Bifidobacteria: Genetic Modification and the Study of Their Role in the Colon

Mariët J. van der Werf* and Koen Venema

Department of Applied Microbiology and Gene Technology, TNO Nutrition and Food Research, P.O. Box 360, 3700 AJ Zeist, The Netherlands

Bifidobacteria are among the most common bacteria in the human intestine and are thought to have a positive effect on human health. Therefore, there is an increasing interest in using these microorganisms as probiotics, either in fermented dairy products or formulated as tablets. However, convincing scientific data supporting their health claims are scarce. The study of the role of bifidobacteria in the colon is complicated by the fact that they are part of a complex ecosystem also interacting with the human host and by the fact that their in vivo study encounters many ethical constraints. Several tools have been developed at TNO with which the role of bifidobacteria can be studied. These include (i) an efficient transformation protocol for the introduction of foreign DNA into *Bifidobacterium* strains and (ii) in vitro models of the stomach/small intestine (TIM-1) and large intestine (TIM-2), creating an environment closely resembling that of the in vivo situation. With these tools, biomarkers from bifidobacteria quantifying their positive effect on gut health can be identified.

Keywords: *Bifidobacterium; probiotics; prebiotics; genetic engineering; transformation; in vitro gastrointestinal model*

INTRODUCTION

The flora of the gastrointestinal tract is a host- and location-specific complex strictly anaerobic ecosystem. The composition and activity of this microflora influence the host's health, including nutrition, physiologic function, drug efficacy, carcinogenesis, and aging as well as the host's immunological responses, resistance to infection, and responses to endotoxin and various other stresses (*1*). The intestinal flora comprises >500 different bacterial species. Although the stomach and upper small intestine contain relatively few resident microorganisms, numbers increase approaching the colon, where there are $10^{10}-10^{11}$ bacteria per gram. The predominant species are *Bacteroides*, *Bifidobacterium*, and *Eubacterium* (Table 2); the potential pathogens among them, *Escherichia coli*, *Veillonella*, *Staphylococcus*, *Clostridium*, and *Klebsiella*, are much less numerous (*2*).

Overall, the microflora has a positive effect on human health. It is generally assumed that *Bifidobacterium* species are the most important bacteria for this health-promoting effect. This is based on the observation that bifidobacteria are normal inhabitants of the human intestinal tract throughout the life cycle, beginning just days after birth. Furthermore, breast-fed babies have higher numbers of *Bifidobacterium* than bottle-fed children, and they are less at risk for diarrheal disease/susceptible to infections than formula-fed infants (*3*). In contrast, in elderly persons the *Bifidobacterium* numbers decrease, whereas clostridial numbers significantly increase.

* Author to whom correspondence should be addressed (telephone +31-30-6944071; fax +31-30-6944466; e-mail vanderWerf@voeding.tno.nl).

Table 1. Transformation of Different Bifidobacterial Species (from Reference *20*)

species	transformants per μg of plasmid
B. animalis ATCC 27536	5×10^3
B. breve 4	1.3×10^4
B. breve AS	2×10^2
B. bifidum U3	3×10^2
B. bifidum ATCC 15696	7.4×10^3
B. infantis U1	2.5×10^2
B. infanctis ATCC 27920	4×10^4
B. longum U2	2.6×10^3
B. longum Wiesby 2	7×10^4

Bifidobacteria are Gram-positive, anaerobic, nonmotile, and nonsporulating fermentative rods, which are often Y- or V-shaped. Traditionally, bifidobacteria are considered as members of the lactic acid bacteria, although this classification is not unanimously accepted. Bifidobacteria produce acetate and lactate (3:2) as their major end products. The mole percent G+C of the DNA of bifidobacteria is high and ranges between 55 and 64%. On the basis of DNA−DNA hybridization measurements and sugar fermentation patterns, 32 species are distinguished within the genus *Bifidobacterium* in the present classification. Bifidobacteria are also host specific; in humans, mainly *B. bifidum*, *B. longum*, *B. breve*, *B. infantis*, and *B. adolescentis* are found, and these are also the five species applied as probiotics. The specific *Bifidobacterium* species alter from predominantly *B. infantis* and *B. breve* in infants to *B. adolescentis* and *B. longum* in adults (*4*).

Since the recognition of the beneficial effects of bifidobacteria, there has been considerable interest in their use as probiotics, either in dairy products (e.g., yogurts) or formulated as tablets. A probiotic has been defined as "a live microbial feed supplement which

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beneficially affects the host animal by improving its intestinal microbial balance" (*5*). Food manufacturers claim that, upon daily intake, probiotic bifidobacteria colonize the gastrointestinal tract and exert their beneficial effects on the host by (i) maintaining a healthy microflora (prevention of diarrhea), (ii) inhibiting the growth of pathogenic bacteria by producing bacteriocins and lowering the pH [by acetate and lactate production], (iii) improving lactose tolerance, (iv) reducing serum cholesterol levels, (v) reducing blood ammonia concentrations, (vi) the production of vitamins (e.g., folate; *6*), (vii) stimulating the immune system, and (viii) having an anticarcinogenic and antimutagenic activity (*1*, *7*).

The scientific community has not yet reached a consensus over the effect of probiotic strains. Some studies show no effect of probiotics on certain parameters measured; other studies show clear effects. The fact that not all in vivo studies are random, double-blind, and placebo controlled does not help to reach the consensus needed for (consumer and) scientific acceptance of the health claims of these products. As probiotic *Bifidobacterium* strains with a scientifically supported health claim have an increased market potential, currently much effort is directed toward proving the claims of probiotics by sound scientific evidence.

The study of the role of bifidobacteria in the colon is complicated by the fact that they are part of a complex ecosystem also interacting with the human host and by the fact that their in vivo study encounters many ethical constraints. At TNO, we have developed several tools with which the role of bifidobacteria can be studied. Here we describe (i) an efficient transformation protocol for *Bifidobacterium* and (ii) in vitro models of the stomach/small intestine (TIM-1) and large intestine (TIM-2).

GENETIC MODIFICATION OF BIFIDOBACTERIA

Molecular biology provides powerful tools with which the mechanisms behind the activities and interactions of *Bifidobacterium* in the intestine can be elucidated. Moreover, these tools provide a means by which the claims of probiotic bifidobacteria can be substantiated; comparison of genetically characterized bifidobacteria altered in specific properties, that is, by knock out or overexpression of a specific gene, will give definitive support to the role these bacteria have in the intestine and will result in proof for their probiotic properties, required by both scientific and regulatory bodies. Moreover, information concerning the molecular basis of probiotic strains has a great potential to be exploited in screening for novel *Bifidobacterium* strains with safe and effective novel probiotic effects. Instead of the present random natural strain selection procedures, which partly rely on luck, more directed and faster genetic screening approaches may be followed, resulting in more well-defined and reliable results. Furthermore, genetic tools allow the monitoring of metabolic activities of strains after processing into products and consumption. To be able to use genetic tools with bifidobacteria, there are two basic requirements: (i) a *Bifidobacterium* replicating vector and (ii) an efficient transformation system.

Vectors for *Bifidobacterium*. Potentially, three types of vectors can be used that replicate in bifidobacteria: (i) vectors that are based on a replicon derived from *Bifidobacterium*; (ii) plasmids derived from bacteria closely related to *Bifidobacterium*; or (iii) broad host range plasmids that replicate in a wide variety of Gram-positive and Gram-negative bacteria.

Up to now, *B. longum* and *B. breve* are the only bifidobacteria from the human intestinal tract that have been shown to harbor plasmids (*8*−*13*). The incidence of plasmids in *B. longum* was 70% and in *B. breve* 40% (*8*, *10*). Moreover, multiple plasmids, small in size (1.25−9.5 kb), were common in these plasmid-containing strains. Three of these plasmids, pMB1, pClBb1, and pKJ50, have been fully sequenced (*13*−*15*). The latter two plasmids replicate via the rolling-circle mechanism, involving single-stranded intermediates. Several authors have subsequently constructed *Escherichia coli*−*Bifidobacterium* shuttle vectors derived from pMB1 an pKJ50 by cloning the endogenous *Bifidobacterium* plasmid and an antibiotic resistance selection marker in a commercial *E. coli* vector (*14*−*19*). pMB1-derived shuttle vectors replicated only in *E. coli* and *Bifidobacterium* but not in *Bacillus subtilis*, *Lactobacillus reuteri*, or *Lactobacillus casei* (*16*, *20*). These shuttle vectors are good candidates for the construction of improved cloning vectors, such as food-grade cloning and expression vectors, which can be used in *Bifidobacterium*.

We found that also two plasmids from *Corynebacterium* spp., a genus phylogenetically closely related to *Bifidobacterium*, replicated in *Bifidobacterium* (*20*). In contrast, two broad host range plasmids from *Lactobacillus* and *Lactococcus*, two AT-rich microorganisms, did not replicate in *Bifidobacterium* (*20*).

Transformation of Bifidobacteria. The presence of a thick (multilayered) cell wall generally forms a barrier for the uptake of exogenous DNA molecules. Bifidobacteria are known to have very thick and complex cell walls composed of a variety of peptidoglycan layers, in addition to significant amounts of polysaccharides, lipoteichoic acids, and proteins (*21*). A considerable amount of research was carried out by several industrial and academic laboratories to develop a transformation procedure for bifidobacteria, without success. Although the electroporation technique has proven to be widely applicable to genetically transform bacterial strains from several genera of lactic acid bacteria, all *Bifidobacterium* strains examined so far have proved to be refractory to efficient and reproducible transformation. At TNO we have developed an efficient and reproducible transformation procedure (Patent Application WO 95/35389) for members of the genus *Bifidobacterium*. This method is based on electroporation of bifidobacteria made competent by preincubation in electroporation buffer at 4 °C for 4 h (*20*). Longer incubation times at 4 °C resulted in lower transformation frequencies. *Bifidobacterium* cells obtained with this treatment were completely viable and showed no morphological changes. The inclusion of a high concentration of sucrose in the growth medium and electroporation buffer proved to be essential. This procedure was applicable to all *Bifidobacterium* species tested so far, although the efficiencies varied from strain to strain (Table 1). Also, Missich et al. (17) reported low-efficiency transformation by electroporation of a strain of *B. longum*. The procedure described involves freezing of the bacteria at −135 °C for 1 h in 10% glycerol and storage at −70 °C, prior to electroporation. We assume that preincubation at 4 °C, as well as freezing and

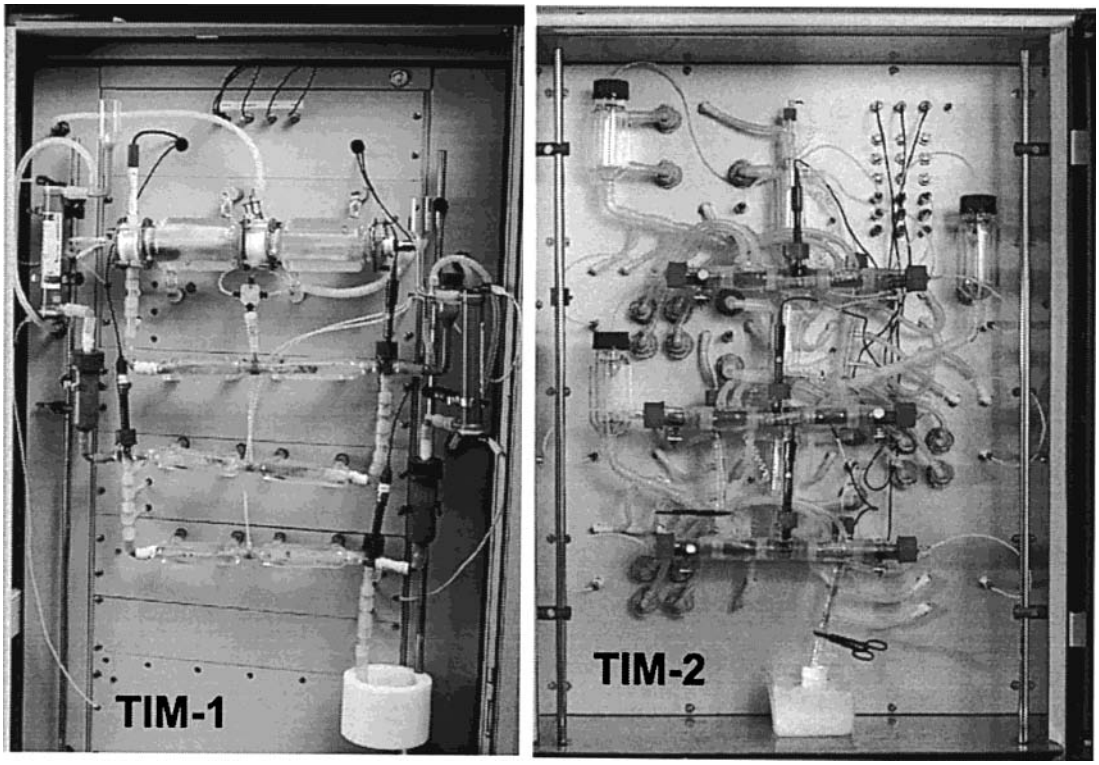

Figure 1. TNOs in vitro gastrointestinal models.

thawing, of bifidobacteria mimics limited autolysis resulting in an altered permeability of the cell wall for the exogenous DNA molecules.

TNO IN VITRO GASTROINTESTINAL MODELS

Another tool we have developed over the past decade for studying bacteria in general, and *Bifidobacteria* in particular, is TNO's in vitro gastrointestinal models (nicknamed TIM) (U.S. Patent 5,525,305; European Patent 0642382). In nutritional research, there is a great need for validated laboratory models with high predictive value because it is simply not possible to perform studies on human volunteers with all varieties of new food ingredients or products to study their physiological effects. Laboratory models have several advantages over research in humans or laboratory animals. The most important is that they do not carry ethical constraints. In addition, they are fast and not labor intensive, are relatively cheap, and have a large sample capacity.

The TIM gastrointestinal models are versatile computer-controlled in vitro systems to determine the fate of ingested products (including live bacteria) in an environment closely resembling the conditions in the stomach, small intestine, and large intestine (*22*−*24*). TIM-1 simulates the stomach and small intestine; TIM-2 is a simulation of the large intestine or colon. These models are accurate and reproducible, and therefore limited numbers of replicates are necessary, and only small amounts of test and control compounds are needed. Separate models exist for babies, adults, and the elderly. For animals, models for pigs, preruminant calves, and dogs exist.

Obviously, limitations of such models should also be considered, for example the absence of hormonal and neurological feedback mechanisms, immunological mechanisms, and other interactions and crosstalks with the (epithelium of the) host. Therefore, we are currently developing the application of monolayers of cultured mucosal and immunocompetent cells on membranes, which can be used in combination with the TIM models. However, it is important to realize that there is a serious drawback in making the system more and more complex.

Description of TIM-1 and TIM-2. The stomach−small intestinal model (TIM-1) consists of a number of linked glass units with flexible walls inside (Figure 1). Peristaltic movements are achieved by pumping water of body temperature into the space between the glass jacket and the flexible wall at regular intervals. The computer controls the sequential sqeezing of the walls, causing the chime to mix and move. The model is equipped with hollow fiber membranes to remove digestion products and prevent product inhibition due to buildup of metabolites. The pH of every compartment (stomach, duodenum, jejunum, and ileum) is controlled continuously. Emptying of the stomach and small intestine is controlled by gastric and ileal emptying curves, respectively, based on average data from in vivo data, although extremes in a population can easily be simulated.

TIM-2, the colon model, also consists of a number of linked glass units with flexible walls inside (Figure 1). The model is equipped with hollow fiber membranes running through the model to remove microbial fermentation products such as short-chain fatty acids (SCFAs). Also here, the dialysis system maintains physiological concentrations of small molecules such as electrolytes and SCFAs and prevents product inhibition due to buildup of microbial metabolites. The environment in the model is strictly anaerobic (achieved by flushing with nitrogen gas) to allow for the growth of a high density, complex, active microflora (Table 2). The flora can be of human or animal origin. Several sampling ports are present, allowing the study of differences in the microflora at the beginning and the end of the colon

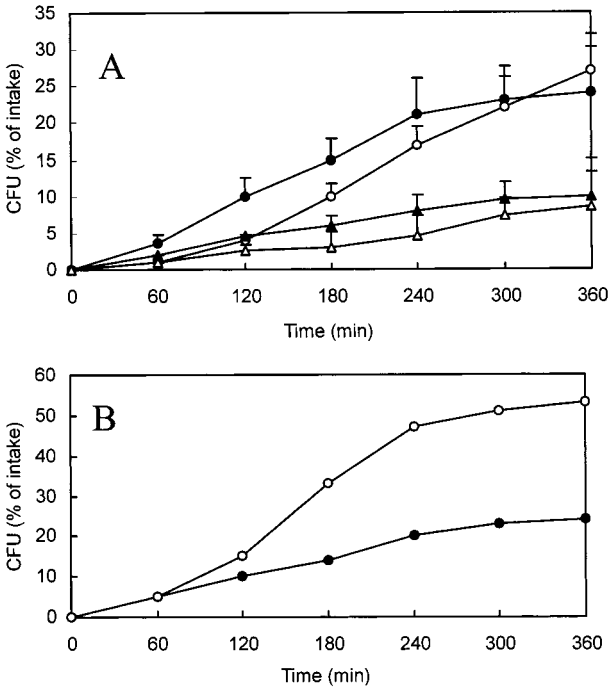

Figure 2. Survival of bifidobacteria in TIM-1 (from ref *26*): (A) comparison of survival of *B. bifidum* (△) and *L. acidophilus* (○) in in vivo (open symbols) experiments and in TIM-1 (solid symbols); (B) survival of bifidobacteria in the presence of a normal (●) or low concentration of bile acids (○).

Table 2. Typical Composition of Microflora in TIM-2 and in Vivo (Fecal Material)

genus	cfu/mL in TIM-2	cfu/g in vivo
Bifidobacteria	10^9-10^{10}	10^9-10^{11}
Bacteroides	10^9-10^{10}	10^9-10^{10}
Lactobacilli	10^5-10^7	10^5-10^7
Enterobacteriaceae	10^5-10^6	10^4-10^6
Enterococci	10^5-10^6	10^5-10^6
sulfite-reducing *Clostridia*	10^4-10^5	10^4-10^5

model. Especially, the peristaltic mixing, the physiological density of microorganisms, with natural production rate of metabolites, and the hollow fiber membranes make the TIM models unique.

Validation. Any in vitro method or model should be thoroughly validated to ensure its applicability and assess its limitations. TIM-1 was validated for various applications. Protein digestibility in vivo of different feeds in pigs and preruminant calves was compared with digestibility in TIM (*24*). Furthermore, digestibility of carbohydrates (*24*), absorption of calcium (*24*), availability of minerals (*25*), and survival of bifidobacteria and lactobacilli during passage through the model (see below and ref *26*) were compared with in vivo studies.

Validation of TIM-2 was done with regard to the composition of the microflora (Table 2), the enzymatic activity of the microflora, and the production and concentration of SCFAs, gases, and other metabolites (*24*).

Survival of Bifidobacteria during Passage through the Gastrointestinal Tract. TIM-1 is ideally suited to investigate the survival of probiotic strains during passage through the stomach and small intestine. During passage through this part of the gastrointestinal tract, microorganisms encounter multiple forms of hostile activities from the host. These are the acid environment of the stomach, the secretion of bile in the duodenum, and the peristaltic movements of the intestine.

Studies investigating the survival of probiotic strains under these conditions show that *B. bifidum* survive relatively well compared with, for instance, *L. acidophilus* (Figure 2a). The percentage of survival is dependent on host factors such as the amount of bile salt secretion. This is exemplified in Figure 2b, in which the effect of low bile salt secretion is shown. Whereas in relative physiological concentrations of bile salt the survival is ∼25%, at low concentrations of bile this is increased to ∼50%. Figure 2a in addition shows that the values found in vitro (TIM-1) are very similar to those obtained in vivo in intubated human volunteers (*26*).

Effect of Prebiotics on the Microflora in TIM-2. Bifidobacteria are known to be able to grow on a variety of oligosaccharides. These are considered prebiotics when they "selectively stimulate the growth and/or activity of one or a limited number of beneficial endog-

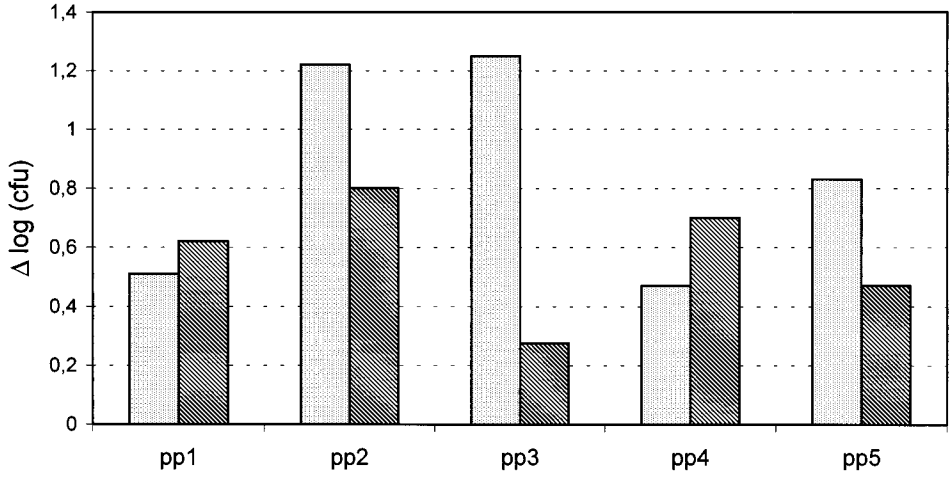

Figure 3. Increase in bifidobacterial counts in vivo and in TIM-2 in fecal samples of different test persons after lactulose treatment. Postmenopausal women (55−65 years) provided a fecal inoculum 10 days prior to and after 7 days of lactulose feeding (10 g of lactulose/day). Fecal material was collected on day 8 (no lactulose treatment) and day 17 (after 7 days of lactulose treatment) and inoculated separately in TIM-2. In vitro also 10 g of lactulose/day was added, spread over the complete day. The experiments in vitro lasted only 48 h. Samples were analyzed for microbial composition using classical plating techniques on (s)elective plates in combination with DGGE and FISH. Light bars represent data from in vivo experiments, and dark (shaded) bars represent changes in bifidobacterial counts in the in vitro experiments.

enous bacteria in the colon" (*27*). Examples of these oligosaccharides are fructo-oligosaccharides (*28*, *29*) and galacto-oligosaccharides (*30*, *31*). Also, lactulose has been shown to stimulate bifidobacteria (*32*, *33*).

We investigated the effect of lactulose on the colonic microflora in TIM-2 and compared it to the in vivo change in microbial composition of the feces (the full report on these data will be published elsewhere). The effect of lactulose on the in vivo and in vitro fecal bifidobacterial counts in five of these test persons is shown in Figure 3. On average, an increase in the in vivo bifidobacteria counts was observed in all women fed lactulose. Also in vitro, the average *Bifidobacterium* counts increased. Both the absolute increase in bifidobacteria numbers and the increase in the in vivo and the in vitro studies depended on the person tested (Figure 3). Comparison of the other microorganisms shows that lactobacilli, bacteroidaceae, and enterobacteriaceae follow the same trend in TIM-2 as in vivo (results not shown). The bifidogenic effect of lactulose, both in vivo and in vitro, is in agreement with the results reported by Terada et al. (*32*) and Mizota (*33*).

DISCUSSION

The tools described in this paper are valuable for substantiating the health claims of bifidobacteria by sound scientific experiments. Furthermore, they can help in finding or developing improved probiotic *Bifidobacterium* strains. Moreover, these tools can help in finding solutions for the stability of bifidobacteria during processing, that is, their oxygen sensitivity and viability upon storage.

In the future, the tools of applied genomics [i.e., transcriptomics (DNA array technology), proteomics, and metabolomics] will undoubtedly further enlarge our understanding of probiotic bifidobacteria and help in discovering the molecular basis for their presumed health-promoting effect(s). We are currently developing different arrays that will allow the study of the variation in the composition of the microflora and the induction of specific bifidobacterial genes upon consumption of prebiotics or other health-promoting food substances.

ABBREVIATIONS USED

TIM, **T**NO's in vitro gastro**I**ntestinal **M**odel; SCFA, short-chain fatty acid.

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