand onderzoek naar aspecten zoals veiligheid en functionaliteit van de gebruikte stammen, alsook naar de technologie en etikettering van het product zelf. De

resultaten bekomen in het kader van dit doctoraatswerk benadrukken de nood aan een grondige microbiële analyze van probiotische producten, waarbij een gecombineerde aanpak van cultuur-afhankelijke en cultuur-onafhankelijke methoden zeer geschikt bleek. Bovendien laten de *in vitro* technieken geoptimaliseerd in deze studie toe om op een snelle en adequate manier *Bifidobacterium* stammen correct te identificeren en te screenen naar de aanwezigheid van atypische antibioticum resistenties, hun gastrointestinale transit tolerantie en immuunmodulerende eigenschappen. Op die manier wordt fundamentele informatie bekomen die hun veiligheid en functionaliteit toelichten en een weloverwogen keuze toelaten van potentiële kandidaten voor verder *in vivo* onderzoek. De bekomen resultaten tonen tevens aan dat naast de stammen die reeds gebruikt worden in de functionele voedingsindustrie ook andere *Bifidobacterium* stammen van humane oorsprong zouden kunnen aangewend worden in de productie van probiotische producten voor humane consumptie.

Table A: Origin and taxonomic diversity of *Bifidobacterium* strains studied in this work

Species (determined using BOX-PCR fingerprinting)	**Original Strain No.**	**R-number**	**Source of Isolation** (type[a], producer/distributor, country)	**PFGE type**[b] (within each species)
B. adolescentis	LMG 10502^T		Adult, intestine	I
	LMG 10733		Adult, intestine	II
	LMG 10734		Adult, intestine	III
	LMG 11579		Bovine, rumen	IV
	LMG 18897		Human, faeces	V
	LMG 18898		Human, faeces	VI
B. angulatum	LMG 10503^T		Human, faeces	I
	LMG 11568		Sewage	II
B. animalis subsp. *animalis*	LMG 10508^T		Rat, faeces	I
	LMG 18900		Rat, faeces	I
B. animalis subsp. *lactis*	LMG 18314^T		Yoghurt	I
	LMG 11580		Chicken, faeces	I
	LMG 18906		Rabbit, faeces	I
	LMG 11615		Infant, faeces	V
	LM 2	R-33222	Yogosan (Y, Lidl, Germany)	I
	LM 33	R-33223	Yogosan (Y, Lidl, Germany)	I
	LM 13	R-33224	Vitality (Y, Müller, Germany)	I
	LM 21	R-33225	Vitality (Y, Müller, Germany)	I
	LM 155	R-33226	Crema actidrink (FD, Müller, Germany)	I
	LM 41	R-33227	Ecoflor (FS, Walthers, The Netherlands)	I
	LM 79	R-33228	Bifidus nature (Y, Grand Jury, France)	I
	LM 90	R-33229	Bio con bifido activo (Y, Asturiana, Spain)	I
	LM 104	R-33230	Bio con bifido activo (Y, Asturiana, Spain)	I
	LM 198	R-33231	Bio drink (FD, Asturiana, Spain)	I
	LM 118	R-33232	Nature bifidus & acidophilus (Y, Coop, Switzerland)	I
	LM 146	R-33233	Kyr (Y, Parmalat, Italy)	I
	LM 165	R-33234	Mio (Y, Nestlé, France)	I
	LM 175	R-33235	B'A vanille (Y, BA, France)	I
	LM 441	R-33236	B'A citron (Y, BA, France)	I
	LM 185	R-33237	Actilus (Y, Champion, France)	I

Table A *(continued)*

Species (determined using BOX-PCR fingerprinting)	**Original Strain No.**	**R-number**	**Source of Isolation** (type[a], producer/distributor, country)	**PFGE type**[b] (within each species)
B. animalis subsp. *lactis*	LM 209	R-33238	Bifidus y acidophilus (Y, Hacendado, Spain)	I
	LM 216	R-33239	Yomo (FD, Yomo, Italy)	I
	LM 232	R-33240	Nutrigen (FD, Mamee-double Decker, Malaysia)	I
	LM 241	R-33241	Benecol (Y, McNeil Consumer Nutritionals, UK)	I
	LM 252	R-33242	Bifidus (Y, Match, Belgium)	I
	LM 281	R-33243	Bifidus (Y, Delhaize, Belgium)	I
	LM 286	R-33244	Pronopal plus (FS, Phytonic, France)	I
	LM 298	R-33245	Bifidus (Y, GB, Belgium)	I
	LM 334	R-33246	Proflora (FS, Chefaro, Belgium)	I
	LM 635	R-33247	Proflora (FS, Chefaro, Belgium)	I
	LM 350	R-33248	Joghurt Gold (Y, Bunte Berte, Germany)	I
	LM 391	R-33249	Debaflor (FS, Deba Pharma, Belgium)	I
	LM 400	R-33250	Debaflor (FS, Deba Pharma, Belgium)	I
	LM 430	R-33251	Bio Life (Y, Yeo Valley organic company, UK)	I
	LM 450	R-33252	Natural bio yoghurt (Y, Tesco, UK)	I
	LM 574	R-33253	Biodophilus (FS, AOV, UK)	I
	LM 586	R-33254	Biodophilus (FS, AOV, UK)	I
	LM 594	R-33255	Culturelle (FS, Pharma Dynamics, South-Africa)	I
	LM 624	R-33256	Activia (Y, Danone, France)	I
	LM 135	R-33257	Teddi (Y, Fattoria Scaldasole, Italy)	I
	LM 125	R-33258	Teddi (Y, Fattoria Scaldasole, Italy)	II
	LM 271	R-33259	Hygiaflora (FS, Laboratoire Super Diet, France)	III
	LM 371	R-33260	Friendly bifidus (FS, Bio-Life, Malaysia)	IV
B. bifidum	LMG 11041^{T}		Breast-fed infant, faeces	IV
	LMG 11582		Adult, intestine	V
	LMG 11583		Adult, intestine	VI
	LMG 13195		Infant, intestine	VII
	LMG 13200		Not known	VIII

Table A ***(continued)***

Species (determined using BOX-PCR fingerprinting)	**Original Strain No.**	**R-number**	**Source of Isolation** (type[a], producer/distributor, country)	**PFGE type**[b] (within each species)
B. bifidum	LM 311	R-33261	Infloran Berna (PP, Berna, Italy)	I
	LM 381	R-33262	Friendly bifidus (FS, Bio-Life, Malaysia)	II
	LM 588	R-33263	Biodophilus (FS, AOV, UK)	III
B. breve	LMG 13208^T		Infant, intestine	I
	LMG 10645		Not known	II
	LMG 11040		Nursling, stool	III
	LMG 11084		Blood	IV
	LMG 11613		Infant, intestine	V
	LMG 13194		Infant, intestine	VI
	LM 646	R-33264	Yakult bifiel (FD, Honsha, Japan)	VII
B. catenulatum	LMG 11043^T		Adult, intestine or faeces	I
	LMG 18894		Sewage	I
B. dentium	LMG 11045T		Dental caries	I
	LMG 11585		Dental caries	I
	LMG 10507		Human, faeces	II
B. gallicum	LMG 11596^T		Adult, intestine	ND
B. longum (biotype infantis)	LMG 8811^T		Infant, intestine	I
	LMG 11570		Infant, intestine	II
	LMG 11588		Infant, faeces	III
	LMG 13204		Infant, intestine	IV
	LMG 18901		Infant, faeces	V
	LMG 18902		Infant, faeces	VI
	LM 418	R-33265	Probiotical (PP, Phacobel, Belgium)	VII
B. longum (biotype longum)	LMG 13197^T		Adult, intestine	III
	LMG 11047		Human	IV
	LMG 11589		Calf, faeces	V
	LMG 13196		Infant, intestine	VI
	LMG 18899		Adult, faeces	VII

Table A *(continued)*

Species (determined using BOX-PCR fingerprinting)	**Original Strain No.**	**R-number**	**Source of Isolation** (type[a], producer/distributor, country)	**PFGE type**[b] (within each species)
B. longum (biotype longum)	LM 257	R-33266	Beneflora (FS, Ortis, Belgium)	I
	LM 613	R-33267	Combiforte (FS, Scipharm, South-Africa)	I
	LM 614	R-33268	Infantiforte (FS, Scipharm, South-Africa)	I
	LM 655	R-33269	Lactoferrin yoghurt (Y, Morinaga Milk Industry co., Japan)	I
	LM 669	R-33270	Lactoferrin active (Y, Morinaga Milk Industry co., Japan)	I
	LM 676	R-33271	Lola (FS, not known, Japan)	II
B. pseudocatenulatum	LMG 10505^T		Infant, faeces	I
	LMG 11593		Sewage	II
	LMG 18903		Human, faeces	III
	LMG 18904		Human, faeces	IV
	LMG 18910		Sewage	V
B. scardovii	LMG 21589^T		50-year-old woman, blood	I
	LMG 21590		44-year-old woman, hip	II

ND: Not Determined

[a] Y: Yoghurt; FS: Food Supplement; FD: Fruit Drink; PP: Pharmaceutical Preparation

[b] Based on macrorestriction analysis with *Spe* I

Table B: Tetracycline resistance properties of *Bifidobacterium* strains tested in this study[a]

Species	Original Strain No.	MIC (µg ml^{-1}) Tetracycline	Minocycline	*tet* gene
B. adolescentis	LMG 10502^{T}	≤ 0.5	ND	NF
	LMG 10733	≤ 0.5	ND	ND
	LMG 10734	≤ 0.5	ND	ND
	LMG 11579	32	32	*tet* (W)
	LMG 18897	≤ 0.5	ND	ND
	LMG 18898	≤ 0.5	ND	ND
B. angulatum	LMG 10503^{T}	≤ 0.5	ND	ND
	LMG 11568	≤ 0.5	ND	ND
B. animalis subsp. *animalis*	LMG 10508^{T}	1	2	NF
	LMG 18900	1	ND	NF
B. animalis subsp. *lactis*	LMG 18314^{T}	16	8	*tet* (W)
	LMG 11580	4	4	*tet* (W)
	LMG 18906	4	4	*tet* (W)
	LMG 11615	16	4	*tet* (W)
	LM 2	ND	ND	ND
	LM 33	ND	ND	ND
	LM 13	8	4	*tet* (W)
	LM 21	16	16	*tet* (W)
	LM 155	ND	ND	ND
	LM 41	ND	ND	ND
	LM 79	ND	ND	ND
	LM 90	ND	ND	ND
	LM 104	ND	ND	ND
	LM 198	32	16	*tet* (W)
	LM 118	ND	ND	ND
	LM 146	ND	ND	ND
	LM 165	ND	ND	ND
	LM 175	ND	ND	ND
	LM 441	16	8	*tet* (W)
	LM 185	ND	ND	ND
	LM 209	ND	ND	ND
	LM 216	ND	ND	ND
	LM 232	ND	ND	ND
	LM 241	ND	ND	ND
	LM 252	ND	ND	ND
	LM 281	32	4	*tet* (W)
	LM 286	ND	ND	ND
	LM 298	32	4	*tet* (W)
	LM 334	ND	ND	ND
	LM 635	ND	ND	ND
	LM 350	16	8	*tet* (W)
	LM 391	ND	ND	ND
	LM 400	ND	ND	ND
	LM 430	ND	ND	ND
	LM 450	ND	ND	ND
	LM 574	ND	ND	ND
	LM 586	ND	ND	ND
	LM 594	ND	ND	ND
	LM 624	8	16	*tet* (W)
	LM 135	ND	ND	ND
	LM 125	16	8	*tet* (W)
	LM 271	16	8	*tet* (W)
	LM 371	8	4	*tet* (W)

Table B *(continued)*

Species	Original Strain No.	MIC (µg ml^{-1}) Tetracycline	Minocycline	*tet* gene
B. bifidum	LMG 11041^T	ND	ND	ND
	LMG 11582	ND	ND	ND
	LMG 11583	ND	ND	ND
	LMG 13195	ND	ND	ND
	LMG 13200	≤ 0.5	ND	NF
	LM 311	1	≤ 0.5	NF
	LM 381	4	2	*tet* (W)
	LM 588	4	4	*tet* (W)
B. breve	LMG 13208^T	ND	ND	ND
	LMG 10645	ND	ND	ND
	LMG 11040	ND	ND	ND
	LMG 11084	≤ 0.5	ND	NF
	LMG 11613	≤ 0.5	ND	ND
	LMG 13194	≤ 0.5	≤ 0.5	NF
	LM 646	ND	ND	ND
B. catenulatum	LMG 11043^T	≤ 0.5	ND	ND
	LMG 18894	≤ 0.5	ND	ND
B. dentium	LMG 11045T	≤ 0.5	ND	NF
	LMG 11585	≤ 0.5	ND	NF
	LMG 10507	≤ 0.5	ND	ND
B. gallicum	LMG 11596^T	≤ 0.5	ND	NF
B. longum (biotype infantis)	LMG 8811^T	2	1	NF
	LMG 11570	ND	ND	ND
	LMG 11588	ND	ND	ND
	LMG 13204	ND	ND	ND
	LMG 18901	ND	ND	ND
	LMG 18902	ND	ND	ND
	LM 418	1	ND	NF
B. longum (biotype longum)	LMG 13197^T	ND	ND	ND
	LMG 11047	ND	ND	ND
	LMG 11589	ND	ND	ND
	LMG 13196	ND	ND	ND
	LMG 18899	≤ 0.5	ND	ND
	LM 257	ND	ND	ND
	LM 613	≤ 0.5	ND	ND
	LM 614	ND	ND	ND
	LM 655	ND	ND	ND
	LM 669	ND	ND	ND
	LM 676	ND	ND	ND
B. pseudocatenulatum	LMG 10505^T	32	1	*tet* (W)
	LMG 11593	32	1	*tet* (W)
	LMG 18903	1	ND	NF
	LMG 18904	1	ND	NF
	LMG 18910	32	2	*tet* (W)
B. scardovii	LMG 21589^T	≤ 0.5	ND	NF
	LMG 21590	≤ 0.5	ND	NF

ND: Not Determined

NF: None of the tested *tet* genes were Found

[a] All bifidobacteria tested possessed a natural resistance to gentamicin, polymyxin B and sulphamethoxazole. They were uniformely susceptible to amoxicillin, chloramphenicol, clindamycin, erythromycin, rifampicin, quinupristin/dalfopristin and vancomycin

Susceptibility to ciprofloxacin, minocycline, tetracycline, trimethoprim and trimethoprim/sulphamethoxazole was variable and strain-specific.

Table C: Immunological and survival properties of *Bifidobacterium* strains tested in this study

Species	Original Strain No.	Immunological properties		Survival properties[a]	
		IL-10/IL-12	IL-10/TNF-α	Tests performed	Bile Salt Hydrolase
B. adolescentis	LMG 10502^T	19,0 ± 7,59	0,065 ± 0,030	GJ, P, CBS	+
	LMG 10733	ND	ND	GJ, P, CBS	+
	LMG 10734	ND	ND	GJ	+
	LMG 11579	ND	ND	GJ, P, CBS	+
	LMG 18897	17,7 ± 4,04	0,173 ± 0,115	GJ	+
	LMG 18898	ND	ND	GJ	+
B. angulatum	LMG 10503^T	18,0 ± 4,32	0,946 ± 0,577	GJ, P, CBS	+
	LMG 11568	18,6 ± 3,97	0,401 ± 0,164	GJ, P, CBS	+
B. animalis subsp. *animalis*	LMG 10508^T	ND	ND	ND	ND
	LMG 18900	ND	ND	ND	ND
B. animalis subsp. *lactis*	LMG 18314^T	26,6 ± 7,65	0,084 ± 0,028	GJ, P, CBS	+
	LMG 11580	ND	ND	GJ	+
	LMG 18906	ND	ND	GJ	+
	LMG 11615	13,5 ± 3,01	0,039 ± 0,012	GJ, P, CBS	+
	LM 2	32,4 ± 4,60	0,092 ± 0,031	GJ, P, CBS	+
	LM 33	ND	ND	ND	ND
	LM 13	34,3 ± 11,44	0,305 ± 0,176	GJ	+
	LM 21	ND	ND	ND	ND
	LM 155	ND	ND	ND	ND
	LM 41	ND	ND	ND	ND
	LM 79	ND	ND	ND	ND
	LM 90	ND	ND	ND	ND
	LM 104	ND	ND	ND	ND
	LM 198	33,7 ± 8,59	0,380 ± 0,156	GJ	+
	LM 118	15,2 ± 3,78	0,045 ± 0,015	GJ	+
	LM 146	ND	ND	ND	ND
	LM 165	33,3 ± 8,02	0,084 ± 0,028	GJ, P, CBS	+
	LM 175	ND	ND	ND	ND
	LM 441	24,1 ± 7,47	0,080 ± 0,030	GJ	+
	LM 185	ND	ND	ND	ND
	LM 209	ND	ND	ND	ND
	LM 216	38,2 ± 9,06	0,075 ± 0,023	GJ	+
	LM 232	30,9 ± 7,10	0,066 ± 0,016	GJ	+
	LM 241	30,4 ± 5,61	0,077 ± 0,021	GJ	+
	LM 252	ND	ND	ND	ND
	LM 281	ND	ND	ND	ND
	LM 286	ND	ND	ND	ND
	LM 298	ND	ND	ND	ND
	LM 334	ND	ND	ND	ND
	LM 635	21,4 ± 6,79	0,056 ± 0,022	ND	ND
	LM 350	34,3 ± 8,99	0,091 ± 0,029	GJ	+
	LM 391	9,6 ± 4,06	0,040 ± 0,017	ND	ND
	LM 400	ND	ND	ND	ND
	LM 430	ND	ND	ND	ND
	LM 450	ND	ND	ND	ND
	LM 574	ND	ND	ND	ND

Table C: ***Continued***

Species	Original Strain No.	Immunological properties		Survival properties[a]	
		IL-10/IL-12	IL-10/TNF-α	Tests performed	Bile Salt Hydrolase
	LM 586	17,5 ± 6,07	0,053 ± 0,020	ND	ND
	LM 594	13,2 ± 3,61	0,042 ± 0,011	ND	ND
	LM 624	27,7 ± 9,88	0,326 ± 0,172	GJ, P, CBS	+
	LM 135	17,1 ± 6,45	0,058 ± 0,026	GJ	+
	LM 125	24,2 ± 8,84	0,089 ± 0,032	GJ	+
	LM 271	8,2 ± 3,43	0,045 ± 0,016	GJ	+
	LM 371	13,3 ± 3,36	0,044 ± 0,015	GJ	+
B. bifidum	LMG 11041^T	12,5 ± 5,54	0,414 ± 0,077	GJ, P, CBS	+
	LMG 11582	ND	ND	GJ	+
	LMG 11583	ND	ND	GJ	+
	LMG 13195	ND	ND	GJ	+
	LMG 13200	26,6 ± 11,33	2,531 ± 1,994	GJ	+
	LM 311	4,3 ± 1,39	3,270 ± 1,526	GJ	+
	LM 381	11,5 ± 4,81	0,049 ± 0,010	GJ, P, CBS	+
	LM 588	7,4 ± 2,85	1,358 ± 0,521	GJ, P, CBS	+
B. breve	LMG 13208^T	42,9 ± 5,30	0,177 ± 0,061	GJ, P, CBS	+
	LMG 10645	10,1 ± 4,88	0,247 ± 0,189	GJ	+
	LMG 11040	ND	ND	GJ, P, CBS	+
	LMG 11084	ND	ND	GJ	+
	LMG 11613	ND	ND	GJ	+
	LMG 13194	ND	ND	GJ	+
	LM 646	24,4 ± 5,86	2,808 ± 2,005	GJ, P, CBS	+
B. catenulatum	LMG 11043^T	24,5 ± 2,75	0,062 ± 0,020	GJ, P, CBS	+
	LMG 18894	12,1 ± 7,51	0,052 ± 0,016	GJ, P, CBS	+
B. dentium	LMG 11045T	23,5 ± 5,44	0,061 ± 0,019	ND	ND
	LMG 11585	ND	ND	ND	ND
	LMG 10507	ND	ND	ND	ND
B. gallicum	LMG 11596^T	13,5 ± 3,71	5,031 ± 4,689	GJ, P, CBS	-
B. longum (biotype infantis)	LMG 8811^T	5,2 ± 3,05	0,030 ± 0,011	GJ, P, CBS	-
	LMG 11570	ND	ND	GJ, P, CBS	+
	LMG 11588	ND	ND	GJ	-
	LMG 13204	ND	ND	GJ	+
	LMG 18901	ND	ND	GJ	+
	LMG 18902	25,1± 10,50	0,107 ± 0,027	GJ, P, CBS	+
	LM 418	41,5 ± 8,71	0,304 ± 0,178	GJ, P, CBS	+
B. longum (biotype longum)	LMG 13197^T	9,7 ± 1,50	5,347 ± 2,506	GJ, P, CBS	+
	LMG 11047	ND	ND	GJ	+
	LMG 11589	ND	ND	GJ	+
	LMG 13196	ND	ND	GJ, P, CBS	+
	LMG 18899	26,8 ± 4,09	0,259 ± 0,113	GJ	+
	LM 257	30,5 ± 8,00	0,161 ± 0,067	GJ, P, CBS	+
	LM 613	ND	ND	ND	ND
	LM 614	42,4 ± 11,12	0,258 ± 0,098	GJ	+
	LM 655	ND	ND	ND	ND
	LM 669	33,4 ± 4,53	0,134 ± 0,052	GJ	+
	LM 676	25,2 ± 7,32	0,128 ± 0,058	GJ, P, CBS	+

Table C: ***Continued***

Species	Original Strain No.	Immunological properties		Survival properties[a]	
		IL-10/IL-12	IL-10/TNF-α	Tests performed	Bile Salt Hydrolase
B. pseudocatenulatum	LMG 10505^T	23,7 ± 4,16	0,349 ± 0,132	GJ, P, CBS	+
	LMG 11593	ND	ND	GJ	+
	LMG 18903	ND	ND	GJ, P, CBS	+
	LMG 18904	28,5 ± 9,09	0,341 ± 0,116	GJ	+
	LMG 18910	ND	ND	GJ, P, CBS	+
B. scardovii	LMG 21589^T	5,2 ± 1,32	0,029 ± 0,010	ND	ND
	LMG 21590	6,4 ± 3,41	0,083 ± 0,073	ND	ND
Lactobacillus salivarius	LS 33	26,1 ± 15,48	0,082 ± 0,019	X	X
Lactococcus lactis	MG1363	0,8 ± 0,45	0,019 ± 0,005	X	X

ND: Not Determined

[a] All strains tested showed good survival capacity in presence of pancreatin (P). Survival in presence of gastric juice (GJ) and conjugated bile salts (CBS) was variable and strain-specific.

Curriculum Vitae

1. General information

Full name: Liesbeth Noella Alex Masco

Date of Birth: July 25th, 1979

Place of Birth: Gent, Belgium

2. Educational background

1985-1991: Primary School - Gemeenteschool, Sint-Martens-Latem

1991-1997: Secondary School - Koninklijk Atheneum III, Gent

- Latijn-wiskunde

1997-2005: University - Ghent University

- 1997-1999: Candidate in Biology
- 1999-2001: Licentiate in Biotechnology

  Dissertation:

  "Molecular characterisation of tetracycline-resistance genes in lactic acid bacteria isolated from a process line of fermented dry sausage"
- 2001-2005: Ph.D. in Biotechnology

  Dissertation:

  "Identification, antimicrobial susceptibility and functionality of potentially probiotic bifidobacteria"

  Laboratory of Microbiology
  Department of Biochemistry, Physiology and Microbiology
  Faculty of Sciences
  Ghent University

  Specialization grant IWT (Flemish government institution)

3. Scientific activities

Supervision of students:

2002-2003: Marcy Vandewalle

Dissertation:
"Molecular detection and characterization of bifidobacteria isolated from probiotic products."

2003-2004: Koenraad Van Hoorde

Dissertation:
"Research regarding the presence of antibiotic resistances in bifidobacteria isolated from probiotic products."

2004-2005: Charlotte Crokaerts

Dissertation:
"*In vitro* survival of probiotic product isolates and human reference strains of *Bifidobacterium* under simulated conditions of the gastro-intestinal tract."

Assistance in practical courses of microbiology (2002-2005)

Stay in foreign lab:

2004-2005: Laboratoire de Bactériologie des Ecosystèmes, Institut Pasteur de Lille-Institut de Biologie de Lille, France.

Attended courses:

Academic English: conference skills. Katrien Deroey. February - April 2005. Ghent University.

4. List of publications

4.1. Publications in international journals (peer-reviewed)

2002:

Gevers D., Masco L., Baert L., Huys G., Debevere J. and Swings J. (2002). Prevalence and diversity of tetracycline resistent lactic acid bacteria and their *tet* genes along the process line of fermented dry sausages. *Systematic and Applied Microbiology* **26**(2):277-283.

2003:

Masco L., Huys G., Gevers D., Verbrugghen L. and Swings J. (2003). Identification of *Bifidobacterium* species using rep-PCR fingerprinting. *Systematic and Applied Microbiology* **26**(4):557-563.

Temmerman R., Masco L., Vanhoutte T., Huys G. and Swings J. (2003). Development and validation of a nested PCR- Denaturing Gradient Gel Electrophoresis method for taxonomic characterization of bifidobacterial communities. *Applied and Environmental Microbiology* **69**(11):6380-6385.

2004:

Masco L., Ventura M., Zink R., Huys G. and Swings J. (2004). Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology* **54**:1137-1143.

2005:

Masco L., Huys G., De Brandt E., Temmerman R. and Swings J. (2005). Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. *International Journal of Food Microbiology* **102**(2):221-230.

Accepted:

Masco L., Van Hoorde K., De Brandt E., Swings J. and Huys G. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products.*The Journal of Antimicrobial Chemotherapy*.

Submitted:

Masco L., Vanhoutte T., Temmerman R, Swings J. and Huys G. Evaluation of real-time PCR targeting the 16S rRNA and recA genes for the enumeration of bifidobacteria in probiotic products. *International Journal of Food Microbiology.*

Masco L., Pot B., Foligné B., Grangette C., Swings J. and Huys G. *Bifidobacterium* strains induce *in vitro* cytokine production by human peripheral blood mononuclear cells in a strain-specific way. *FEMS Immunology and Medical Microbiology.*

Masco L., Huys G., Crockaert C. and Swings J. *In vitro* assessment of the gastrointestinal transit tolerance of human reference strains and probiotic isolates of *Bifidobacterium*. *International Journal of Food Microbiology.*

4.2. Publications in international journals (not peer-reviewed)

2003:

Temmerman R. and Masco L. (2003). Probiotics: Health aspects and applications. *Innovations in Food Technology* **18:**16-18.

4.3. Book chapters

2004:

Temmerman R., Masco L., Huys G. and Swings J. (2004). In search of new probiotics: diversity, selection and exploitation of health-promoting organisms. *In: Microbial Genetic Resources and Biodiscovery* (I. Kurtböke & J. Swings, Eds.). Queensland Complete Printing Services, Queensland. pp. 220-234.

2005:

Temmerman R., Masco L., Huys G. and Swings J. (2005). Application of repetitive element sequence-based (rep-) PCR and denaturing gradient gel electrophoresis for the identification of lactic acid bacteria in probiotic products. *In: Probiotics in Food Safety and Human Health* (I. Goktepe & V. uneja, Eds.). Marcel Dekker Inc., New York. *In press.*

5. Poster presentations

Masco L., Huys G., Gevers D., Verbrugghen L. and Swings J. (2003). Identification of *Bifidobacterium* species using rep-PCR fingerprinting. The Food, GI-tract Functionality and Human Health Cluster PROEUHEALTH Workshop 2, 3-5 March 2003, Taormina, Italy.

Masco L., Huys G., Temmerman R. and Swings J. (2004). Qualitative analysis of *Bifidobacterium* strains originating from probiotic products following a culture-dependent and culture-independent approach. The Food, GI-tract Functionality and Human Health Cluster PROEUHEALTH Workshop 3, 15-17 March 2004, Sitges, Spain.

Masco L., Temmerman R., Vanhoutte T., Huys G. and Swings J. (2004). Real-time quantitative detection of *Bifidobacterium* strains in probiotic products. 13ème Colloque du club des bacteries lactiques. 8-10 September 2004, Nantes, France.

Masco L., Vancanneyt M., Van Hoorde K., De Brandt E., Huys G. and Swings J. (2005). Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. The 8th symposium on Lactic Acid Bacteria. 28 august-1 september, Egmond aan Zee, The Netherlands.

Masco L., Pot B., Foligné B., Grangette C., Huys G. and Swings J. (2005). Effect of human reference strains and commercial probiotic isolates of *Bifidobacterium* on *in vitro* cytokine production by human peripheral blood mononuclear cells. The 3rd Probiotics, Prebiotics and New Foods Congress. 4-6 September 2005, Rome, Italy.