ProDigest BVBA
Technologiepark 3
B-9052 Gent, Belgium
Tel: +32 9 241 11 90
Fax: +32 9 241 11 91
info@prodigest.eu

In vitro evaluation of prebiotic fibers in short-term experiments

Final report – October 2013

Project coordinators:

Massimo Marzorati

Nikki Van de Genachte

ProDigest
Gastrointestinal Expertise

Prof. Isam Siddig
Nature Gums
Building 24, Block 24
Street 37, Khartoum 2
Sudan

Our Ref: 2013/1/Short-term study

Date: November 01st, 2013

In vitro evaluation of prebiotic fibers in short-term experiments

Executive summary

The aim of this study was to screen the potential prebiotic activity of two products (i.e. PreBioM and PreBioT) by means of a short-term batch experiment.

The most interesting findings can be summarized as follows:

- **SCFA: PreBioM led to an increase of all the three main SCFAs (proportionally more propionate) while PreBioT was mainly correlated to a butyrogenic effect.**
- **Lactate: PreBioT led to a higher lactate production as compared to PreBioM.**
- **Ammonium: PreBioT led to 34% lower production as compared to PreBioM.**
- **Intestinal pH: A higher pH decrease was found in the incubation with PreBioT.**
- **Gas production: PreBioM led to a higher gas production as compared to PreBioT.**
- **qPCR: Both products led to an increase in the concentration of total bacteria and were correlated with a bifidogenic and a lactobacillogenic effect.**

Both products gave clear indications of potential prebiotic activity, however leading to different fermentation profiles. PreBioM: higher SCFA production and bifidogenic/lactobacillogenic effect. PreBioT: mainly butyrogenic, lower ammonium and gas production and bifidogenic/lactobacillogenic effect.

Project description

Concept

In vitro approaches to study the gastrointestinal tract and intestinal microbial processes offer an excellent experimental setup to study possible prebiotic properties of selected food ingredients. Not only is it 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.

Screening for prebiotic properties in short-term batch experiments

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 (pH 2, pepsin);

2. Small intestine: addition of porcine pancreatic enzymes and bile salts

3. Large intestine with a representative bacterial inoculum. This bacterial inoculum will be 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 prebiotic under study was 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 was assessed upon simulated colon incubation.

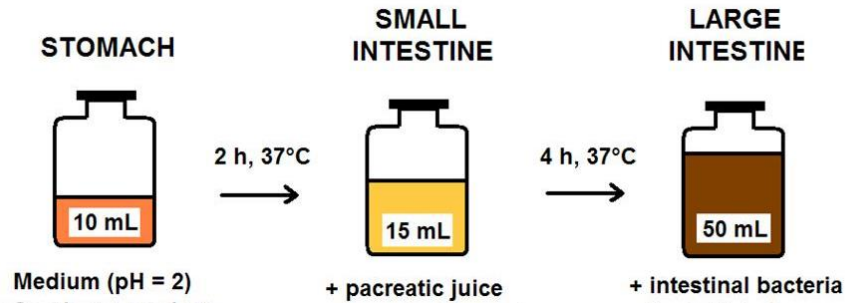


Figure 1: The typical experimental setup for short-term batch experiments (volumes are only indicative)

The goal of this experiment was to obtain a detailed estimation of the potential prebiotic properties of the studied compounds based on changes in both microbial composition and activity. Although these experiments only simulated the intestinal environment up to a certain degree, careful evaluation of the data obtained of the different compounds allowed to evaluate the potential effect of the lead compounds in a highly cost-effective way.

info@prodigest.eu
www.prodigest.eu

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

- **Community composition**:
  - Samples were taken at the beginning, after 24h and 48h for DNA extraction. As the experiment made use of the same bacterial inoculum, the microbial community composition at the beginning of the colon incubation was quantified only once (in triplicate) and not for each individual incubation.
  - qPCR protocols were used to measure the concentration of total bacteria, bifidobacteria and lactobacilli.

- **Community activity**:
  - Samples were collected at time 0, 4, 24 and 48h. This allowed to compare the kinetics in the production of bacterial metabolites depending on the tested compound.
  - Short chain fatty acid analysis:
    - The pattern of SCFA and lactate 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 normal GI microbiota.
  - Ammonium analysis:
    - Ammonia is a product of proteolytic degradation and can also work as an indirect marker for substrate availability.
  - pH: as the degree of acidification at the end of the experiment is a measure of the intensity of bacterial metabolism of the potential prebiotic, the pH of the incubations will be determined at the beginning and at the end of the experiment.
  - Gas analysis:
    - Samples were collected at time 0, 4, 24 and 48h.
    - The excessive production of gas is considered as a potentially negative side-effect of increased saccharolytic activity of the intestinal community upon prebiotic administration. The kinetics of the total gas production were analyzed throughout the experiment.

info@prodigest.eu
www.prodigest.eu

Results

Effect on Short-chain fatty acid (SCFA) production

SCFA (acetate, propionate and butyrate) are the main metabolites from carbohydrate metabolism and the specific production of certain SCFA is related with various health effects. Whereas **acetate** can be used as energy source for the host and as a potential substrate for lipid synthesis in the body, **propionate** reduces cholesterol and fatty acid synthesis in the liver (beneficial effect on metabolic homeostasis). **Butyrate** on the other hand, is a major energy source for colonocytes and induces differentiation in these cells (related to cancer prevention). Positive effects of prebiotics on SCFA production can therefore be mainly associated with a relative increase of propionate and/or butyrate. The pattern of the SCFA production is therefore an assessment of a specific fiber metabolism.

The data of the SCFA production with the two different products are presented in Figure 2 and Figure 3. In Figure 2 the absolute values of the measured amount of each SCFA (mmol/L) at the different sampling points are shown, while Figure 3 shows the calculated A/B/P ratio, in which the individual production of each of the three major SCFA is expressed relative to the combined production of the three acids.

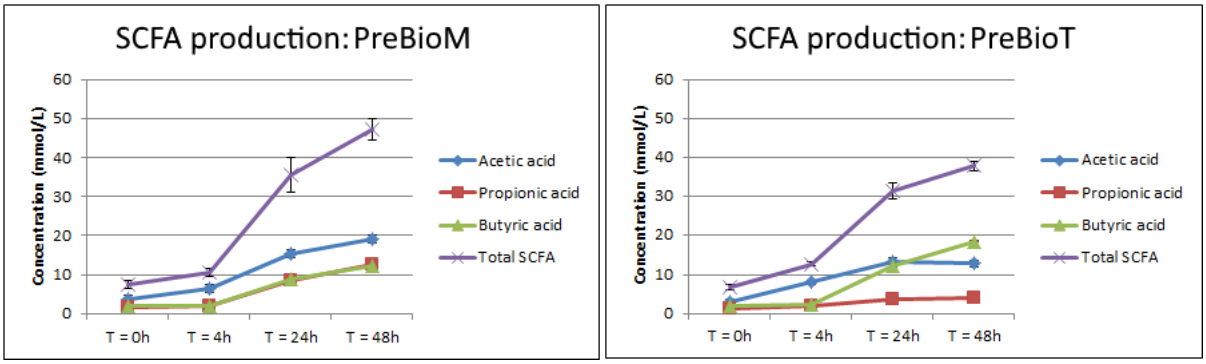


Figure 2: Short-chain fatty acid (SCFA) production for the incubations of PreBioM or PreBioT. Samples were collected at the start and after 4, 24 and 48 hour of incubation. The concentrations of the three major SCFA (acetate, propionate and butyrate) are presented separately, as well as the total SCFA production.

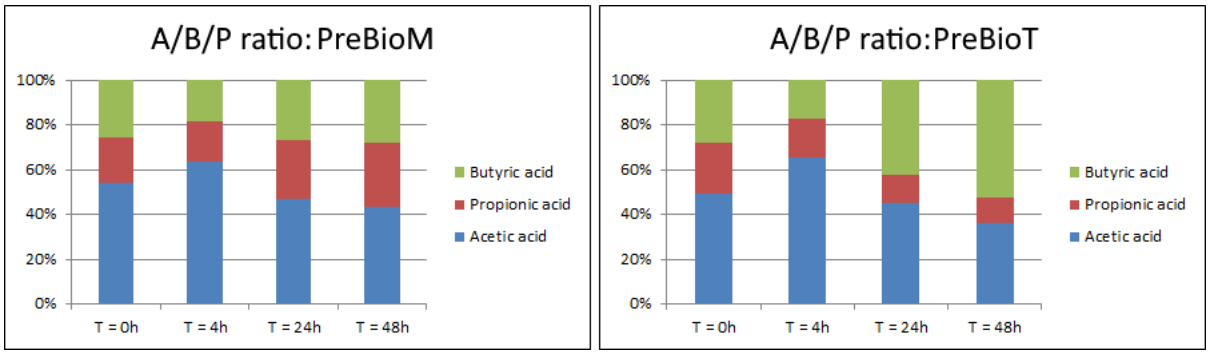


Figure 3: The A/P/B ratios (A = acetate, P = propionate and B = butyrate) in the samples collected after 0, 4, 24 and 48 hours of incubation with PreBioM or PreBioT. This ratio expresses the relative production of the three major SCFA, and is therefore a measure of the importance of each SCFA in the total production from each test product.

info@prodigest.eu
www.prodigest.eu

Observations:

- *PreBioM*

After 4 hour of incubation, only a small amount of SCFA was produced (7% of total SCFA that will be produced after 48 hour incubation). This can be explained by the fact that between 0 and 4 hour, the bacteria need to adapt to the incubation conditions, a characteristic typical of fiber that are selective. The highest production in total SCFA occurred between 4 and 24 hour (53% of total SCFA produced), with acetate being produced in the highest amount. SCFA production between 24 and 48 hour slowed down again.

The relative results showed that there was a shift in the production from acetate (61% of total SCFA to 41% after 48 hour) towards propionate (17% of the total SCFA after 4 hour to 27% after 48 hour) with an improvement of the cross-feeding among bacteria.

- *PreBioT*

Similar for PreBioM, the highest production of SCFA occurred between 4 and 24 hour (50% of total SCFA produced). PreBioT showed a lower production in total SCFA, which implies that the fermentation is more difficult/slower as compared to PreBioM. Moreover, the production was not equal for the different SCFA produced as acetate and propionate were produced in lower amounts (33% and 68% respectively), with a selective effect towards butyrate (50% more butyrate was produced).

As a consequence, the relative results (A/P/B ratio) showed a shift of the SCFA from acetate (63% after 4 hour to 34% after 48 hour) to butyrate (17% after 4 hour to 49% after 48 hour). This is a highly desired characteristic as butyrate is known to be health promoting in the intestinal environment.

Conclusion:

Both fibers can be efficiently degraded by saccharolytic bacteria leading to a different fermentation profile. PreBioM can be fermented more easily, with higher SCFA concentrations at the end of the incubation. PreBioT, on the other hand, had a more desired SCFA profile, with butyrate being present in the highest concentration at the end of the simulation.

Effect on lactate production

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.

The analysis of lactate concentrations in the incubations with the different products is presented in Figure 4.

info@prodigest.eu
www.prodigest.eu

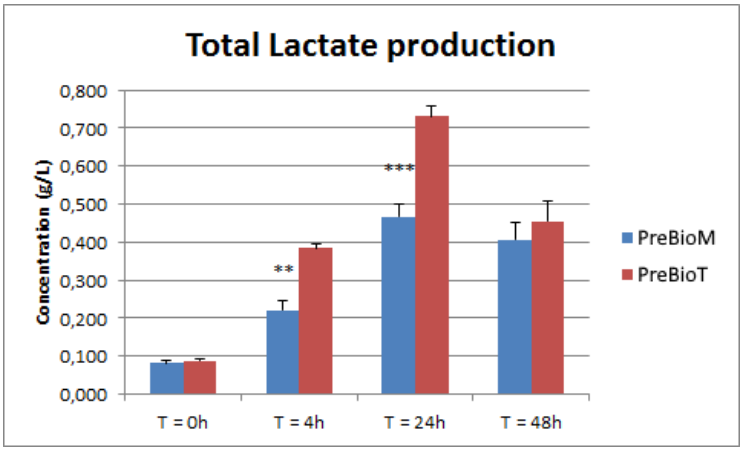


Figure 4: Lactate production in the different incubations. Samples were collected at the start and after 4, 24 and 48 hour of incubation and expressed as g/L. Significant difference between the products as been tested with T-test (: p<0.01; ***: p<0.001).**

Observations:

- *PreBioM*

The results showed that after addition of PreBioM, lactate was produced from the start of incubation. After 24 hour of incubation, the concentration of lactate did not further increase.

- *PreBioT*

The lactate production during fermentation of PreBioT was very different. Significantly more lactate was produced after 4 hour ($p < 0.01$) and 24 hour ($p < 0.001$) when PreBioT was added, as compared to PreBioM. After 48 hour, approximately 1/3 of the lactate that was present at 24 hour was removed. Presumably, PreBioT can be metabolized by lactate-producing bacteria (between 4 and 24 hour) after which the lactate is used by other bacteria for cross-feeding (between 24 and 48 hour).

Conclusion:

The results of the SCFA and lactate production showed that PreBioM and PreBioT are differently fermented by the intestinal bacteria. PreBioM is fermented during 48 hour of colon incubation to acetate, propionate and smaller amounts of butyrate and lactate. PreBioT showed a very different fermentation profile. During the first hour of incubation, the bacteria mainly fermented the fiber to acetate and lactate. The latter were then used to produce butyrate by cross-feeding.

Effect on ammonium production

The amount of ammonium, produced during the incubation, depends on a number of factors:

- Protein content of the sample as nitrogen source.
- Sugar content and availability. If easily fermentable sugars are available for the bacteria, less protein will be degraded. Alternatively, if no, or only a highly specific group of bacteria can use the carbohydrate substrate, most bacteria will need to use alternative energy sources,

info@prodigest.eu
www.prodigest.eu

such as proteins, which will result in increased ammonium production. Ammonium is therefore also a marker of the selectivity of the substrate towards certain bacteria.

Figure 5 shows the values of ammonium concentrations measured at the different time points during the incubation.

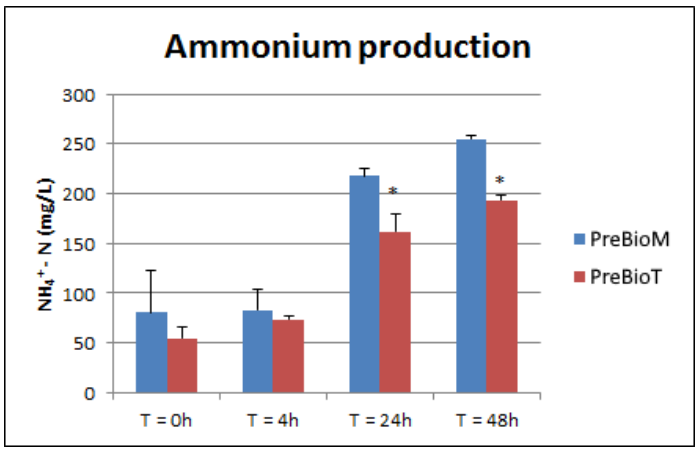


Figure 5: Ammonium production in the different incubations. Samples were collected at the start and after 4, 24 and 48 hour of incubation and expressed as mg NH_4-N/L. Significant difference between the products as been tested with T-test (*: p<0.05).

Observations:

No ammonium was produced between 0 and 4 hour with either of the products. After 24 hour, 34% more ammonium was found in the incubation with PreBioM as compared to the incubation with PreBioT. Between 24 and 48 hour, an equal amount of ammonium is produced in both incubations.

Conclusion:

In absence of a clear specification on the composition of the 2 products (i.e. purity and eventual amount of proteins present) the data can lead to a double interpretation.

In fact, the **lower ammonium production observed with PreBioT** may be an indication of the fact that this product is more pure and fewer proteins are present to be fermented. Conversely, PreBioM can be more easily fermented and the bacteria start to use the peptone and yeast extract present in the medium faster because the fiber depleted earlier as compared to PreBioT.

Effect on intestinal pH

The change in pH during the experiment is correlated with the production of SCFA, ammonium and lactate in the sample.

Data on pH are shown in Figure 6.

info@prodigest.eu
www.prodigest.eu

Observations and conclusions:

Both products led to a pH decrease after 48 hour of incubation. **The Δ pH as a result of the addition of PreBioT was significantly (p < 0.5) more pronounced as compared to PreBioM**. This fits perfectly with the data already presented on SCFA, lactate and ammonium production. In fact, PreBioT led to a higher lactate production and a lower ammonium production as compared to PreBioM and this is reflected in the ΔpH.

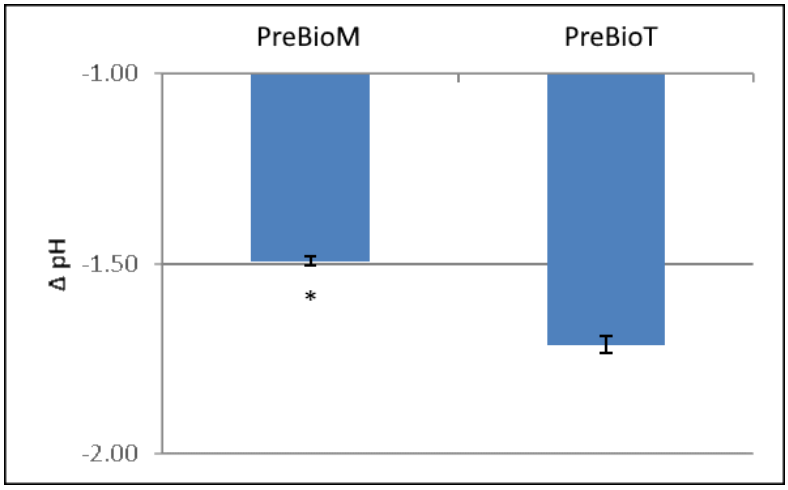


Figure 6: Average pH difference of the replicates for the products ± SD. The pH was monitored at the beginning and after 48 hour of incubation. Significant difference between the products has been tested with T-test (*: p<0.05).

Effect on gas production

The gas production in the samples throughout the experiment can be considered as a **measure of the fermentability of the specific product**. Moreover, the speed of gas production is related to the fermentation rate of the products.

The total gas production profiles are presented in Figure 7 as the increase of gas produced at each time point in comparison with the previous time point (net production). Expressing the data in this way allows to evaluate at which time intervals most gas is produced (i.e. measure for the fermentation rate of each product).

Observations:

The net gas production between two time points during the incubations was similar for both products. Only **between 4 and 24 hour, significantly (p < 0.05) more gas was produced in the incubation with PreBioM as compared to the incubation with PreBioT**.

info@prodigest.eu
www.prodigest.eu

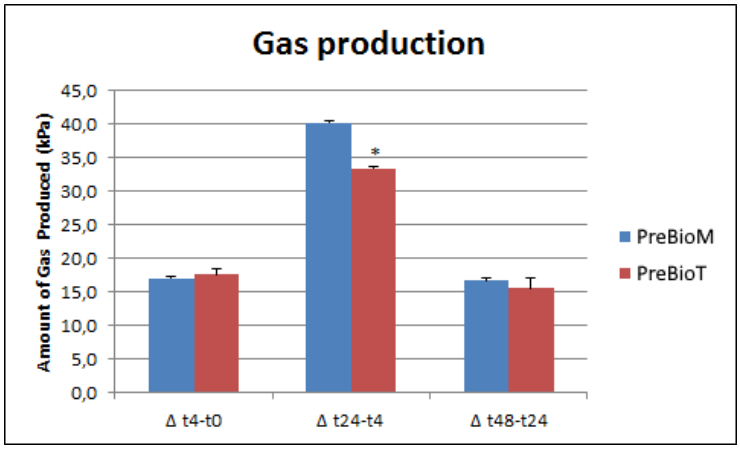


Figure 7: Total gas production in the different incubations. The gas pressure in the incubations was monitored at the start and after 4, 24 and 48 hour. The results are presented as the gas pressure increase in the headspace compared to the previous sampling time. (*: significant difference as tested with T-test ($p<0.05$)).

Conclusion:

PreBioM led to a higher SCFA production and this was reflected in a higher gas production. The latter confirmed that PreBioM can be more easily fermented as compared to PreBioT.

Analysis of the microbial community composition

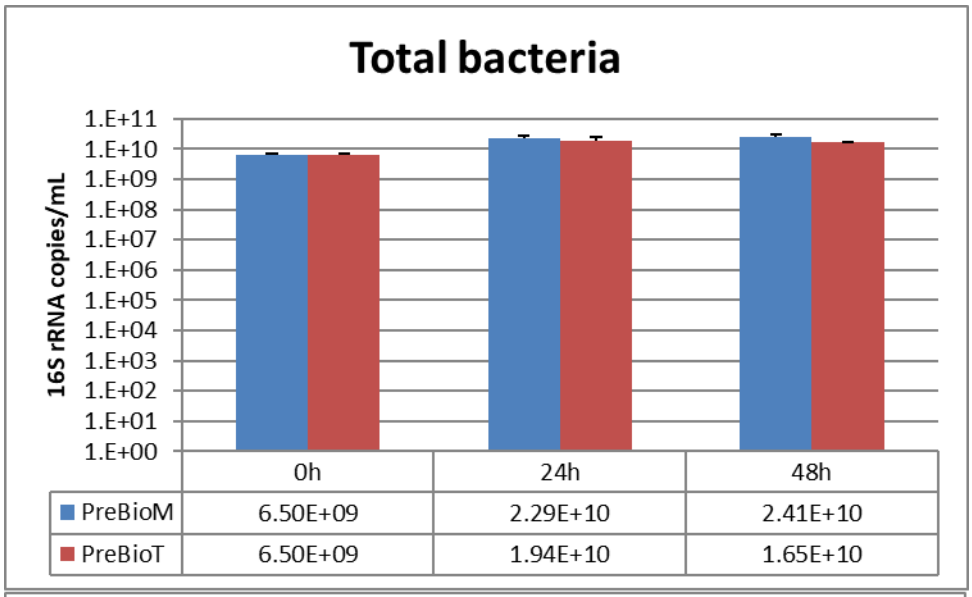
Quantitative Polymerase Chain Reaction

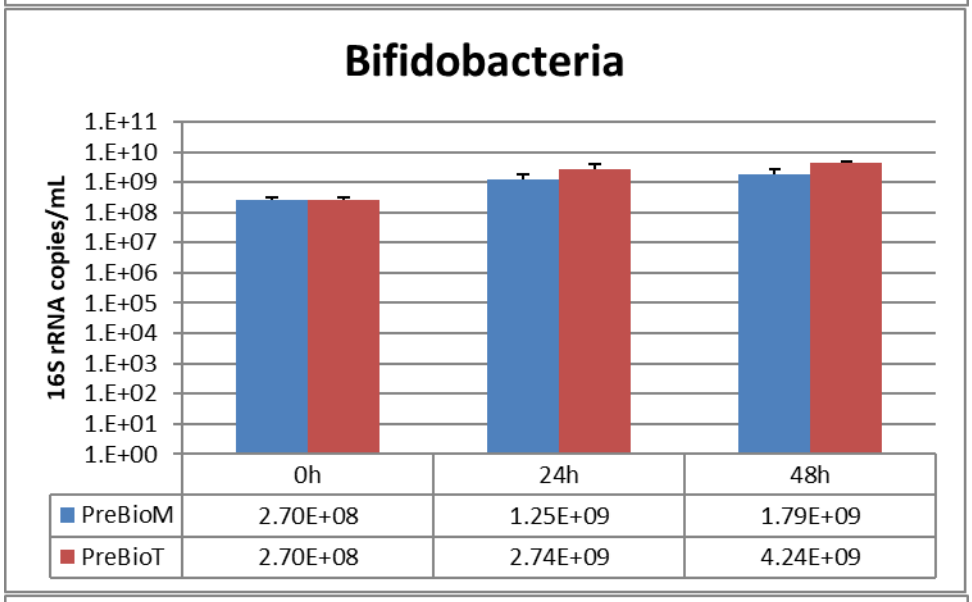
Quantitative PCR (qPCR) is a molecular technique which is based on the amplification of specific bacterial sequences (16S rRNA genes), combined with the quantification of the number of these specific sequences which is present in a microbial ecosystem at different time points.

The effect of the presence of the products on the microbial community composition were followed by means of qPCR protocols specific for:

- total bacteria,
- *Bifidobacterium* spp. and
- *Lactobacillus* spp.

Figure 8 reports the concentration of these microbial groups at three different time points (0h, 24h and 48h) during the simulation of colonic fermentation.





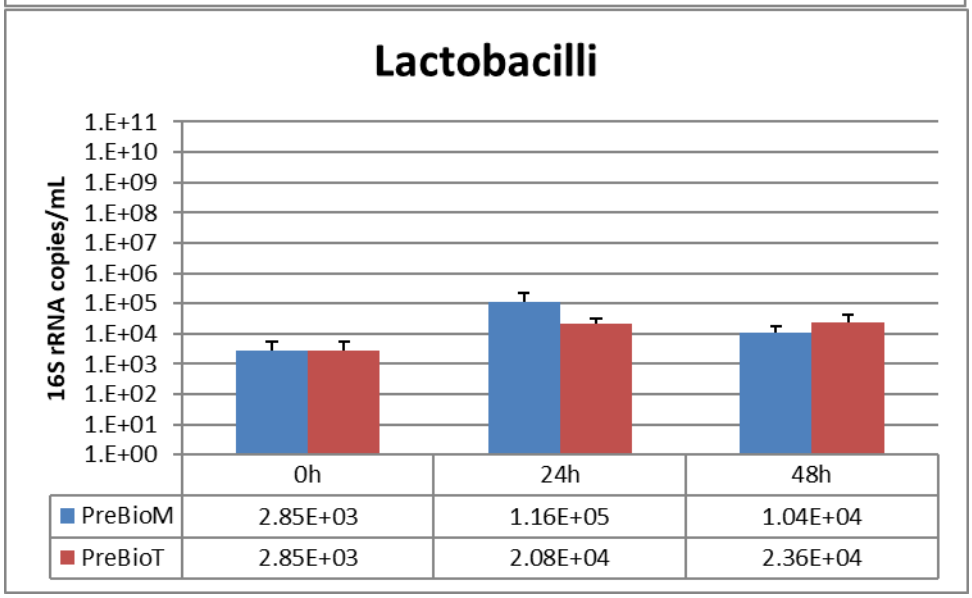


Figure 8: qPCR data specific for total bacteria, bifidobacteria and lactobacilli.

info@prodigest.eu
www.prodigest.eu

Observations:

Both products led to an increase in the concentration of total bacteria (+0.5 Log). Moreover, both products were also correlated with a **bifidogenic effect** (+1 Log for PreBioM and +1.2 Log for PreBioT at 48h) and a **lactobacillogenic effect** (+2 Log for PreBioM at 24h and +1 Log for PreBioT at 48h).

Conclusions

A short-term experiment was performed to test the prebiotic properties of two fibers, PreBioM and PreBioT. Specific analyses were performed to follow up the gut microbial community activity and composition.

Conclusions on microbial community activity:

- SCFA:
  - SCFA are the main metabolites from the carbohydrate metabolism. The pattern of the SCFA production is an assessment of a specific fiber metabolism. Changes over time in the SCFA profile towards less acetate and more propionate and/or butyrate are generally considered healthy.
  - **Both fibers could be efficiently fermented by saccharolytic bacteria, leading however to a different profile. PreBioM led to an increase of all the three main SCFAs (proportionally more propionate) while PreBioT was mainly correlated to a butyrogenic effect. PreBioM led to a higher total SCFA production, as compared to PreBioT.**
- Lactate:
  - Lactic acid is an important metabolite from saccharolytic fermentation. Not only it has direct positive effects on the intestinal environment, but it is also an important intermediate for propionate and butyrate production.
  - **Different lactate production profiles were found for the two products. Results showed that PreBioT led to a higher lactate production as compared to PreBioM. The extra lactate could subsequently be used for butyrate production by cross-feeding.**
- Ammonium:
  - Ammonium production is the result of proteolytic fermentation.
  - **Different ammonium production profiles were observed for the products. Both product led to an increase in ammonium production (this is normal in short-term experiments) but PreBioT led to 34% lower production as compared to PreBioM.**
- Intestinal pH:
  - Acidification of the intestinal environment is a consequence of increased saccharolytic fermentation. It is generally considered as beneficial for health.

info@prodigest.eu
www.prodigest.eu

- **The difference in SCFA, lactate and ammonium production profiles was reflected in the pH profile. Overall, a higher pH decrease was found in the incubation with PreBioT.**

- Gas production:
  - The speed of gas production is a marker for the speed of saccharolytic fermentation (boost vs. gradual fermentation).
  - **PreBioM led to a higher SCFA production and this was reflected in a higher gas production. The latter confirmed that PreBioM can be more easily fermented as compared to PreBioT.**

Conclusions microbial community composition:

- qPCR:
  - Total bacteria and lactobacilli/bifidobacteria (i.e. 2 groups normally associated with health promoting effects) were monitored throughout the experiment by means of qPCR.
  - **Both products led to an increase in the concentration of total bacteria and were correlated with a bifidogenic and a lactobacillogenic effect.**

Both products gave clear indications of potential prebiotic activity, however leading to different fermentation profiles. PreBioM: higher SCFA production and bifidogenic/lactobacillogenic effect. PreBioT: mainly butyrogenic, lower ammonium and gas production and bifidogenic/lactobacillogenic effect.

info@prodigest.eu
www.prodigest.eu