GUT MODEL STUDY SUMMARY

Bacillus Subtilis HU58™

In an effort to validate the function of Bacillus Subtilis HU58™ as a true probiotic, we commissioned and now have completed a very elaborate gut model study of the strain. This study was completed at a 3rd party, independent research company called ProDigest™ who specializes in digestive studies. The company is a spin-off from Ghent University.

One of the key features that make this study unique and highly important is the fact that it is a Continuous Model of the gut. Not just a single dose simulated gastric system. Because it's a continuous model it allows us to study the effects of the probiotic on the intestinal culture over longer periods of time, we can specifically look at each region of the GI and the effects, it allows for repeated ingestion effect vs. single dose and the model allows for studying the effect of the probiotic under fasting and fed conditions. This means we know exactly what our product does in each part of the digestive system and what its lifecycle is like in the GI. This is very unique and not available as a study by most probiotic companies.

The results were remarkable as far as the abilities of this strain, here are some very brief conclusions on the findings:

1) The strain naturally survives the stomach and immediately germinates in the upper GI.

2) The strain is well suited for life in the GI and colonizes effectively.

3) Best germination occurs when taken with food.

4) The strain showed increased saccharolytic fermentation over proteolytic which favors lowered pH (beneficial pH change for the GI) and does not produce gas (which can be an issue for many probiotic supplements).

5) The strain produces significant amounts of short-chain fatty acids (SCFA) from carbohydrate digestion - in particular there was an increased production of acetate initially and then higher levels of butyrate in longer term use. These SCFAs have been shown to be powerful candidates as anti-cancer agents in the GI, reduction of inflammation and increases the growth of favorable endogenous bacteria. There are a number of significant GI benefits from SCFA production and the quantifiable production by these strains shows great therapeutic value.

6) The strain showed the ability to alter the entire microbiota - this is unique for a probiotic to have demonstrated the ability to alter the microbiota. This has profound implications on the types of claims that can be made as far as the probiotic benefits of these strains.

A study like this really sets us apart from other probiotics in our space. The study is attached.

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Evaluation of the Bacillus subtilis strain HU-58 in the SHIME technology platform

Final report – July 2013

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Evaluation of the *Bacillus subtilis* strain HU-58 in the SHIME technology platform

Executive summary

This project report describes the results of a set of experiments in which the SHIME technology platform is used to evaluate the intestinal fate and beneficial properties of *Bacillus subtilis* strain HU-58 under simulated gastrointestinal conditions.

In the next pages, the details of the experiments are described, which consisted of three main study stages:

- Optimization of the different study and analysis protocols
- Evaluation of the intestinal germination, growth and resporulation of the strain
- Evaluation of the effect of the strain on the intestinal environment

Main conclusions: After extensive investment of resources, optimal protocols for the culturing of vegetative cells and spores, performance of the experiments and analysis of the results, were obtained. The germination experiments indicated specific intestinal behavior for *Bacillus subtilis* HU-58 with efficient germination and survival, which was also translated in a positive modulation of the intestinal environment by the strain.

Content

Project description

Concept

In vitro approaches to study the gastrointestinal tract and intestinal microbial processes offer an excellent experimental setup to study the digestibility and possible prebiotic properties of selected food ingredients and probiotic properties of microbial strains. Not only it is possible to screen a large set of lead molecules in a rapid and cost-effective way in short-term batch experiments but the application of well-designed continuous models allows the in-depth study of the biological activity of selected molecules in the gut under representative environmental conditions. Furthermore, recent advances in *in vitro* modeling also allow to combine the study of bacteria-host interactions, such as mucosal adhesion and interaction with the immune system, with the continuous model, thereby further increasing both the scientific output and commercial relevance.

Short-term (batch) studies are a useful tool to rapidly screen the effects of the intestinal conditions on the Bacillus strains and on selected parameters related to the intestinal environment. On the other hand, they represent a very simplified environment where control of the changing conditions is not possible, with the result that only short-term experiments can be conducted.

Final validation of the efficacy of the product should therefore be performed in a long-term administration study, using a continuous model of the gastrointestinal tract. Such models allow to culture the complex intestinal microbial ecosystem over a longer period and under representative conditions of the different intestinal regions. Finally, the model allow to simulate repeated ingestion of the product. This is of extreme importance in order to obtain reliable data for the in vivo conditions as previous *in vivo* studies have shown that the real detection of pre- or probiotic properties may only be evaluated after a repeated administration of the product.

In practice, the intestinal fate of the strains (germination, survival, multiplication, resporulation) and the effect on the resident microbiota will first be evaluated in short-term screening experiments. This will provide the necessary information to set up the long-term full scale experiment in the SHIME, in which the test products will be administered daily for a period of two weeks.

Background on bacilli

Bacillus species are Gram-positive bacteria able to form endospores, i.e. uniquely robust entities that are able to survive extremes of temperature, irradiation and long-term storage. They have been studied extensively in the past as a model system to improve knowledge on unicellular differentiation. As some spore-forming species, such as *B. cereus* play a role in food poisoning, a secondary interest has been to understand methods for their destruction. However, except for *B. cereus* and *B. anthracis*, no other members of this genus are considered harmful to healthy humans.

In contrast, several species of *Bacillus* are even consumed as food ingredients. This includes *B. subtilis*, *B. licheniformis*, *B. clausii*, *B. pumilus* and *B. coagulans*. Typical examples include fermented food products, such as the traditional Japanese Natto, containing soybeans fermented by *B. subtilis*, which has been used for hundreds of years in Japan and is consumed in large quantities by most of the population. Similarly, *B. clausii* spores are the sole component of Enterogermina, an over-the-counter (OTC) medicine for the treatment of childhood diarrhoea (rotavirus). In this particular case, *B. clausii* is used as a licensed medicine and has specific claims attached to it. Other examples are the use of *B. subtilis* and *B. licheniformis* in the animal feed product known as BioPlus 2B™ that is manufactured by Christian Hansen and Clostat™ by Kemin Industries. Together, there is sufficient evidence supporting that the use of ***Bacillus* can be safe for human consumption**.

In addition to the safety, several studies have explored potential health benefits of *Bacillus* strains, such as stimulation of innate immunity. In the case of *B. subtilis* var Natto a serine protease known as Nattokinase is secreted from the vegetative cells, which has been shown to reduce blood clotting by fibrinolysis. Such alkaline protease is produced by most *Bacilli* and most probably, many if not all *Bacilli* carry this same potential health benefit. Currently three strains of *B. subtilis* have been registered with the FDA with self-affirmed GRAS status in the USA in 2012.

Some *Bacillus* species are adapted to survival in the host GIT. In such case, spores ingested with food are able to survive transit through the stomach after which they germinate and proliferate, a phenomenon that has been proven using *in vivo* molecular studies. Following growth and proliferation they re-sporulate as the bacteria pass through the GIT and are shed into the environment. Such germination and re-sporulation would be a necessity for certain health benefits, as was shown for the stimulation of the development of the gut-associated lymphoid tissue (GALT) in infant rabbits by *B. subtilis*.

Compared to other live bacteria that are used as probiotics, *Bacillus* spores are particularly attractive for functional reasons. Indeed, the robust spores are not only able to pass though the gastric barrier without loss of function, they are also stable to many food processing steps and can be stored at room temperature without the need of refrigeration.

Study aim

The aim of this project was to obtain insight in the intestinal fate of *Bacillus subtilis* HU-58, further referred to as strain HU-58. The intestinal fate will be characterized as the capacity to germinate in the stomach and/or small intestine, to replicate and potentially to resporulate before exiting the gastrointestinal tract.

Furthermore, a second aim constitutes to evaluate potential effects of the intestinal passage of the strains on the intestinal environment. Such effect will be characterized by evaluation of changes in the composition and activity of the intestinal microbial community, in relation to potential health effects.

In order to evaluate both endpoints, a set of experimental protocols first had to be optimized.

Step 1: Optimization of the study protocols

Rationale

At the start of the project, a number of experimental protocols were provided by Royal Holloway London, and a number of papers were available in which experimental protocols were described. The idea was therefore that these protocols could be implemented 'as is', without much further optimization work. However, during the protocol implementation, major issues were observed in relation to most of the protocols. Therefore, ProDigest spent a first part of its efforts in developing new protocols for the cultivation of the strain, harvest of spores and detection of the vegetative cells and spores.

Summary of key protocols

Optimization of growth conditions

The following conditions were applied for the cultivation of vegetative cells and spores (Table 1).

Table 1: Conditions allowing optimal growth and sporulation of the test strain

Strain	Vegetative cells	Spores
HU-58	3 hours in TY at 37°C	36 hours in DSM at 37°C

Pasteurization conditions

Optimal pasteurization conditions, in which vegetative cells are killed yet spores remain viable, are essential to distinguish vegetative cells from spores and to purify spore stocks. However, the recommended method (25 min @ 60°C) as modified to give a

more pure spore suspension.

To evaluate the best pasteurization conditions, the following procedures were followed:

Growing of the vegetative cells

DAY 1

- Inoculate TY medium with the spore stock culture.
- Grow overnight at 37°C on a shaking incubator.

DAY 2

- Check the OD of the growing vegetative cells.
- Check the vegetative cells for spores under the phase-contrast microscope.

Pasteurization test

- Make a dilution of the vegetative cells in saline solution.
- Make three aliquots of the dilution in eppendorf tubes.
- Put the aliquots in a warm water bath at the correct temperature.
- Take one sample out of the warm water bath every 15 minutes.
- Make a dilution series and plate the dilutions.
- Plates are grown overnight at 37°C.

Conclusions:

- Pasteurization at 83°C for 15 min is sufficient to kill all vegetative cells yet protecting spore integrity.
- Plate counts of pasteurized and non-pasteurized cells allows to distinguish veg cells from spores.

Quantification of germination

Quantification of the amount of germinated cells can be performed using plate counts on pasteurized and non-pasteurized cells. However, to allow rapid screening and to monitor kinetics of the germination process, an additional method was developed. This spectrophotometrical method makes use of the change in optical density when spores germinate into vegetative cells. The change in OD is measured at 600nm.

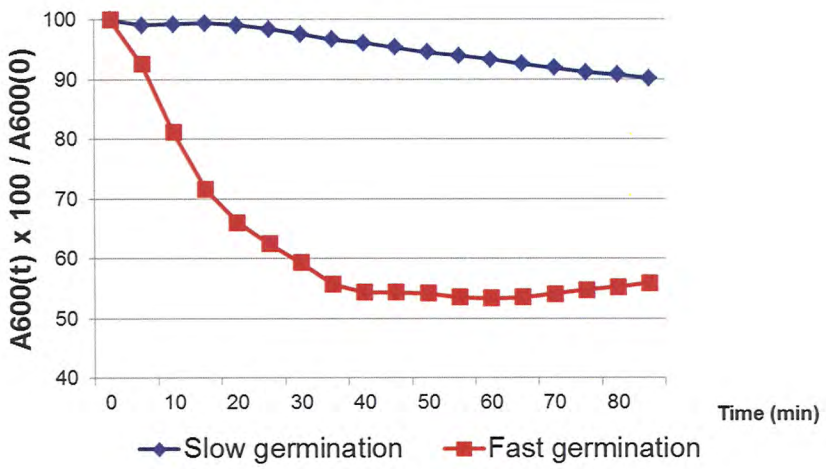


Figure 1 Change in OD in case of rapid and slow germination.

This example shows that germination leads to a decrease in OD values. Changes in such OD values therefore indeed can be used to rapidly monitor the kinetics of the germination process.

Step 2: Evaluation of the intestinal fate of HU-58

Aim

In order to maintain itself in the intestinal tract, administered spores need to have sufficient capacity to germinate in the intestinal tract and to replicate as vegetative cells. Furthermore, for some *Bacillus* strains it is known that health beneficial effects (e.g. immune stimulation) actually depend on factors released during the germination process. Finally, in order to have modulating effects on the intestinal environment, the spores have to become metabolically active vegetative cells. In order to obtain information on the capacity of the strains to have such effects, more information is needed on the intestinal fate of the strains upon oral ingestion as bacterial spores. This is therefore investigated in detail using the ProDigest intestinal model systems.

Test setup

The intestinal fate of strain HU-58 was investigated using the standardized screening setup of ProDigest. In this setup, conditions representing the stomach and small intestine are simulated in a sequential design. Test products can therefore pass the complete upper intestine in sequence, yet may also be incubated separately under either stomach or small intestinal conditions.

Furthermore, ProDigest recently developed a dynamic approach in which the intestinal conditions can be adapted to either the fasted or fed state. As both conditions strongly differ in environmental conditions (pH, retention time, bile salt and enzyme concentrations etc.), the administration of the strains under fasted or fed conditions can have a profound influence on the intestinal fate of the strains. Knowledge on the optimal conditions for germination and survival will allow to develop the best-performing formulation and administration strategy.

The setup of the short-term screening assay, as carried out by ProDigest, is presented below (Figure 2):

1. **Stomach**:
   Fed conditions:
   - Medium composition:
     - High pepsin levels
   - pH: from 4.5 to 2
   - Incubation period: 90 minutes

   Fasted conditions:

- Medium composition:
  - Low pepsin levels
- pH: 2 (constant value for fasted conditions)
- Incubation period: 60 minutes

2. **Small intestine**:

   Fed conditions:

   - Medium composition:
     - Low bile salt and enzyme levels
   - pH: stable at 6.8
   - Incubation period: 180 minutes

   Fasted conditions:

   - Medium composition:
     - High bile salt and enzyme levels
   - pH: stable at 6.8
   - Incubation period: 180 minutes

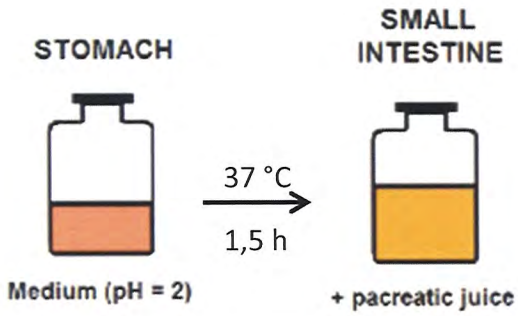


Figure 2: The typical experimental setup for short-term batch experiments.

Preliminary experiments

In practice, a number of preliminary experiments were performed to evaluate which environmental conditions would be most optimal for efficient germination. After this, the full setup under both fasted and fed conditions was applied. A number of different experiments were performed. A summary of the most important results is provided below.

Effect and of activation of the spores by means of pasteurization of medium conditions on germination

Previous experiments with other bacterial spores has shown that the germination of spore can be more efficient when the spores have be 'triggered' by a stress situation. Such stress situation may be simulated by a heat shock prior to the incubation of the strains. In addition, the combination of such trigger with the temperature at incubation or specific germination factor was shown to be of importance.

Aspects related to the activation of spores upon a heat shock, and the influence of temperature and the specific medium composition were therefore also evaluated for strain HU-58. Results related to these experiments are presented in Fig. 3.

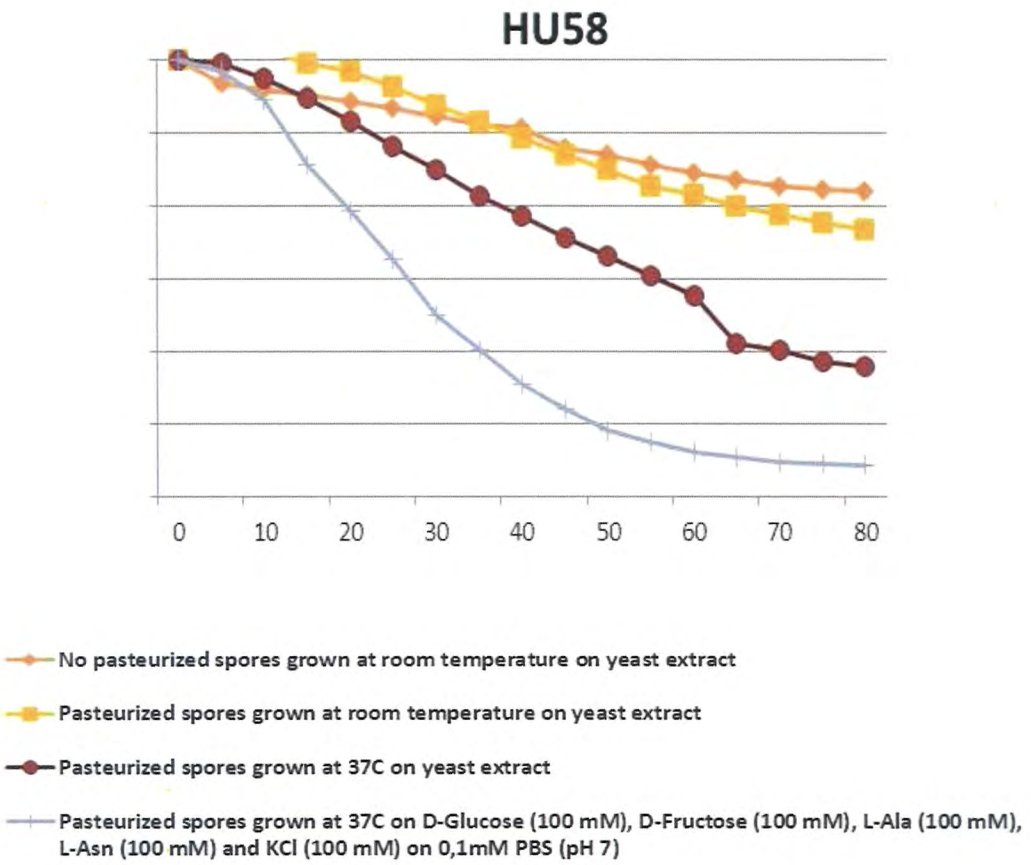


Figure 3: Incubation of HU-58 in different growth media and evaluation of the germination by spectrophotometric analysis (Medium A = 0,9% NaCl, 0,1% casamino acids, 0,1% D-glucose).

The results show that **a trigger of the spores by means of pasteurization indeed improves the germination** under different conditions. Furthermore, **incubation at 37°C leads to better germination** and for **strain HU-58, specific germination factors** increase the germination capacity of the strain.

Effect of specific intestinal environmental factors on germination: pH and bile salts

For many bacteria, low pH conditions and/or high bile salt concentrations represent an important barrier for passage through the upper intestine. Indeed, these factors are among the most important defense mechanisms of the human body towards unwanted colonization and infection by bacterial pathogens. In order to become vegetative cells, the spores therefore have to be able to sustain such conditions. As next preliminary experiment, the effect of different pH conditions and different bile salt levels on the germination of the spores was therefore investigated. The results for strain HU-58 are shown in Fig. 4 and show that the **germination capacity is strongly affected by the environmental pH, yet only into a lesser extent by bile salt concentrations**.

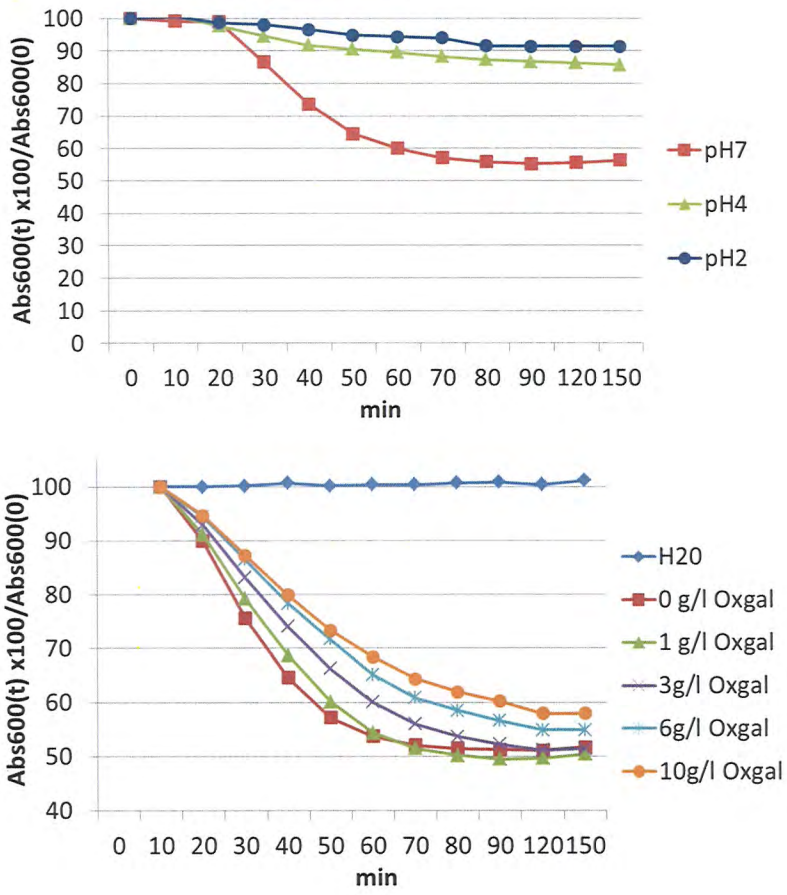


Figure 4: Incubation of HU-58 under different pH conditions and bile salt concentrations and evaluation of the germination by spectrophotometric analysis.

Conclusion preliminary experiments

The **preliminary experiments indicate that the best conditions for germination include**:

- Incubation at 37°C (=body temperature)
- Incubation at pH>4 => no germination to be expected in stomach
- Small effect of bile salt levels => germination may be less efficient in beginning of small intestine (high bile salt levels)
- Specific medium components/co-factors may improve the germination capacity
- A stress situation prior to the incubation may improve the germination capacity

Final test setup

Germination experiments in stomach and small intestine

In the final experiment of this project step, the test product was administered as spores to the simulated stomach and then passed the stomach and small intestine. While the preliminary experiments were performed using spore stocks grown and produced by ProDigest, the experiment below was performed with commercialspore stocks provided by Viridis. The experiments were performed under fasted and fed conditions and the endpoints of the study was the evaluation of germination, survival and resporulation, as quantified using plate count measurements for total bacteria and spores at the beginning of the stomach incubation and at the start, middle and end of the small intestinal incubation.

As the test was performed with products provided by Viridis, the first step was to quantify the number of bacterial spores and vegetative cells in the products.

To do this, the following procedures were followed:

- Weigh 0,3 g of the product in a 2 mL Eppendorf tube.
- Add 1 mL of PBS solution.
- Vortex thoroughly.
- Make four 1/10 dilutions.
- Pasteurize the two 10^{-1} dilutions in screw cap tubes for 15 minutes at 83°C.
- Make a dilution series until 10^{-8} of the dilutions that are not being pasteurized.
- Plate 25 µL of the dilutions on LB agar plates.
- Incubate at 37°C overnight.
- When the pasteurization is finished, briefly put the tubes on ice water to cool them down.
- Dilute these samples until 10^{-8}.
- Plate 25 µL of the dilutions on LB agar plates.
- Incubate at 37°C overnight.
- Count the colonies.

The results are shown in Fig. 5.

This shows that the spore culture contained negligible % of vegetative cells. This information was taken to the next experiments.

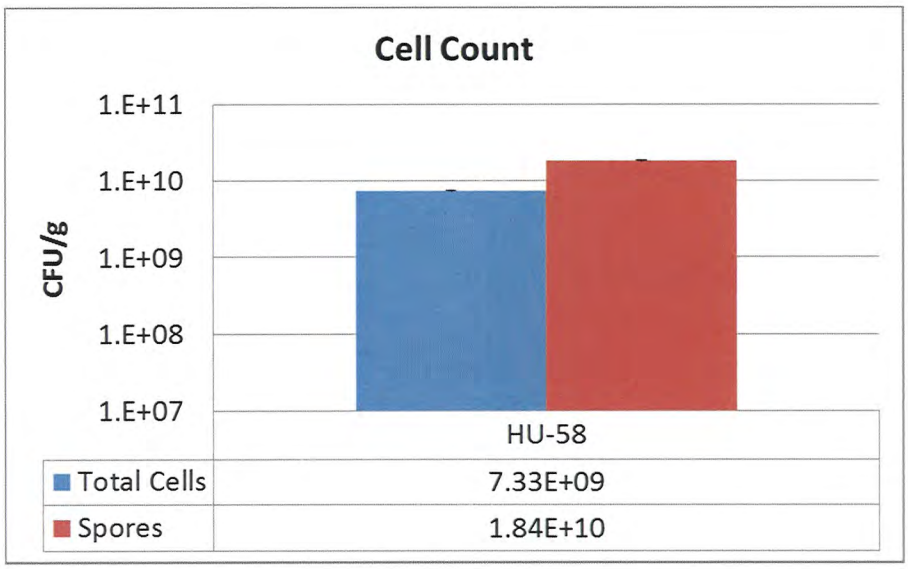


Figure 5: Total counts and counts of bacterial spores in the test products provided by Viridis.

The following procedures were followed in the germination experiment:

Methods fasted experiment

- Add 1g of product to 22,5 mL of gastric juice.
- Sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (S_0).
- Incubate in a 37 °C shaking incubator during 60 minutes.
- Add 8,5 mL of pancreatic juice.
- Sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (SI_0).
- Incubate in a 37°C shaking incubator.
- After 90 minutes, sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (SI_1).
- After 180 minutes, sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (SI_2).

Methods fed experiment

- Add 1 g of product to 24,5 mL of gastric juice.
- Sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (S_0).
- Incubate in a 37 °C shaking incubator during 90 minutes.
- Add 266,5 µL 0,5 M HCl after 5', 15, 25, 35, 45, 55, 65, 75 and 85 minutes.
- Add 9 mL of pancreatic juice and 1 mL 0,5 M NaOH.
- Sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (SI_0).
- Incubate in a 37°C shaking incubator.

- After 90 minutes, sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (SI_1).
- After 180 minutes, sample 1,5 mL for plate counts and 2x 1 mL for DNA extraction (SI_2).

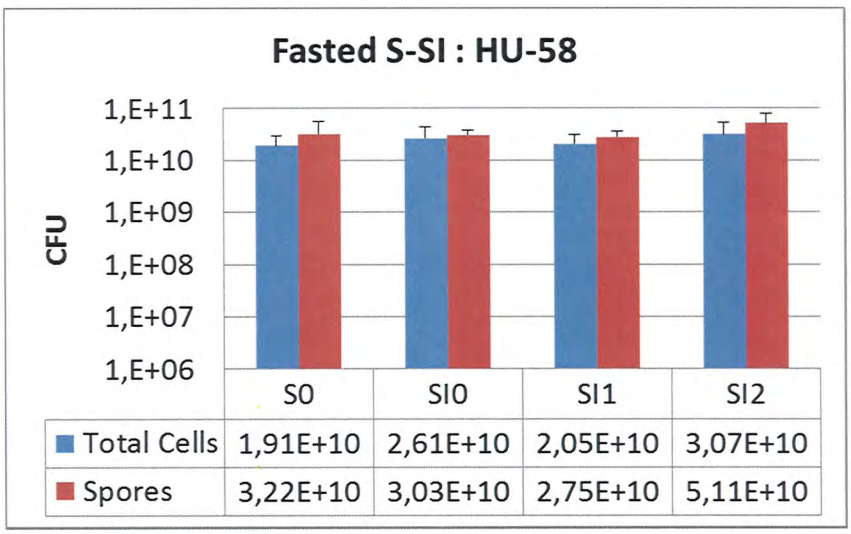
Samples for plate counts

- Make a 1/10 dilution of the sample in PBS buffer.
- Sample 1,4 mL and pasteurize these samples.
- Make a dilution series in duplicate of the 1/10 dilution and plate on LB to count the total amount of cells present.
- After pasteurization, make a dilution series of the two samples and plate on LB to count the amount of spores present.

Samples for DNA extraction

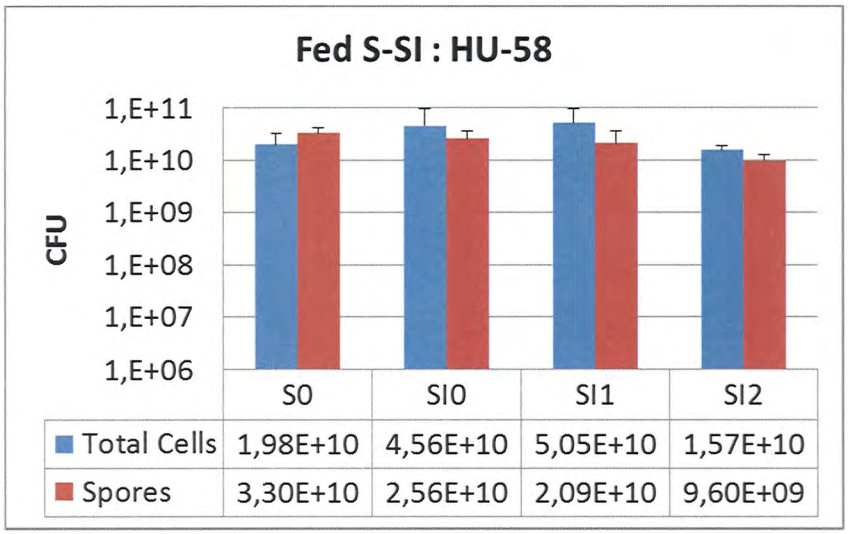
- Centrifuge and remove the supernatant.
- Freeze the pellet.

The results of the incubation experiment with strain HU-58 are presented in Fig. 6 and 7.



	S0	SI0	SI1	SI2
Total Cells	1,91E+10	2,61E+10	2,05E+10	3,07E+10
Spores	3,22E+10	3,03E+10	2,75E+10	5,11E+10

Figure 6: Germination of HU-58 under fasted stomach and small intestinal conditions, as determined by plating.

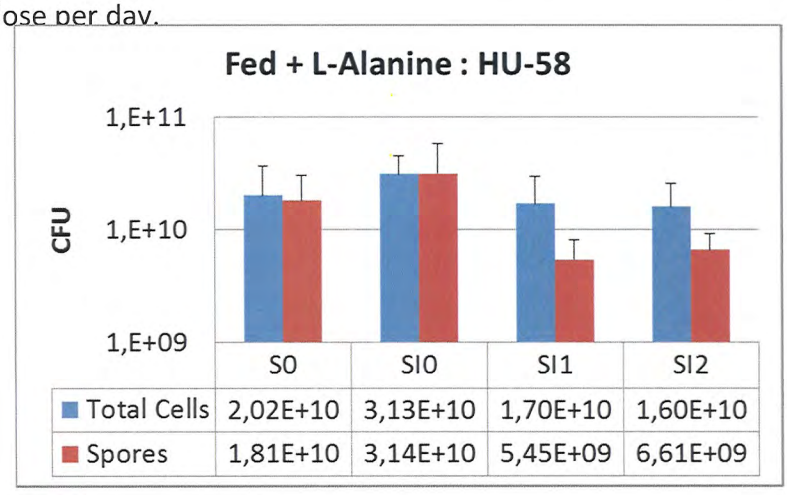


	S0	SI0	SI1	SI2
Total Cells	1,98E+10	4,56E+10	5,05E+10	1,57E+10
Spores	3,30E+10	2,56E+10	2,09E+10	9,60E+09

Figure 7: Germination of HU-58 under fed stomach and small intestinal conditions, as determined by plating.

In contrast with what was initially expected, **no clear germination occurred for strain HU-58**, neither in the fasted nor the fed state. This may be related to the **lack of a necessary co-factor** for germination, as seen before for other spores and in Fig. 3. As this may be related to the absence of a specific co-factor, as seen in the preliminary experiments, the experiment under fed conditions was repeated with addition of L-alanine, an amino acid known to improve germination for other bacterial spores (Fig. 8). Interestingly, the **presence of a low dose of L-alanine led to a germination of about 70%** of the spores. Germination reached its maximum at half of the incubation and no clear indication of resporulation could be observed.

Both the observations lead to show importance of HU58 therapy along with the meals or right after. It is clear that once HU58 colonizes it will arrive at a steady state concentration of 1.04×10^{10} cfu in the gut by a single dose per day.



	S0	SI0	SI1	SI2
Total Cells	2,02E+10	3,13E+10	1,70E+10	1,60E+10
Spores	1,81E+10	3,14E+10	5,45E+09	6,61E+09

Conclusion germination experiments

Based on the sequential stomach-small intestine experiments, the **following conclusions** can be made:

- The **capacity to germinate depends in the feeding state** of the individual.
- No germination was observed for HU-58 under the standard conditions.
- In order **to improve the germination capacity of HU-58, specific co-factors** are required, as shown by the addition of L-alanine.

Step 3: Evaluation of the effect of the *Bacillus* strains on the intestinal environment

Aim

As indicated before, some *Bacillus* strains have been documented as so-called probiotics, i.e. viable bacteria which confer a health effect by selective modulation of the intestinal environment. This may also be the case for HU-58. As shown in Step 2, viable, vegetative cells may indeed reach the colon as sufficient germination occurs in the small intestine. This warrants the evaluation of the probiotic properties of the strain under study.

To evaluate these aspects, a combination of screening experiments and a long-term study were performed.

Short-term screening study

Setup

The typical short-term screening assay, as carried out by ProDigest, consists of the sequential incubation of a representative dose of the selected lead compounds under simulated conditions for:

1. Stomach (Based on the previous experiments, a fed state in the stomach was applied);

2. Small intestine, with the addition of pancreatic enzymes and bile salts

3. Large intestine with a representative bacterial inoculum. This bacterial inoculum was derived from an already '*in vitro* adapted' microbial community from the ascending colon compartment in our SHIME system.

The experiment is designed in such a way that typical residence times of food products in the gastrointestinal tract are maintained. The strains under study were administered at a given concentration at the start of the simulated stomach incubation and, after simulated digestion under stomach and small intestinal conditions, the metabolic and community profile of the intestinal microbiota were assessed upon simulated colon incubation.

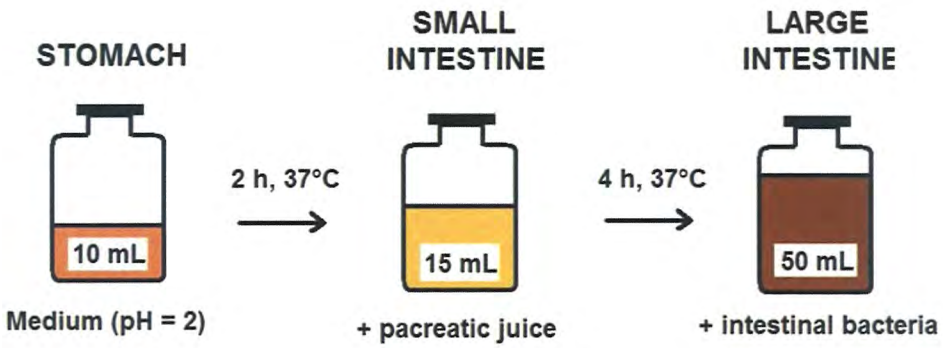


Figure 9: The typical experimental setup for short-term batch experiments (volumes are only indicative)

Each incubation was run in triplicate to control for biological variability.

- Incubation conditions colon:
  - 37 °C, shaking, anaerobic
  - Incubation period: 48h
- Analyses:
  - Quantification of *Bacillus* spores and total counts using plate counts.
  - Evaluation of pH change during the experiment.
  - Evaluation of gas production.
  - Short chain fatty acid analysis:
    - The pattern of SCFA is an assessment of the microbial carbohydrate metabolism (acetate, propionate and butyrate) or protein metabolism (branched SCFA) and can be compared to typical fermentation patterns for the control incubation (without any strain)
  - Ammonium analysis:
    - Ammonia is a product of proteolytic degradation and can also work as an indirect marker for substrate availability.

The experiments were performed with the **test products provided by Viridis**. As the products **contain dextrin** in addition to bacterial spores and as dextrin may have an effect itself on the intestinal environment, **dextrin was also taken up in the experiment as reference and all results of the test products were compared to the dextrin reference**.

The following procedures were followed:

Methods

- Add 1 g of product to 15,7 mL of gastric juice.

- Sample 1 mL for plate counts (S).
- Incubate in a 37 °C shaking incubator during 90 minutes.
- Decrease the pH according to predetermined schedule over 90 minutes
- Add 9 mL of pancreatic juice.
- Incubate 2,5 hours in a 37°C shaking incubator.
- Sample 1 mL for plate counts (SI).
- Add 43 mL of colonic medium.
- Flush the bottles to remove oxygen.
- Add 7 mL SHIME inoculum.
- Sample 2 x 1 mL for DNA extraction, 5 mL for SCFA/NH4/LA analysis and 1 mL for pH quantification (C_0).
- Measure the pressure inside the bottle (C_0).
- Incubate in a 37°C shaking incubator.
- After 24 hours, sample for 1 mL for plate counts, 2 x 1 mL for DNA extraction and 5 mL for SCFA/NH4/LA analysis (C_1). Before and after sampling, measure the pressure inside the bottle (C_1).
- After 48 hours, sample 1 mL for plate counts, 2 x 1 mL for DNA extraction, 5 mL for SCFA/NH4/LA analysis and 1 mL for pH quantification (1 mL) (C_2). Before and after sampling, measure the pressure inside the bottle (C_2).

Samples for plate counts

- Make two 1/10 dilutions of the sample in PBS buffer.
- Pasteurize one of the two 1/10 dilutions.
- Make a dilution series of the other 1/10 dilution and plate on LB to count the total amount of cells present.
- After pasteurization, make a dilution series of the sample and plate on LB to count the amount of spores present.

Samples for DNA extraction

- Centrifuge and remove the supernatant.
- Store the pellet at -20°C.

Samples for SCFA/NH4/LA analysis

- Store the samples at -20°C.

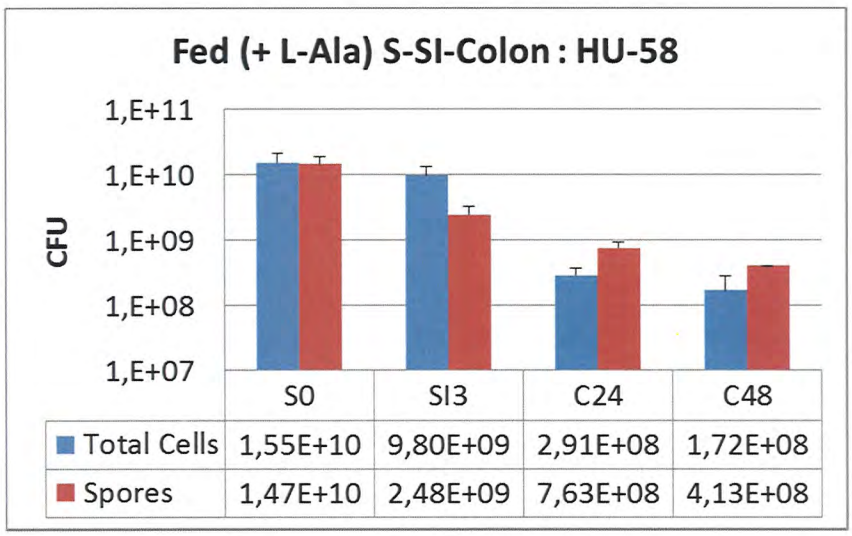
Samples for pH quantification

- Measure the pH.

Results

Germination under colonic conditions

The results of the specific plate counts for **HU-58** are shown in Fig. 10. Again, the levels of spores in the incubation medium continued to go down during the colonic incubation, indicating **further germination under colonic conditions**. Germination occurs continuously during colonic incubation. Viable counts are however low, as indicated by a 2 log decrease in total counts, which may indicate **HU-58 is as such not highly adapted to complex colonic conditions**. Upon a single administration.



	S0	SI3	C24	C48
Total Cells	1,55E+10	9,80E+09	2,91E+08	1,72E+08
Spores	1,47E+10	2,48E+09	7,63E+08	4,13E+08

Figure 10: Germination of HU-58 under stomach, small intestinal and colonic conditions, as determined by plating

Effect on metabolic parameters

The **pH decrease during the colonic incubation** experiment is shown in Fig. 11. The evolution of the pH in the incubation is the result of the balance between saccharolytic fermentation, producing acids, and proteolytic fermentation, producing ammonia. As a pH decrease is typically associated with a positive modulation of the intestinal environment, comparison of pH decreases between incubations provides a first insight in specific metabolic effects induced by the bacterium under study.

Interestingly, **strain HU-58 did not induce a stronger effect on intestinal pH profiles as compared to the dextrin reference**. This is in line with the observation of a lower capacity of HU-58 to maintain itself in the colonic environment, upon a single administration.

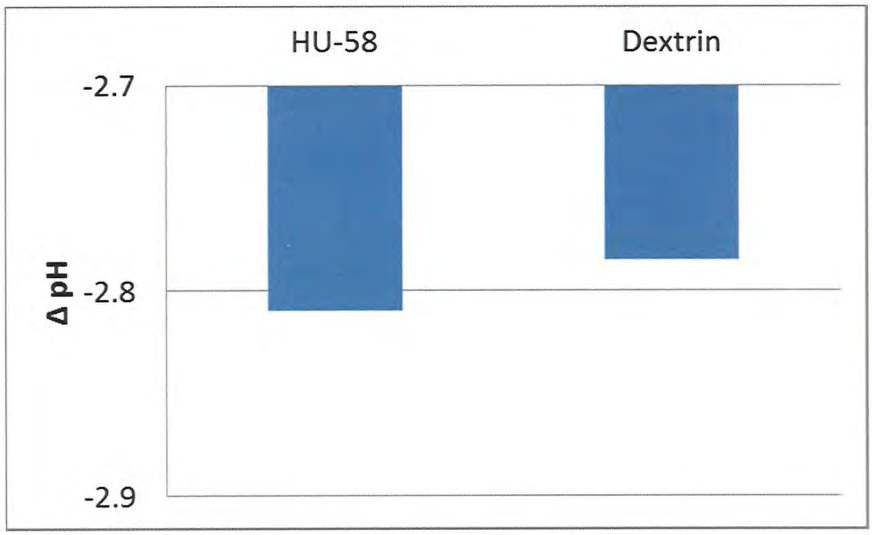


Figure 11: PH decrease during the colonic incubation with HU-58 and the dextrin reference.

The results of the SCFA analysis are presented in Fig. 12 and 13. SCFA are mainly produced during sugar fermentation.

- Evaluation of the absolute amounts of the different SCFA produced during incubation with a bacillus strain as compared to incubation with dextrin allows to evaluate changes in production rates (Fig. 12).
- Evaluation of the relative increase of one SCFA in the total amount of SCFA produced during incubation with a bacillus strain as compared to incubation with dextrin allows to monitor selective changes in SCFA profiles (Fig. 13).

As compared to the dextrin reference, **an increase in total SCFA production was noted with about 38% for HU-58**, indicating additional metabolic activity in the colon. Interestingly, maximum levels were however already reached after 24h and no further increase was observed between 24h and 48h. This may again be related with a lower capacity of HU-58 to maintain itself in the colonic environment, upon a single administration. In this short-term experiments, acetate was the main metabolite for HU-58.

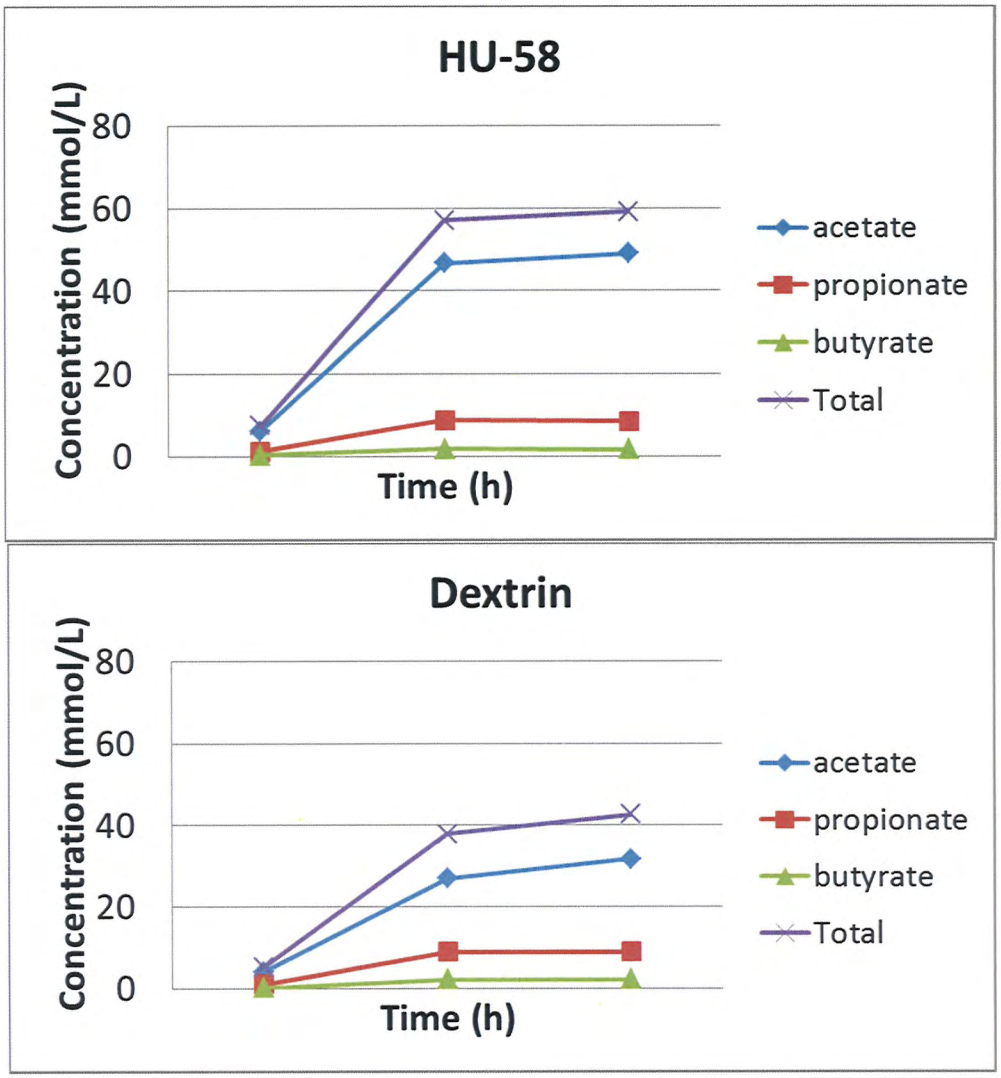


Figure 12: SCFA production profiles during the colonic incubation of HU-58 or the dextrin reference.

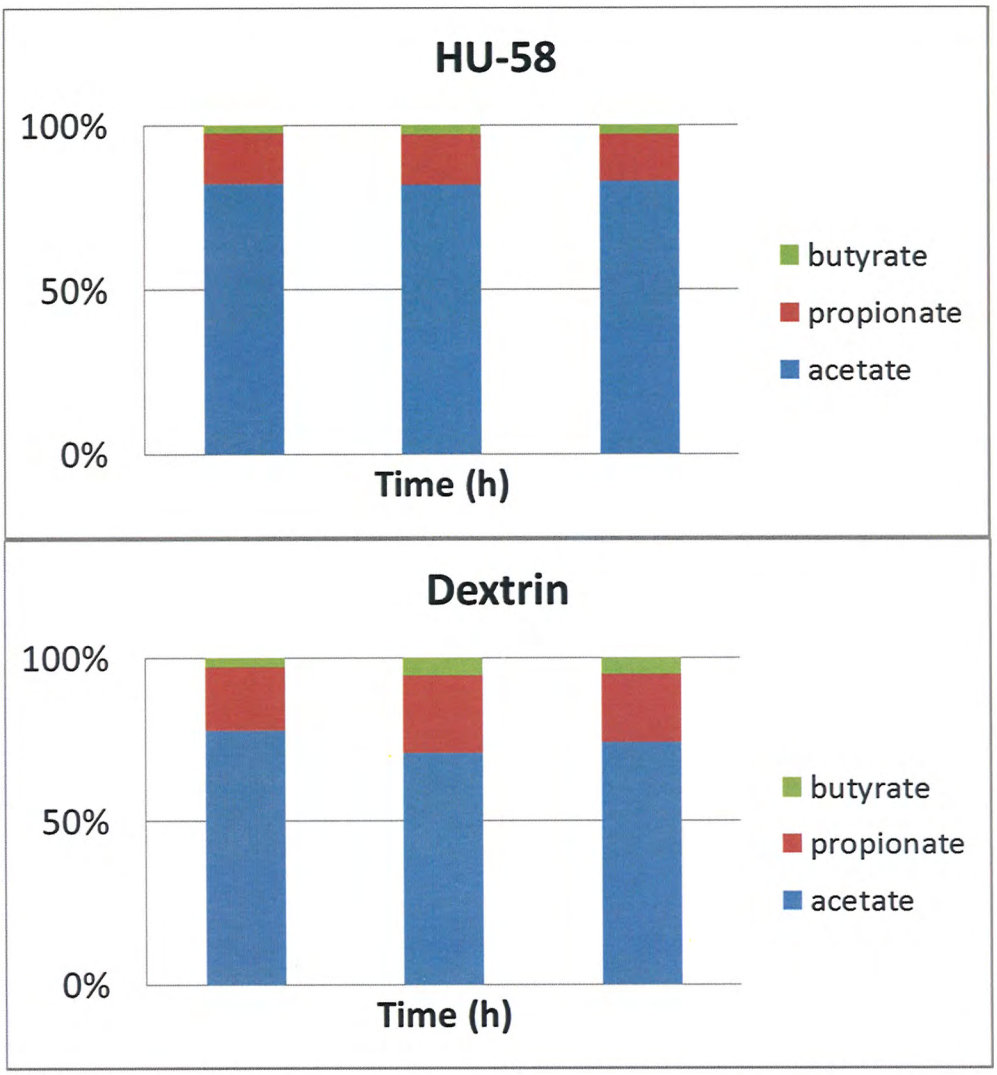


Figure 13: Relative production of acetate, propionate or butyrate as compared to the total during the colonic incubation of one of strain HU-58 or the dextrin reference.

The results of the **ammonium analysis** are presented in Fig. 14. In line with the very high and rapid acetate production during the first 24h, ammonia production was low in that time period. Later on, ammonium production increased, yet, overall a **decreased proteolytic fermentation was noted**.

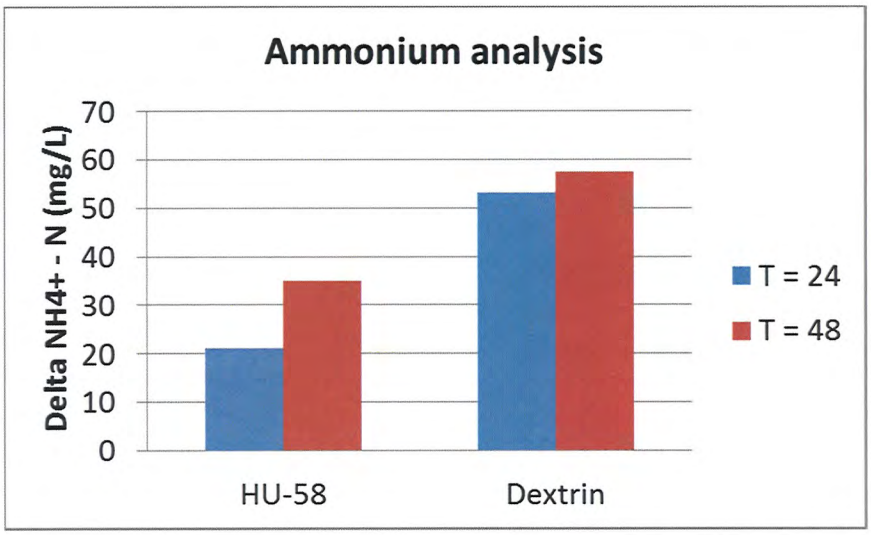


Figure 14: Ammonia production profiles (expressed as delta from T=0h) during the colonic incubation of HU-58 or the dextrin reference.

The results of the **gas pressure analysis** are presented in Fig. 15. Since gasses are a major endpoint of fermentative activity, measurement of changes in the gas pressure in the closed reactors gives a further indication of the fermentation profile. As excessive gas production can lead to intestinal discomfort, a lower gas production is generally considered positive. In this case, **gas production indeed decreased for HU-58**, despite the additional metabolic activity which was induced. This is therefore considered as a positive result.

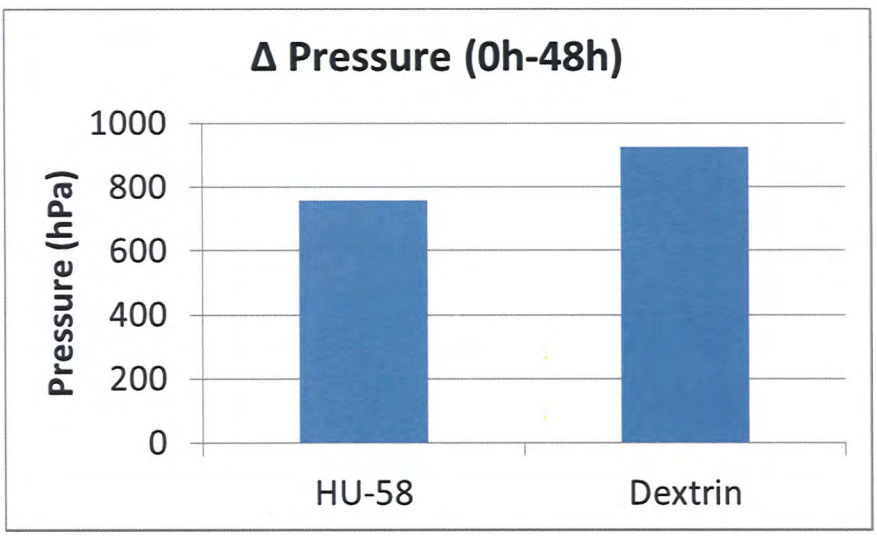


Figure 15: Gas production profiles (expressed as delta from T=0h) during the colonic incubation of HU-58 or the dextrin reference.

Conclusion screening experiment

Based on the screening experiments, it can be concluded that **HU-58 has a limited capacity to maintain itself under colonic conditions upon a single administration**, as shown by the lower survival and limited metabolic activity in the second part of the colonic incubation. However, the increased saccharolytic and decreased proteolytic fermentation which was observed, indicates that repeated administration of the strain may have promising properties in relation to improvement of the intestinal environment.

Long-term SHIME experiment

General setup

The reactor setup was adapted from the SHIME®, representing the gastrointestinal tract of the adult human, as described by Molly *et al.* (1993). The SHIME® consists of a succession of five reactors simulating the different parts of the human gastrointestinal tract. The first two reactors are of the fill-and-draw principle to simulate different steps in food uptake and digestion, with peristaltic pumps adding a defined amount of SHIME feed (140 mL 3x/day) and pancreatic and bile liquid (60 mL 3x/day), respectively to the stomach (V1) and duodenum (V2) compartment and emptying the respective reactors after specified intervals. The last three compartments are continuously stirred reactors with constant volume and pH control. Retention time and pH of the different vessels are chosen in order to resemble *in vivo* conditions in the different parts of the gastrointestinal tract. The overall residence time of the last three vessels, simulating the large intestine, is 70 h. Upon inoculation with fecal microbiota, these reactors simulate the ascending (V3), transverse (V4) and descending (V5) colon.

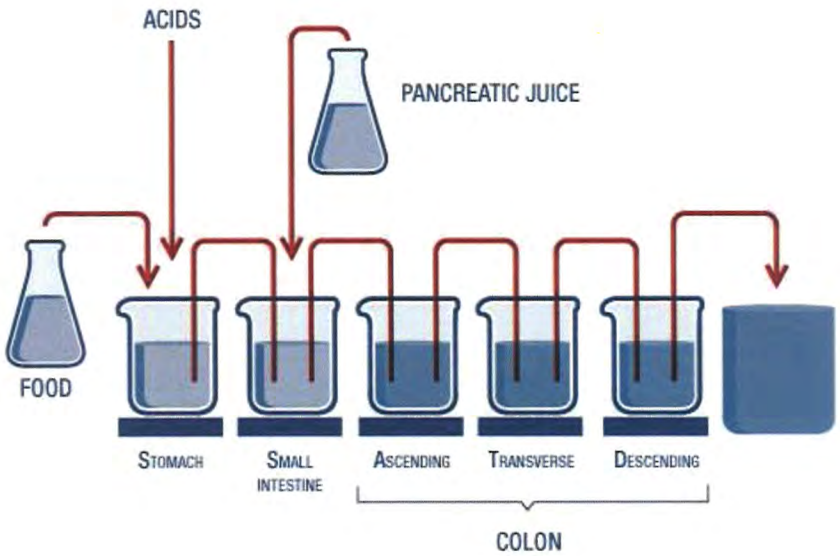


Figure 16: Standard setup of the Simulator of the Human Intestinal Microbial Ecosystem, consisting of 5 sequential reactors, which simulate the different regions of the human intestinal tract.

To facilitate cost-effective evaluation of repeated dosing of HU-58, the setup was adapted to an alternative setup, containing a stomach, small intestine and one colon compartment.

The SHIME experiment consisted of the following periods:

- **Startup**: After inoculation of the colon reactors with an appropriate fecal sample, a two-week startup period allows the microbial community to differentiate in the different reactors depending on the local environmental conditions.
- **Control period**: This is the actual start of the experiment, in which standard SHIME nutrient matrix is dosed to the model for a period of 14 days. Analysis of samples in this period allows to determine the baseline microbial community composition and activity in the different reactors, which are used as control to compare with the results from the treatment.
- **Treatment**: In this 2-week period, the SHIME reactor is operated under nominal conditions, in which standard SHIME feed is dosed to one stomach vessel with the addition of the *Bacillus* strain.

In practice, 0.5 g test product was administered during the treatment period to the stomach reactor (1x/day; at morning nutrition), corresponding to the following dose:

- HU-58: $5x10^9$ CFU

An important (unique) characteristic of the SHIME is the possibility to regularly collect samples from the different intestinal regions for further analysis. The large volumes in the colonic regions allow to collect sufficient volumes of liquids each day, without disturbing the microbial community or endangering the rest of the experiments. Typical analyses relate to the quantification of specific microbial strains (for probiotics) and effects on the resident microbial community composition and activity.

The following parameters were followed up:

- Microbial community activity
  - Short-chain fatty acids (SCFA): samples were taken 3x/week from all colon compartments to analyze the concentration of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid and caproic acid.
  - Ammonium (marker for proteolysis): samples were taken 3x/week from all colon compartments.
  - Lactate analysis: The human intestine harbors both lactate-producing and lactate-utilizing bacteria. Lactate is produced by lactic acid bacteria and decreases the pH of the environment acting also as an antimicrobial agent. It

can also be rapidly converted to acetate, butyrate, and propionate by other microorganisms. Samples were taken 3x/week from all colon compartments.

- Microbial community composition
  - Quantification of *Bacillus* spores and total counts using plate counts.
  - Quantification of 6 bacterial groups, to obtain a general idea on effects in community composition.
  - Denaturing Gradient Gel Electrophoresis (DGGE). DGGE is a fingerprinting technique which creates barcodes of microbial communities and allows to evaluate changes in the gut microbial community structure over time due to a specific treatment.

Results

Effect on metabolic parameters

The results of the SCFA analysis are presented in Fig. 17.

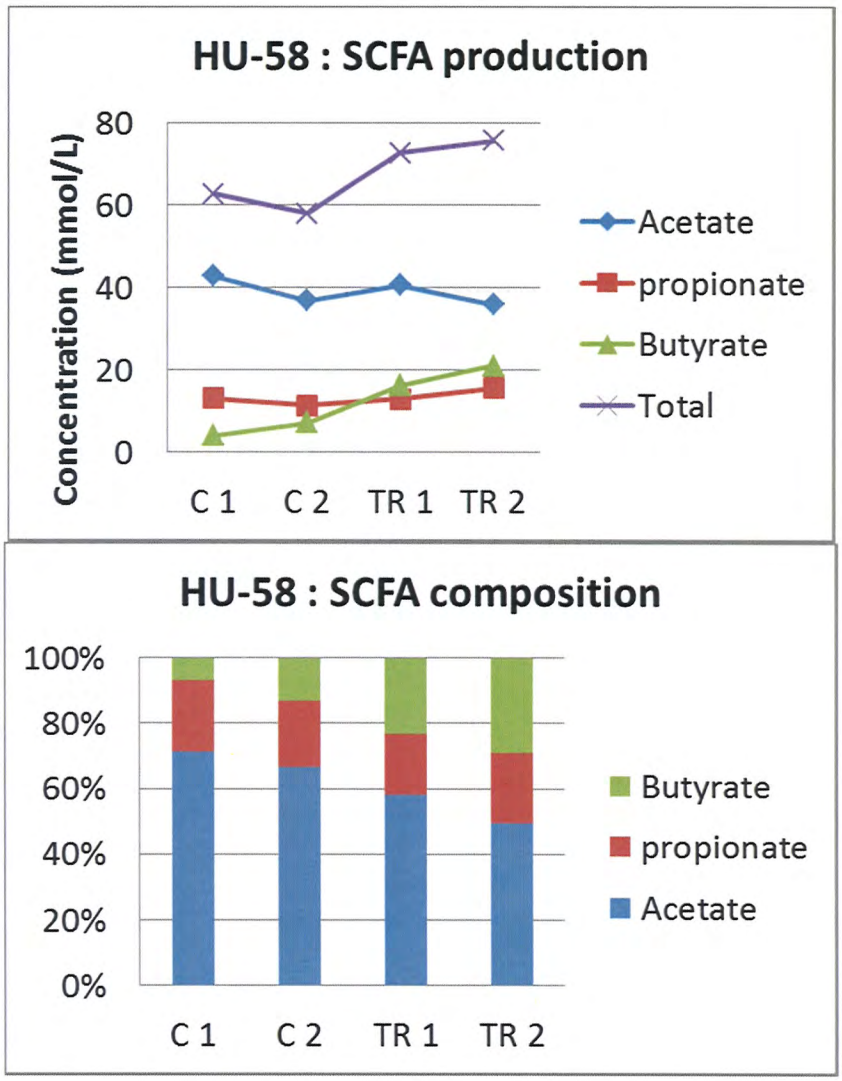


Figure 17: SCFA profiles in the SHIME experiments with HU-58. C=Control period; TR=Treatment period.

From the SCFA profiles, the following conclusions can be made:

- Administration of HU-58 induced clear changes in the SCFA profiles. Despite the fact that the same amount of total carbon source was provided to the experiment, a strong increase in saccharolytic fermentation could be observed.
- The effect on SCFA production was mainly associated with a very strong increase in butyrate production, which was already observed during the first week of treatment.

- Butyrate is generally known as a health-beneficial substrate in the intestine. On the one hand, it is efficiently used as energy source by the intestinal epithelium, thereby protecting it from excessive proliferation and uncontrolled growth (i.e. tumor development). On the other hand, it has a well-described protective effect on the host immune system and is considered as an immunomodulatory bacterial agent.
- An increase in butyrate levels is therefore considered highly beneficial for host health.

The results of the lactic acid analysis are presented in Fig. 18. Lactic acid is typically a precursor for butyrate production. The **decrease in lactate production is therefore related to the increased butyrate levels** observed.

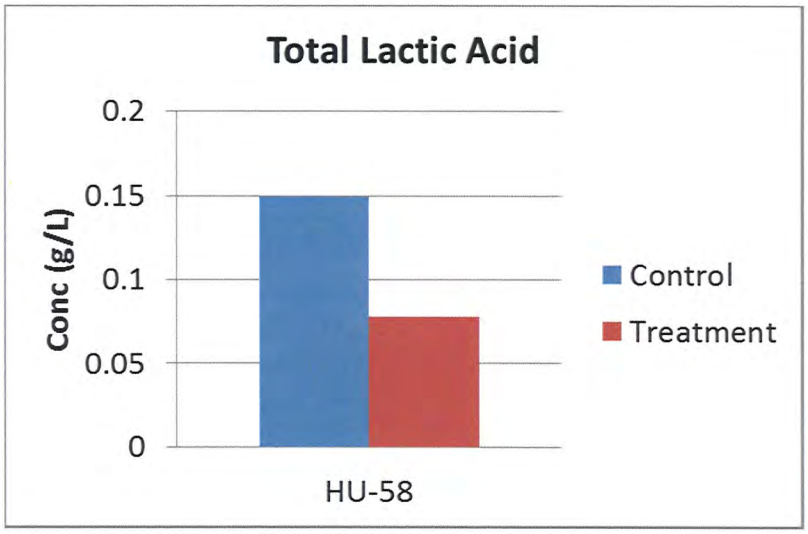


Figure 18: Lactic acid profiles in the SHIME experiments with HU-58.

The results of ammonium production are presented in Fig. 19. Generally, only **little or no effect on ammonium** production was observed upon repeated administration of any of the strains. This indicates that the strains mainly affected saccharolyic fermentation rather than proteolyic fermentation.

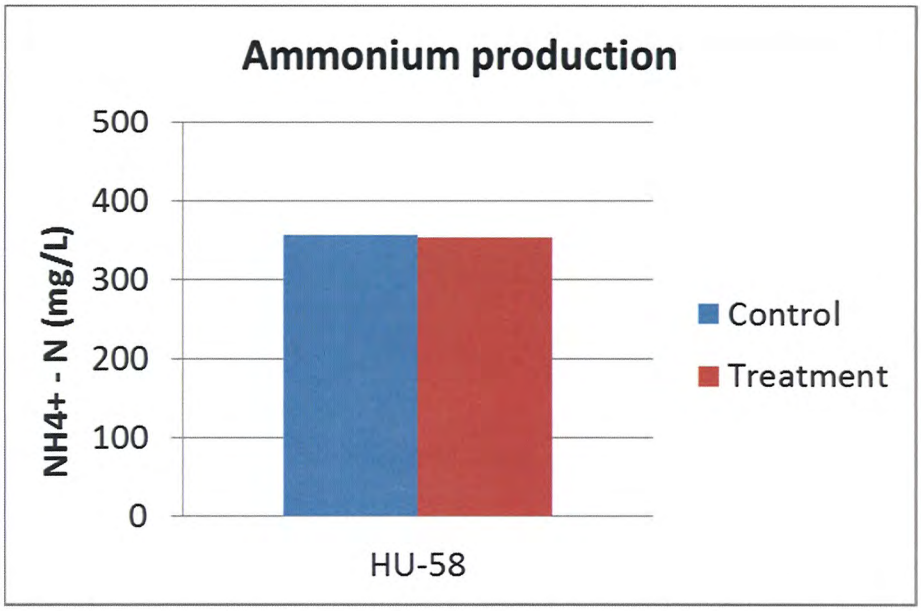


Figure 19: Ammonium profiles in the SHIME experiments with HU-58.

Effect on Bacillus germination and survival

As indicated before, the strain was daily dosed to the SHIME at a daily dose of 5E9 CFU. Total counts and spore counts on *Bacillus* specific culture media allowed to evaluate the colonic fate of the strains after 2 weeks of administration (Table 2). This showed similar total counts as the administered dose were recovered in addition to slightly lower spore counts.

Table 2: Administered daily dose of bacterial spores and total *Bacillus* counts and spore counts in the SHIME after 2 weeks of daily administration.

Product	Added	Counted	
	Spores	Total CFU	Spores
HU-58	5,00E+09	5,02E+09	1,55E+09

Effect on resident microbiota composition

In order to evaluate whether the administration of the *Bacillus* strain induced additional changes in the resident gut microbial community, **plate counts for 6 bacterial groups** were used as initial screening tool, as shown in Fig. 20. However, these plate counts **did not reveal clear differences** between microbiota from the control and treatment period in the SHIME.

As plate counts only have a limited resolution and don't allow to investigate subtle changes in the gut microbiota in detail, a **molecular fingerprinting technique** was used.

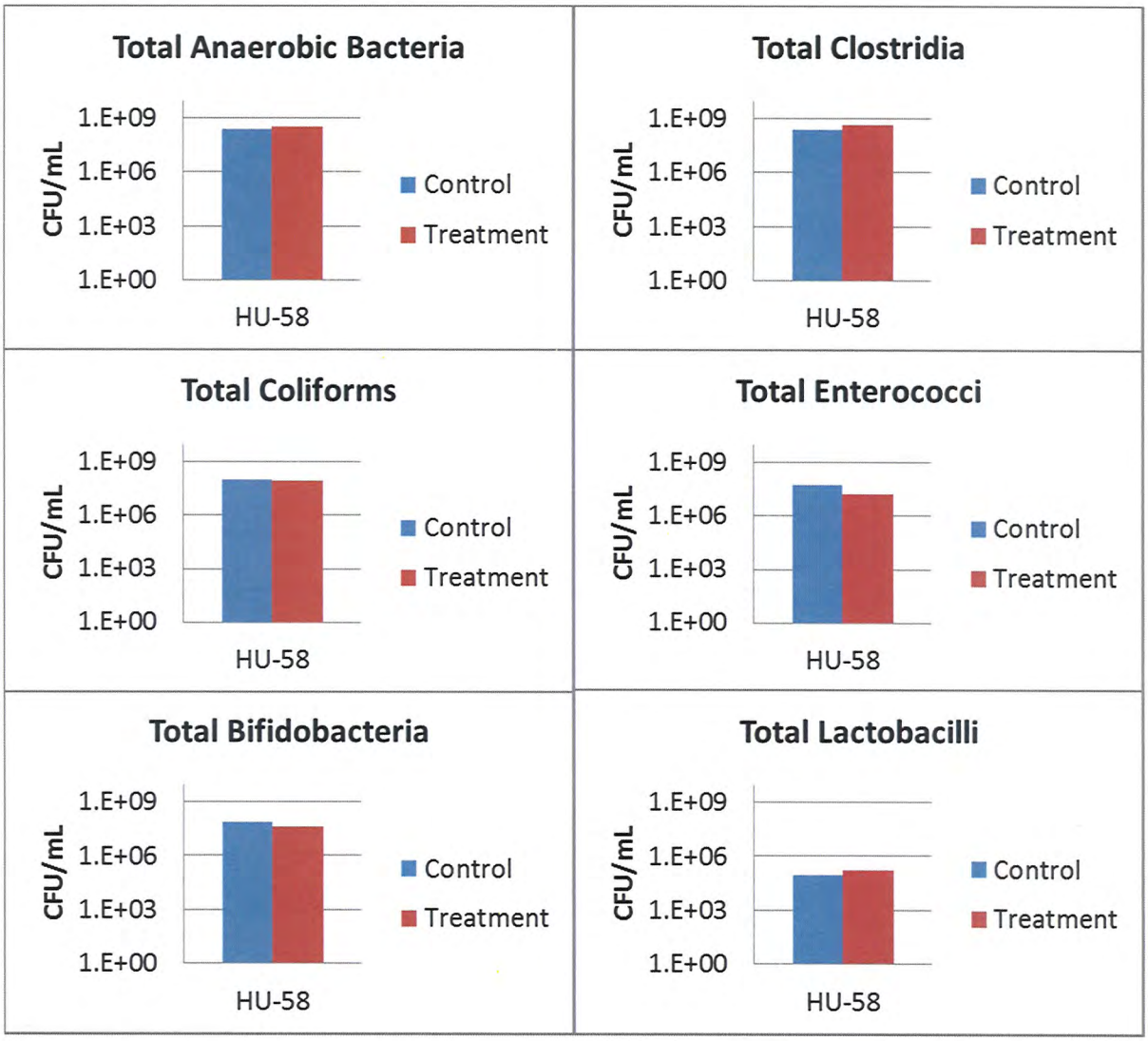


Figure 20: Plate counts of 6 bacterial groups in the SHIME experiments with HU-58.

Denaturing Gradient Gel Electrophoresis (DGGE) creates a barcode from a microbial community, in which roughly on band in the barcode corresponds to one type of species. By creating such barcodes from samples collected at different time points during the SHIME

experiment, and by comparing these profiles, qualitative changes in the microbiota over time (due to a specific treatment) can be evaluated.

The results of DGGE analysis are presented in Fig. 21. The principle of the analysis is that the similarity between the 'barcodes' from different samples is investigated. Samples with similar microbial community composition will group together, while samples with a different microbial community composition will be positioned separately from the other samples. In the figure, each horizontal barcode represents the microbiota composition of a sample collected from the SHIME experiment, during the control and treatment period.

The following conclusions can be drawn from the figure:

- Similarity analysis of the barcodes shows that the 2 samples from the control period (C1 and C2) from the HU-58 experiment, i.e. before administration of the strain, group together with a similarity of about 85%. This shows that the microbial community composition was therefore highly stable during the control period.
- Administration of HU-58 induces a clear change in the microbiota composition. This is shown by the fact that the 2 samples from the treatment period (T1 and T2) group separately from the control samples, with a similarity of only 70%.
- **This confirms the previous observations that repeated intake of strain HU-58 has a profound effect on the intestinal environment, both in terms of microbiota community composition and activity**.

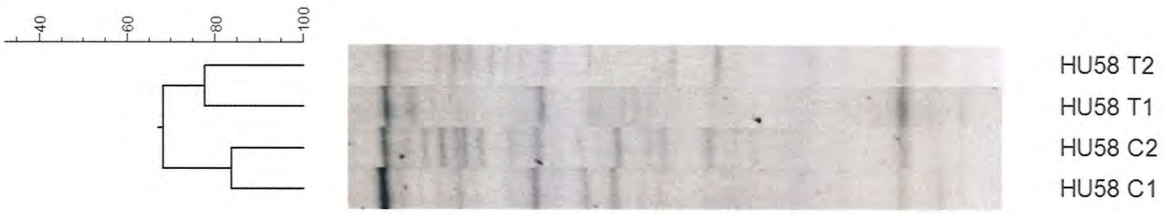


Figure 21: DGGE fingerprint of the microbiota in the SHIME experiments with HU-58. C=Control period; TR=Treatment period

Conclusion

The SHIME experiments provide a representative summary of the effects which can be expected upon repeated intake of HU-58. The main conclusions are:

- HU-58 was able to germinate under colonic conditions and could maintain itself in doses similar as the ingested dose.
- HU-58 affected the intestinal environment, as shown by increased levels of SCFA. Interestingly, butyrate was an important end product. Given the known effects of butyrate on the development of a healthy immune system and the prevention of colon cancer, these are important findings in relation to potential probiotic properties of the strain. The strain mainly affected saccharolytic fermentation, without influencing strongly proteolytic fermentation.
- HU-58 induced specific changes in the gut microbiota composition as indicated by specific changes in the DGGE community fingerprints.

GENERAL CONCLUSION

From this project, the following conclusions can be made:

- Optimization of the experimental, procedures led to the development of a **toolbox for the efficient evaluation of the intestinal fate of *Bacillus*** spores and vegetative cells.
- Upon simulated oral intake, **spores from *Bacillus subtilis* HU-58 efficiently passed the stomach** (good survival, no germination) and reached the small intestine intact.
- **In the small intestine, efficient germination** occurred under fed conditions.

- Cells reaching the colon upon a **single administration** were metabolically active, yet only in the first hours after administration. No further metabolic activity was noted after 24h and cell counts of HU-58 gradually decreased. This indicates that ***Bacillus subtilis* HU-58 needs to be administered regularly to obtain probiotic effects.**.
- **Repeated intake** of HU-58 for a period of 2 weeks (daily dose $5*10^9$ CFU) resulted in levels of 1.04×10^{10} CFU in the colon. These high bacterial counts confirm the survival of the strain under colonic conditions and the fact that ***Bacillus subtilis* HU-58 can maintain itself under colonic conditions, when administered repeatedly over longer periods**.
- Repeated intake of *Bacillus subtilis* HU-58 resulted in a **promising modulation of the intestinal environment**, both in terms of microbial community composition and activity. The most interesting observation was the increased saccharolytic fermentation with a **potent increase in butyrate production**. As butyrate is well-known for its protective effects towards the intestinal epithelium and for it stimulation of the host immune system, the available data suggest **potential probiotic properties of *Bacillus subtilis* HU-58**.

Summarized, the combination of all data suggests that daily intake of commercially relevant doses of *Bacillus subtilis* HU-58 could lead to a probiotic modulation of the intestinal environment.