

APPENDIX



Ghent University
Laboratory of Microbial Ecology and Technology
Coupure L 653
B-9000 Ghent
Tel.: +32 09 264 59 76
Fax.: +32 09 264 62 48
E-mail: Willy.Verstraete@Ugent.be

Report : Survivability of encapsulated *Bifidobacterium longum* under *in vitro* gastrointestinal conditions

Ir. Ellen Eeckhaut
Ir. Selin Bolca
Ir. Lynn Vanhaecke
Dr. ir. Tom Van de Wiele

Ghent, February 17, 2007

Introduction

Probiotics are living microorganisms that, when ingested, beneficially affect the host's health. They are commonly dosed as food supplements under the form of yoghurts or capsules. There are different mechanisms by which probiotic organisms have positive effects towards the host. The group of probiotic lactobacilli for instance produce lactic and acetic acid by which they lower the pH in the intestinal environment, thus creating growth-inhibiting conditions for opportunistic pathogens. Some lactobacilli also produce bacteriocins by which they have a direct inhibitory effect against pathogens and other probiotic organisms go into competition with pathogens for nutrients. Some probiotic strains adhere to the intestinal epithelium by which they prevent the adherence of pathogenic bacteria. Moreover, some probiotics stimulate the host's immune system by increasing the epithelial production of immunoglobulins.

The most important criteria for microorganisms to come into consideration for being applied as a probiotic are survival in the acidic environment of the stomach, resistance against digestive enzymes and bacteriostatic bile salts in the small intestine, survivability in the complex microbial community of the large intestine and ability to decrease the adherence and/or growth of pathogenic bacteria in the gastrointestinal tract.

Bifidobacterium longum is one of the best studied probiotic bacteria from the group of bifidobacteria. The genome has been fully sequenced and its probiotic effects in human have been well-described. For instance, *Bifidobacterium longum* has been successfully applied to abate lactose intolerance, to relieve gastrointestinal complaints from radiation sickness and increase the colonization resistance against pathogens. Up till now, *Bifidobacterium longum* has been typically applied in a dosage range of 9 to 11 log CFU/mL. This way, enough bacterial cells survived the stomach and small intestine passage in order to ensure a high enough concentration reaching the large intestine environment that may then elicit health-promoting effects.

Yet, increasing the survival of this probiotic strain during gastrointestinal passage may still enhance the effectiveness of this probiotic. One of the novel techniques that is under study is encapsulation which would make a strain stable, convenient to administer and amenable to widespread use. In this study, it was evaluated to what extent encapsulation of *Bifidobacterium longum* increased its survival during stomach and small intestine passage and to what extent the added strain was able to maintain itself in the complex microbial community from the large intestine. For this, the Simulator of the Human Intestinal Microbial Ecosystem was used.

Material and methods

Products of *Bifidobacterium longum*

Two products were tested and compared to one another. A first product contained *Bifidobacterium longum* in lyophilized form and is further referred to as Bif L. The second product contained *Bifidobacterium longum* in the encapsulated form and is further referred to as Bif E.

Reactor setup

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a dynamic model of the human gastrointestinal tract. It consists of 5 double-jacketed vessels maintained at a temperature of 37 °C, respectively simulating the stomach, small intestine, ascending colon, transverse colon and descending colon, with a total retention time of 76 h. The colon vessels harbour a mixed microbial community and pH controllers (pH controller R301, Consort, Turnhout, Belgium) maintain the pH in the range 5.6-5.9, 6.2-6.5 and 6.6-6.9 in the ascending, transverse and descending colon simulations, respectively. There is no gas exchange between the different vessels and the headspace of the culture system was flushed twice a day for 15 min with N₂ to ensure anaerobic conditions. The growth medium for the microbial inoculum consisted of a carbohydrate-based medium containing arabinogalactan (1 g l⁻¹), pectin (2 g l⁻¹), xylan (1 g l⁻¹), starch

(4.2 g l⁻¹), glucose (0.4 g l⁻¹), yeast extract (3 g l⁻¹), peptone (1 g l⁻¹), mucin (4 g l⁻¹) and cysteine (0.5 g l⁻¹).

For the survival of both *Bifidobacterium* products, a worst case scenario was envisaged. Here, a stomach pH of 2 was maintained during 2 hours after which the stomach suspension with Bif L or Bif E was gradually transferred to the small intestine compartment where digestion with pancreatic enzymes and bacteriostatic bile salts takes place.

Experimental setup

The SHIME reactor was operated during 12 weeks and consisted of the following periods:

- Stabilization period: week 1, 2 and 3
- Control period: week 4 and 5
- Treatment period Bif L: week 6
- Washout period Bif L: week 7
- Treatment period Bif E: week 8
- Washout period Bif E: week 9
- Prolonged washout and monitoring period: week 10, 11 and 12

At the beginning of the stabilization period, the SHIME colon vessels were inoculated with isolated fecal microorganisms. During the first 3 weeks of the SHIME run, different microbial communities develop in the respective colon vessels, due to changes in parameters such as incoming digested food, pH, redox potential... After three weeks, the microbial communities in the ascending, transverse and descending colon vessels differ from one another in composition and metabolic activity and resemble the microbial community from the respective colon compartments *in vivo*.

The major objective during the control period was monitoring the baseline levels of fermentation activity and determination of the microbial community in the different colon vessels. This way, it could be assessed to what extent the supplementation of Bif L or Bif

E influenced the residing microbial community or Bifidobacteria population in the colon vessels. During the treatment period Bif L, 1 g of Bif L was supplemented daily to the SHIME reactor. It was subjected to the stomach and small intestine digestion steps and was then transferred to the colon compartments. During the washout period Bif L, no supplementation of the *Bifidobacterium* product was carried out. During the treatment period Bif E, 1 go of Bif E was supplemented daily to the SHIME reactor. It was subjected to the stomach and small intestine digestion steps and was then transferred to the colon compartments. During the washout period Bif E, there was no more supplementation of Bif E to the SHIME. The washout period was prolonged to see whether Bif E had an long term effect on the metabolic activity or community composition of the SHIME colon compartments.

Analyses

Short chain fatty acids (SCFA). Liquid samples were collected and frozen at -20 °C for subsequent analysis. The SCFA were extracted from the samples with diethyl ether and determined with a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands). The GC was equipped with a capillary free fatty acid packed column (EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 mx0.53 mm; film thickness 1.2 µm), a flame ionization detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL min⁻¹. The column temperature and the temperature of the injector and detector were set at 130 °C and 195 °C, respectively.

Ammonia. Using a 1026 Kjeltex Auto Distillation (FOSS Benelux, Amersfoort, The Netherlands), ammonium in the sample was liberated as ammonia by the addition of an alkali (MgO). The released ammonia was distilled from the sample into a boric acid solution. The solution was back-titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm).

Microbial community analysis.

Plate counting. The following bacterial groups were quantified by growth on specific media (Oxoid, Hampshire, UK): lactobacilli (rogosa agar), bifidobacteria (raffinose *Bifidobacterium* agar), enterococci (*Enterococcus* agar), enterobacteria (MacConkey agar), and clostridia (tryptose sulfite cycloserin agar). Liquid samples were withdrawn from the culture system and serially diluted in saline solution (8.5 g NaCl l⁻¹). Three plates were inoculated with 0.1 ml sample of three dilutions, and incubated at 37 °C (43 °C for enterobacteria) under aerobic or anaerobic conditions where necessary. Anaerobic incubation of plates was performed in jars with a gas atmosphere (84% N₂, 8% CO₂, and 8% H₂) adjusted by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium).

PCR-DGGE. The bifidobacteria population of the colon was analysed. A nested PCR approach was used to amplify the 16S ribosomal RNA genes of the bifidobacteria. In brief, one µl of the DNA was amplified using the primers BIF164f-BIF662r for bifidobacteria. When the first PCR round produced a clearly visible band, a second amplification round with forward primer P338F (with a GC-clamp of 40 bp) and reverse primer P518r was used. The 16S rRNA genes of all bacteria were amplified applying primers P338F with GC-clamp and P518r on total extracted DNA. Denaturing gradient gel electrophoresis was performed as described earlier using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). On each gel, a home made marker of different PCR fragments was loaded, which was required for processing and comparing the different gels (Boon *et al.* 2002). The polyacrylamide gels were made with denaturing gradient ranging from 45% to 60%. The electrophoresis was run for 16 h at 60 °C and 38 V. Staining and analysis of the gels was performed as described previously described.

Real-time PCR. For quantification of bifidobacteria by real-time PCR, amplification was performed in 25 µl reaction mixtures by using buffers supplied with the qPCRe Core

Kit for Sybre Green I as described by the suppliers (Eurogentec, Liège, Belgium) in Micro-Amp Optical 96-well reaction plates with optical caps (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Primers BIF164f-BIF662r for 16S ribosomal RNA genes were used for the quantification of bifidobacteria at a concentration of 1 $\mu\text{mol l}^{-1}$. PCR temperature program was as follows: 50°C for 2 min, 95 °C for 10 min, followed by 40 cycles of 94°C for 1 min, 62°C for 1 min and 60°C for 1 min. The template DNA in the reaction mixtures was amplified (n = 3) and monitored with an ABI Prism SDS 7000 instrument (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). DNA was extracted from a 6.4×10^7 CFU/ml culture of *Bifidobacterium breve* (LMG11042). Standard curves were constructed after real-time PCR amplification of four different DNA concentrations (n = 4) ranging from 1.28×10^7 to 1.28×10^4 cell equivalents/well. The standard curve had a R^2 value of 0.99 and the slope was -3.3.

Results and discussion

Specific

It should be mentioned that on during the treatment period Bif E, a white precipitate was formed in the stomach compartment. This white precipitate was transferred to the small intestine compartment and clogged the tubings on day 3 of treatment period Bif E which lead to a technical breakdown of the SHIME. All tubings from the stomach and small intestine vessels were replaced and the SHIME experiment was resumed that same day.

Survival of the strain after stomach and small intestine digestion

To find out to what extent Bif L was able to survive the gastrointestinal passage, samples were taken from the stomach and small intestine compartments from the SHIME after the digestions in those respective compartments had finished. These experiments were repeated 3 times: on day 1, day 4 and day 7 of the treatment period Bif L. The inoculum concentration, as measured by plate counts was 8.4 log CFU/mL (Figure 1). After the stomach digestion a very significant decrease of Bif L to values between 5.5 and 5.8 log

CFU/mL was observed. In the small intestine, a higher survival of Bif L was noted with values between 6.6 and 7.6 log CFU/mL of small intestine suspension (Figure 1).

The same measurements were done during treatment period Bif E. As the product Bif E was delivered at LabMET at the end of the day, plate counts of Bif E in the stomach and small intestine were not made. The survival of Bif E was therefore measured two times: on day 5 and day 7. It was immediately noted that the plate count measurement of Bif E in the inoculum was only 4.7 log CFU/mL. This was probably due to the fact that not all bacterial cells in the product could be measured by plate counting as they were still encapsulated. Nevertheless, a very high survival of this strain was noted in the stomach compartment both on day 5 (4.8 log CFU/mL) as on day 7 (5.4 log CFU/mL).

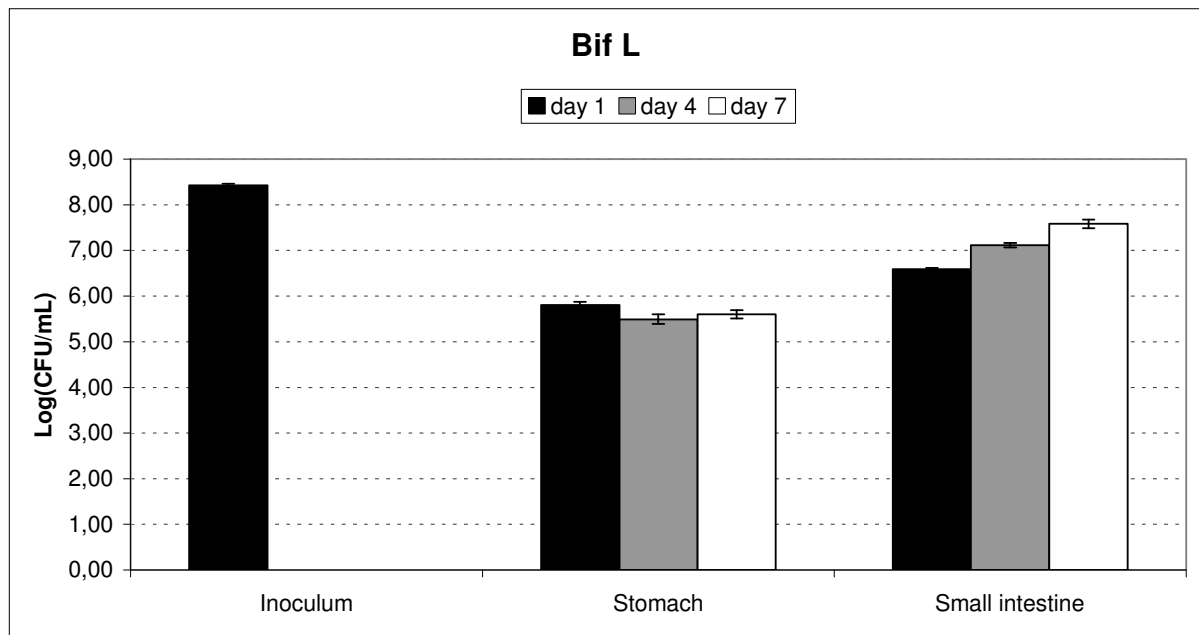


Figure 1. Plate count measurements of *Bifidobacterium longum* in product Bif L after inoculation and after the stomach and small intestine passage on day 1, day 4 and day 7 of treatment period Bif L

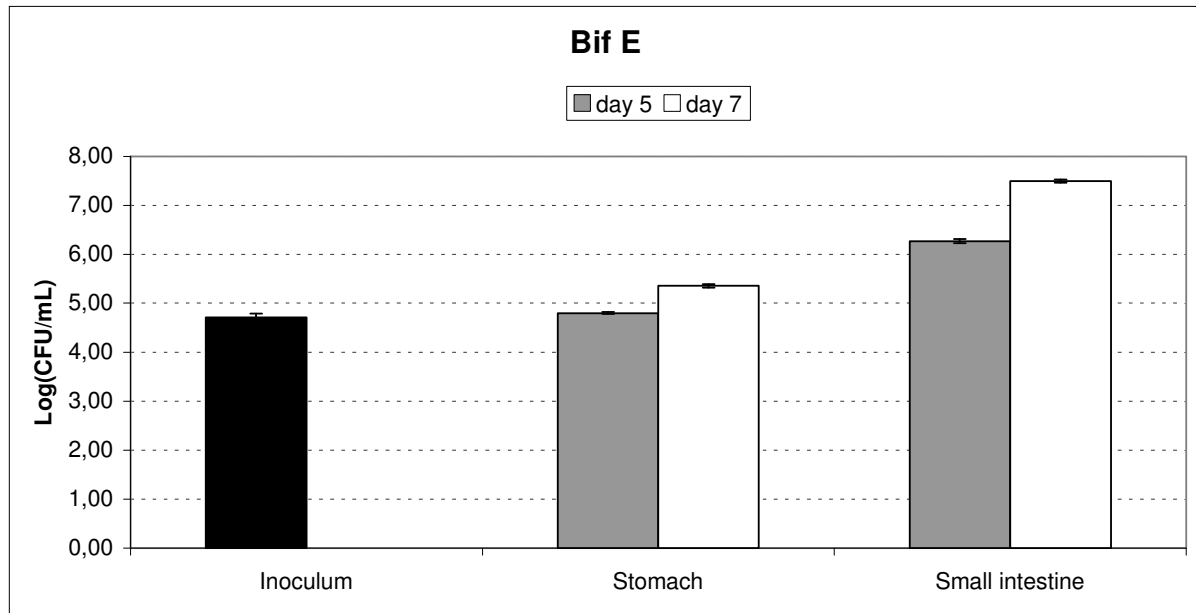


Figure 2. Plate count measurements of *Bifidobacterium longum* in product Bif E after inoculation and after the stomach and small intestine passage on day 1 (inoculum concentration solely), day 5 and day 7 of treatment period Bif E

As with Bif L, a higher concentration of *Bifidobacterium longum* was observed in the small intestine, 6.3 and 7.5 log CFU/mL on day 5 and 7 of treatment period Bif E, respectively. As not all bifidobacteria cells in the suspension may have been counted – due to the encapsulation process – these values may be an underestimation of the actual amount of bifidobacteria in the suspension.

To deal with the fact that not all bifidobacteria may have been counted from the encapsulate product Bif E, real-time PCR was carried out on the samples from the stomach and small intestine. For Bif L, a similar trend as with the plate counts was observed. For Bif L in the stomach sample a concentration of around 5 log copies/mL was noted, whereas in the small intestine, a higher concentration of around 7 log copies/mL was observed (Figure 2). Interestingly, the encapsulated product Bif E showed a very high concentration of bifidobacteria both in the stomach as the small intestine sample (Figure 2). This indicates that the encapsulation process for product Bif E has actually worked very well and that the strain is able to survive the harsh conditions

from the stomach and small intestine. It should be noted that product Bif L eventually also had a substantial survival in the small intestine. This product as well would have a chance of reaching the colon environment in a high concentration. Yet, the survival of the encapsulated *Bifidobacterium longum* surpasses that of the lyophilized strain by several orders of magnitude.

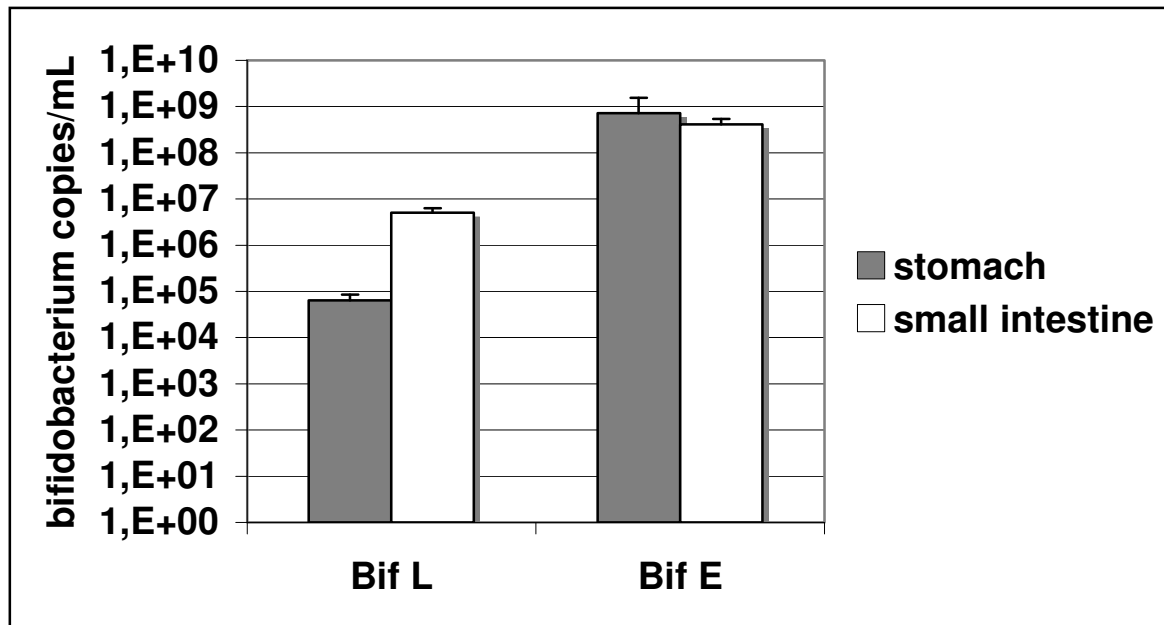


Figure 2. Real-time PCR analysis for bifidobacteria in the samples from the stomach and small intestine during the treatment with product Bif L and product Bif E.

Influence of Bif L and Bif E supplementation on the colon microbial community

Fermentation activity

During the entire SHIME run, no specific differences were observed in short chain fatty acid production (Figure 3). At the end of washout period Bif E, a temporary increasing trend in propionic and acetic acid production was observed (Figure 3A and B). There is however no evidence that this was attributed to the prolonged presence of the encapsulated Bif E product. Similarly, during the entire SHIME run, no specific differences were observed in ammonia production (Figure 4). This indicates that the

supplementation of Bif E or Bif L, if they survived the colon during the SHIME run, did not interfere too much with the basic metabolic activities from the intestinal microbiota.

Microbial community composition

Plate count measurements from the most important microbial groups indicated no significant changes in microbial composition that could be explained by the treatment with Bif L or Bif E (Figure 5). This indicates that the supplementation of Bif E or Bif L did not interfere too much with different microbial groups of relevance in the SHIME colon compartments. No significant increase in bifidobacteria counts was observed during week 6 and 8 (the two weeks of treatment). Probably, the added *Bifidobacterium longum* did not reach the colon compartments in a high enough concentration to be detectable amongst the other bifidobacteria in the colon suspension. Therefore, a molecular strategy was applied to investigate whether the strain was detectable in the colon suspension.

Bifidobacteria DGGE

Using PCR-DGGE, it was attempted to visualize the added *Bifidobacterium longum* in the bifidobacteria population from the different colon compartments. Bacterial DNA was extracted from the intestinal suspension of the colon compartments during the SHIME run and PCR was carried out with specific bifidobacteria primers. Then, a DGGE was made which generates a distinctive fingerprint profile of the bifidobacteria population in the intestinal suspension. Typically, each band in such a DGGE profile roughly corresponds to 1 *Bifidobacterium* species.

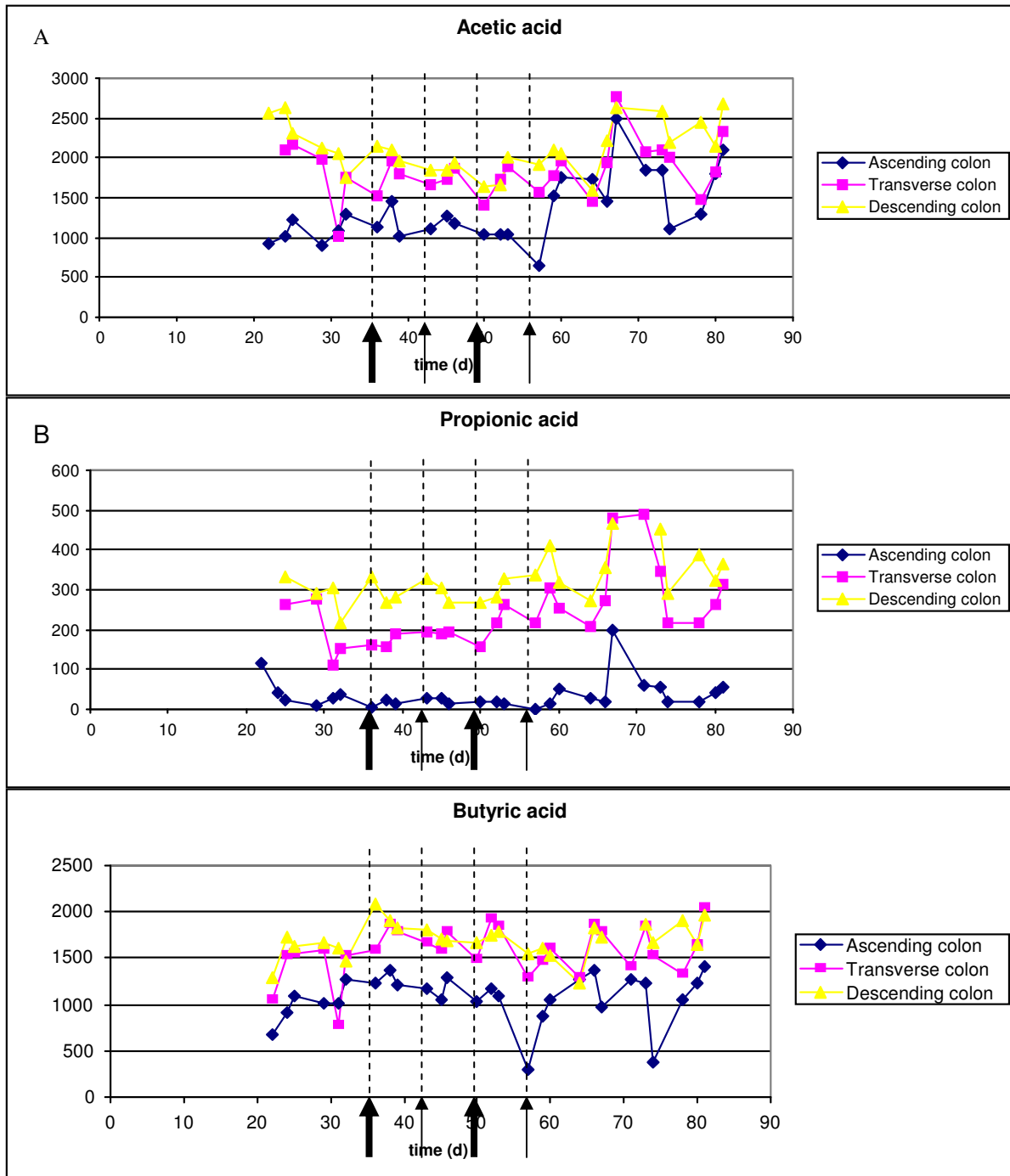


Figure 3. Concentrations of acetic (A), propionic (B) and butyric (C) acid in the ascending, transverse and descending colon vessels of the SHIME reactor. The two bold arrows in each graph indicate the start of the treatment period with Bif L, respectively Bif E. The two normal arrows indicate the start of the respective washout periods.

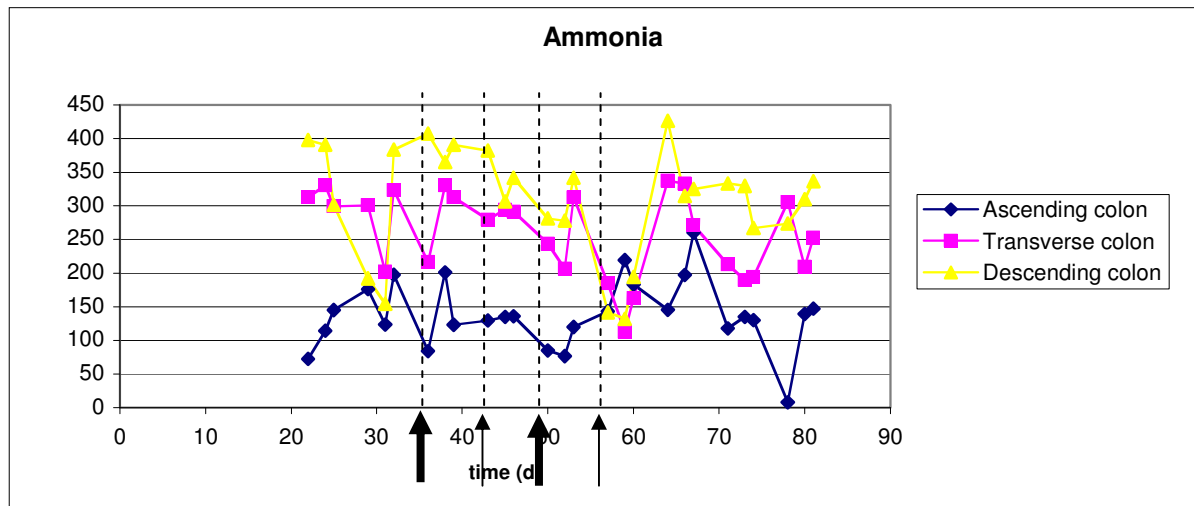


Figure 4. Concentrations of ammonia in the ascending, transverse and descending colon vessels of the SHIME reactor. The two bold arrows in each graph indicate the start of the treatment period with Bif L, respectively Bif E. The two normal arrows indicate the start of the respective washout periods.

It was noted from the sample of all colon compartments of week 5 (control week, before treatment with Bif L), that quite some bifidobacteria species were visualized on the DGGE (Figure 6). Remarkably, in week 6 (treatment period Bif L) the intensity of one of the DGGE bands was much higher, indicating a higher predominance of that specific *Bifidobacterium* species. The band is marked with a circle in Figure 5. In the following washout period (week 7), the intensity of that specific band got weaker which points to a lower predominance of that species in the washout week. In the following week (week 8), treatment period Bif E, the specific band got more intense again in all 3 colon compartments. The following washout period (sample of week 9) brought again a decrease in the strain.

Presumably, the bacterial species that is responsible for the more intense DGGE band, is the added *Bifidobacterium longum* from the Bif L and Bif E products. This was confirmed by another DGGE analysis where *Bifidobacterium longum* from product Bif L was put on gel together with the colon samples from the treatment period (data not shown). Bif L generated a band that appeared on DGGE at the same height as the bands

that got more intense in samples from the colon compartments during the treatment periods Bif L and Bif E.

Further research is currently underway to quantify the supplemented *Bifidobacterium longum* by means of real-time PCR. The development of this protocol is however still underway.

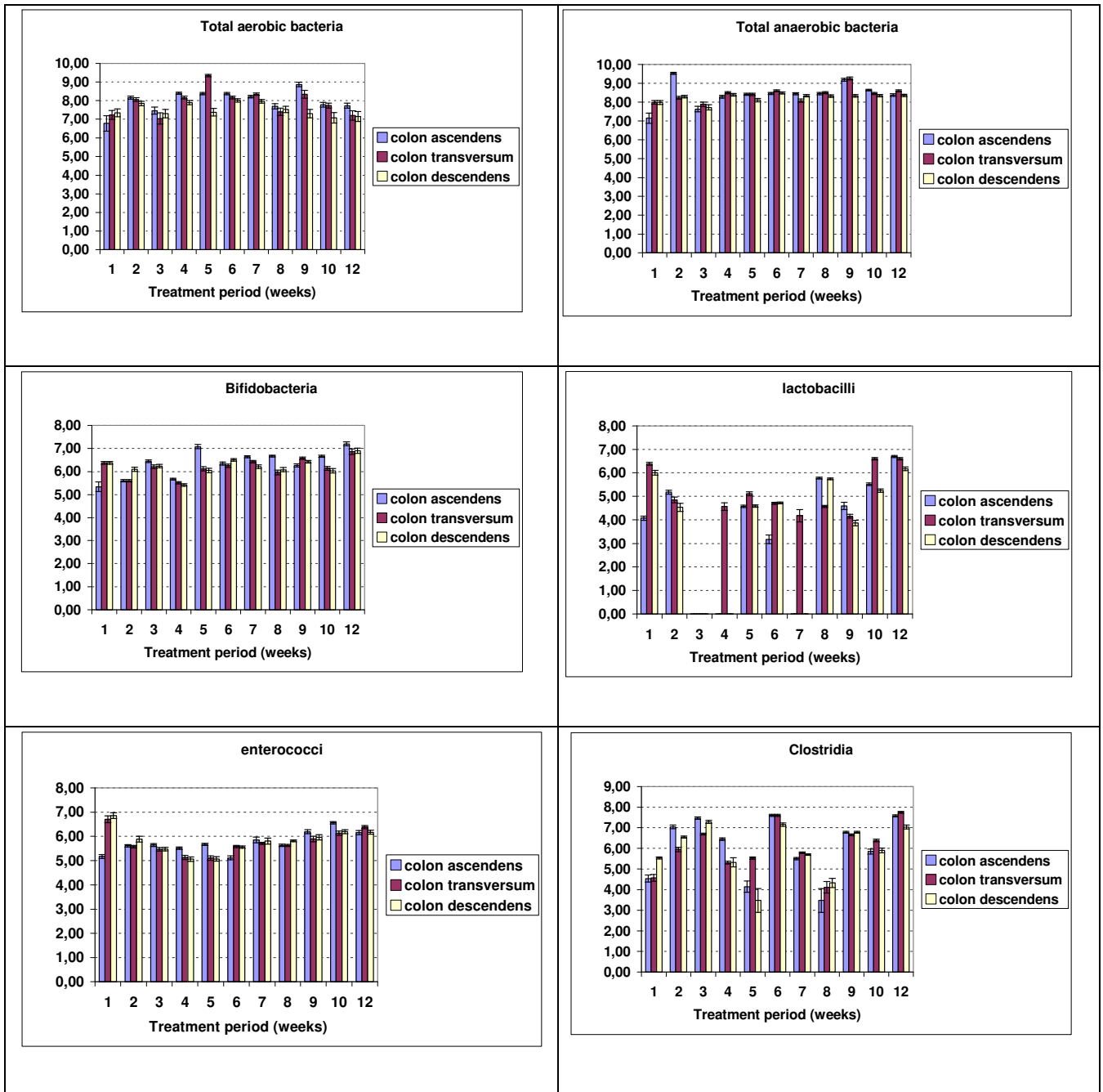


Figure 5. Plate count measurements of the most relevant microbial groups in the ascending, transverse and descending colon vessels of the SHIME reactor. Week 6 and week 8 were the treatment periods with product Bif L and Bif E, respectively.

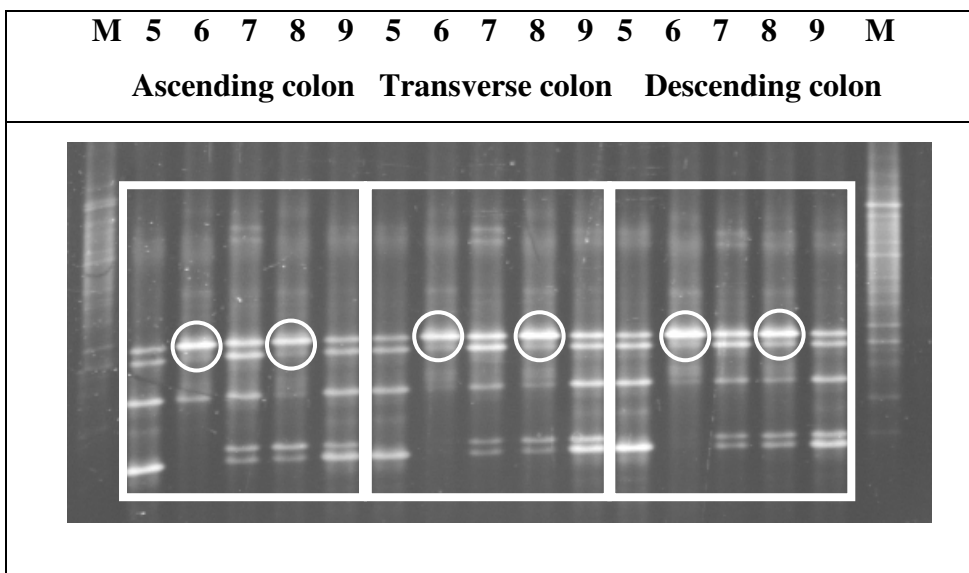


Figure 6. DGGE pattern of bifidobacteria in the colon compartments of the SHIME reactor. “M” indicates marker lanes. Samples from the respective colon compartments are grouped together. Week 6 and 8 were the treatment weeks with product Bif L and Bif E respectively. The band that was more intense in the DGGE profile during the treatment periods is indicated with a white circle.

Conclusion

Two products of *Bifidobacterium longum* were tested in this SHIME experiment: a lyophilized form of this bacterium Bif L and an encapsulated form Bif E. When Bif E was added, a recalcitrant precipitate was formed in the stomach compartment for which caution should be taken in future research steps. Bif L showed a moderate degree of survival during stomach passage and a higher degree of survival during small intestine passage. In contrast Bif E showed a very high survival under stomach conditions as well as under small intestine conditions. Although plate counts showed that not all bifidobacteria were measurable in the samples that contained Bif E (due to the

encapsulation process), real-time PCR data showed strong evidence that survival of Bif E during the stomach passage was almost complete.

With regard to the influence of Bif L and Bif E on the colon microbial community, no interference with the most important metabolic processes in the colon compartments was observed. Although it was expected that the higher survival of Bif E would have resulted in beneficial shifts in the fermentation pattern, no changes in short chain fatty acid or ammonia production were noted. Likewise, no significant changes in microbial community were observed by plate count measurements, nor in the health-promoting population (bifidobacteria and lactobacilli) nor in the more pathogenic population (clostridia).

Interestingly, DGGE profiles of the bifidobacteria population showed that the added probiotic strain was enriched in the population, both during addition of Bif L as Bif E. It should however be remarked that the PCR-DGGE analysis also detects inactive bacteria. It can therefore not be assessed to what extent the added *Bifidobacterium longum* maintained itself in the colon suspension. No prolonged presence of the strain was observed in the periods following the treatment, indicating that the supplemented strain had probably washed out. However, real-time PCR analysis for the *Bifidobacterium longum* strain is currently under development for a better quantification of the specific strain in the colon suspension. This will allow to assess to what extent the supplemented probiotic strain persists in the colon compartments during the treatment, but also during the washout periods.