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To pool or not to pool? Impact of the use of individual and pooled fecal samples for *in vitro* fermentation studies

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ABSTRACT

This study investigated the stability and the activity of the microbiota from a single and a pool of donors in the TNO *in vitro* model of the colon (TIM-2 system). Our findings demonstrate the suitability of the preparation of a pool of fecal sample to be used for fermentation experiments.

1. Introduction

Monitoring the *in vivo* fermentation of carbohydrates in humans is difficult, due to the inaccessibility of the proximal colon, the ethical considerations and the medical supervision required. Validated *in vitro* models using human feces as an inoculum are much simpler systems that allow the study of the fermentation of any compound accompanied by the assessment of metabolites such as short-chain fatty acids (SCFAs) and other bacterial products. Moreover, such systems constitute a powerful tool to follow disruptions in the microbial equilibrium in detail. Therefore, it is crucial to guarantee the development of a representative gut community in any of these systems without any confounding factor, unrelated with the aim of the study that would affect the composition of the microbiota.

TIM-2, the TNO *in vitro* model of the colon, is such an *in vitro* model that closely mimics the fermentation by the microbiota in the human large intestine allowing the growth of a highly complex, stable and dense (~10^{11} CFU/ml) active microbiota (De Graaf et al., 2010). Studies performed in this system generally use a standardized inoculum which is derived from a pool of subjects of interest and is stored frozen. However, the use of an inoculum prepared from either a single donor or a pool of donors remains debatable among experts. The main argument relies in the concern about how representative such inoculum is in regard to the colonic ecosystem taking into account the abundance and the variety of bacterial species. Consequently, the use of an individual or a mixed inoculum is believed to lead to a degree of variation among experiments, even when the single inoculum is repeatedly taken from the same individual over time. Although it has been shown that the microbiota of adults is relatively stable over time, differences in the composition of the microbiota of a single individual over a 4 year period have been observed (Rajilic-Stojanovic et al., 2012). With respect to the TIM-2 system, no studies have been performed in order to find out differences related to the individual or pooling preparation of the inoculum in its composition and activity during a standard fermentation process with the use of a Standard Ileal Efflux Media (SIEM) as substrate. Still, previous data in this system using the individual inocula from 10 donors has shown that the microbial activity of the individual donors was extremely similar in functionality, despite a different microbiota composition (Venema, 2012; Venema et al., 2003) corroborating the hypothesis that a standardized pool of gut microbiota can be used for these type of experiments.

It is also important to consider the report in which the effect of arabinoxylan and inulin has been tested using an inoculum prepared from a pooled donation (in TIM-2) and from a single volunteer

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(in SHIME; Simulator of the Human Intestinal Microbial Ecosystem; another *in vitro* model) where a similar metabolic activity of the microbiota in both setups was observed (Van den Abbeele et al., 2013).

Thus, the purpose of this study was to compare both ways of preparing a fecal inoculum to be used in *in vitro* studies. Changes were identified by monitoring the composition and activity of the microbiota under standard fermentation experiments in TIM-2. To the best of our knowledge this is the first time that this direct comparison has been performed.

2. Methods

2.1. Microbiota: source, collection and processing

Individual fecal homogenates were prepared from a group of 4 healthy volunteers (age range 29–62; 3 males, 1 female). A homogenate with the pool of the microbiota from these individuals was also used. The group of participants was recruited at TNO (Zeist, The Netherlands). The individuals were non-smokers and had not used antibiotics, prebiotics, probiotics or laxatives 3 months prior to the donation. Informed consent was provided by each volunteer prior to the participation in the study.

Fresh fecal samples were directly collected in a tightly closed box with an anaerobic strip (AnaeroGen™, Oxoid, Cambridge, UK) inside. Within 1 h after being collected, the donations were homogenized under strict anaerobic conditions in an anaerobic chamber (Bactron IV, Sheldon Manufacturing, Cornelius, OR, USA) containing 5% H_2, 5% CO_2, and 90% N_2. 8.75 g of the fecal donation (individually as well as pooled) was mixed with a Turrax (IKA Ultra Turrax T25 digital, Fisher Scientific Nederland) with a physiological saline preparation/dialysate (content per liter: 4.7 g $K_2HPO_4 \cdot 3H_2O$, 8.4 g NaCl, 0.009 g $FeSO_4 \cdot 7H_2O$, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.8 g $CaCl_2 \cdot 2H_2O$, 0.7 g ox bile and 0.3 g cysteine hydrochloride) and glycerol (as cryo-protective agent).

2.2. Feed used for fermentation: source and preparation

The feeding substrate used for fermentation in TIM-2 simulates the average non-digestible carbohydrates consumed in a normal Western diet (Maathuis et al., 2012). SIEM is prepared with 0.5 g pectin, 0.5 g xylan, 0.5 g arabinogalactan, 0.5 g amylopectin and 4.5 g starch (Tritium Microbiology; Veldhoven, The Netherlands) per day. Specific details have been previously described (Maathuis et al., 2009; Van Nuenen et al., 2003).

2.3. TIM-2 experimental protocol

The TIM-2 system was flushed for 4 h with N_2 prior to the introduction of the inoculum and it was maintained under this condition at 37 °C for 96 h with the pH kept at or above 5.8 by automatic titration with 2 M NaOH. Water and fermentation products were removed from the lumen with a dialysate system (described in the following section) consisting of a semi-permeable hollow membrane which ran through the lumen. For all the experiments, the speed of the dialysis was set at 1.5 ml/min.

The feed preparation mentioned above was gradually introduced into the system in a total volume of 45 ml in the adaptation period and 180 ml over the 72 h of the test period at a rate of 2.5 ml/h. Luminal content was maintained at a level of approximately 120 ml in each unit by a level sensor (Liquiphant FTL20-0025, Endress + Hauser).

After 24 and 48 h of fermentation 25 ml of lumen sample was removed (Fig. 1) through the system's sample port using a sterile syringe to mimic the transit of material from the proximal to the distal colon (Maathuis et al., 2009). The dialysate fluid used in the system contained per liter: the dial preparation described under “Microbiota: source, collection and processing” section and 1 ml of vitamin mixture containing per liter: 1 mg menadione, 3 mg D-biotin, 0.8 mg vitamin B-12, 15 mg pantothenate, 7 mg nicotinamide, 7 mg para-aminobenzoic acid, and 6 mg thiamine (all from Tritium Microbiology).

2.4. Design of the study

Approximately 30 ml portions of fecal homogenate (±25% w/v) were used to inoculate the separate TIM-2 units for each experiment. Each unit was then filled to 120 ml with dialysate. Right after the inoculation, the microbiota was left to adapt (16 h) to the new environment. After this adaptation period, the culture was deprived from SIEM for 2 h which aims at full use of fermentable carbohydrates prior to feeding of a test carbohydrate. Minekus et al. (1999) explained that this starvation period was established when the lack of production of acids was observed in the system when the feeding line was turned off.

Next, units were fed with SIEM until the end of the experiment. Samples were taken from the lumen after −16, −14, −8, 0, 24, 48 and 72 h. Dial was collected after 0, 24, 48 and 72 h (Fig. 1). Both luminal and dial samples were first snap frozen in liquid nitrogen (−196 °C) immediately after collection and were stored at −80 °C previous to analysis.

2.5. Analytical methods

With the samples from lumen and dialysate the production of SCFA and branched-chain fatty acids (BCFAs) were calculated. Microbial composition was sequenced at t_{-16} and t_{72}.

2.5.1. SCFA (acetate, propionate, and n-butyrate) and BCFA (iso-butyrate and iso-valerate)

Samples for SCFA and BCFA analyses were determined by analyzing their concentration by GC (Stabilwax-DA, length 15 m, ID 0.53 mm, film thickness 0.1 mm; Varian Chrompack, Bergen op Zoom, The Netherlands). Samples were prepared as previously described by van Nuenen et al. (2003).

2.5.2. Phylogenetic analysis of the microbiota

DNA from the luminal samples was isolated using the AGOWA mag Mini kit (DNA Isolation Kit, AGOWA, Berlin, Germany), according to the manufacturer's instructions.

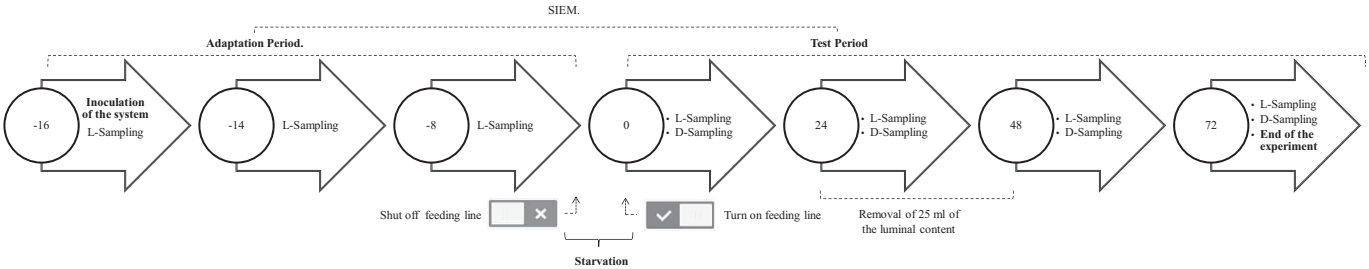

Fig. 1. Experimental setup (not at scale). L-sampling = luminal samples; D-sampling = dialysate.