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Evaluation of an optimal preparation of human standardized fecal inocula for *in vitro* fermentation studies

Marisol Aguirre [a,b,c], Anat Eck [d], Marjorie E. Koenen [c], Paul H.M. Savelkoul [d,e], Andries E. Budding [d], Koen Venema [a,c,*]

[a] Top Institute of Food & Nutrition, PO Box 557, 6700 AA Wageningen, The Netherlands
[b] Maastricht University, School of Nutrition, Toxicology and Metabolism (NUTRIM), Faculty of Health, Medicine and Life Sciences, Department of Human Biology, PO Box 616, 6200 MD, Maastricht, The Netherlands
[c] The Netherlands Organization for Applied Scientific research, TNO, PO Box 360, 3700 AJ, Zeist, The Netherlands
[d] VU University Medical Center, Department of Medical Microbiology and Infection Control, 1081 BT Amsterdam, The Netherlands
[e] Maastricht University Medical Center, Department of Medical Microbiology, PO Box 5800, 6202 AZ, Maastricht, The Netherlands

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ABSTRACT

This study investigated the optimal preservation approach to prepare human feces as inoculum for *in vitro* fermentations as an alternative to the use of fresh feces. The four treatments studied were: Treatment 1) fresh feces resuspended in dialysate solution + glycerol; Treatment 2) fresh feces resuspended in dialysate solution + glycerol and then stored at −80 °C; Treatment 3) fecal sample frozen with 1.5 g glycerol; and Treatment 4) fecal sample frozen. All the treatments contained 8.75 g of feces, 3.5 ml dialysate and 4.9 ml glycerol when inoculated in TIM-2 *in vitro* system. Treatment 1 (fresh fecal preparation) was used as a reference. The effects were evaluated in terms of i) metabolic activity and ii) composition of the microbiota using fermentation experiments in the TIM-2 *in vitro* system. In all treatments, high levels of acetate were produced followed by *n*-butyrate and propionate. However, the metabolic activity of the bacteria, in terms of short-chain fatty acid production, was affected by the different treatments. Microbiota composition was analyzed using the IS-pro profiling technique. Diversity in *Actinobacteria*, *Firmicutes*, *Fusobacteria* and *Verrucomicrobia* and *Proteobacteria* groups seemed to be preserved in all treatments whereas it was observed to decline in the *Bacteroidetes* group. Preparing a human fecal inoculum resuspended in dialysate solution with glycerol and then stored at −80 °C showed high similarities to the results obtained with fresh feces, and is proposed as the optimal way to freeze fecal material as an alternative to fresh feces for in vitro fermentation studies.

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1. Introduction

The human gut harbors a community of microorganisms commonly known as the microbiota. This community is dominated by anaerobic bacteria and consists of at least 10^{14} members with a wide variety of species (±500–1000) (Bäckhed et al., 2004).

The intestinal microbiota in humans has been demonstrated to be highly active and able to ferment indigestible compounds from the host's diet (Flint et al., 2007). The type of diet determines whether the fermentation process occurring in the gut is dominantly saccharolytic or proteolytic (Scott et al., 2013). The metabolites from these two types of fermentation include mainly short-chain fatty acids (SCFA), specifically acetate, propionate and butyrate (Flint et al., 2007), and branched-chain fatty acids (BCFA) including principally *iso*-butyric, *iso*-valeric and 2-methylbutyric acids (Bergman, 1990) Metabolites such as acetate, propionate and butyrate are of particular interest since they have been found to be involved in lipid metabolism, reduction of food intake, improvement of tissue insulin sensitivity and intestinal barrier, and energy balance (Al-Lahham et al., 2010; Al-Lahham et al., 2011; Ferchaud-Roucher et al., 2005; Peng et al., 2009; Roediger, 1982; Scheppach, 1994). As a consequence, increasing evidence shows that the composition and activity of the intestinal microbiota is associated with the overall health state of humans, including obesity. Food components affect the composition and activity of the gut microbiota. Therefore, the fermentation characteristics of an ample number of substrates have been studied (Cardelle-Cobas et al., 2012; Fassler et al., 2006; Hernot et al., 2009).

Part of these studies include experiments performed in in vitro systems which offer a high flexibility in their design since there are less

* Corresponding author at: Johan Karschstraat 3, 6709 TN Wageningen, The Netherlands.
E-mail addresses: marisol.aguirremorales@tno.nl (M. Aguirre), a.eckhauer@vumc.nl (A. Eck), marjorie_koenen@hotmail.com (M.E. Koenen), p.savelkoul@vumc.nl (P.H.M. Savelkoul), D.Budding@vumc.nl (A.E. Budding), koen.venema@outlook.com (K. Venema).

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limitations in regard to costs and ethical constraint when compared to human trials. For such in vitro studies, the use of a well-preserved fecal sample or inoculum is crucial to perform reproducible experiments and to guarantee the robustness and reliability of these experiments. The use of a standardized inoculum provides the opportunity to perform a large number of studies with the same microbiota for different substrates. This contributes to more reproducible assays that can be performed over a long period of time, which is impossible with a single fresh fecal sample.

There is a lack of literature addressing the possible variations in the microbial activity and composition induced by storage and preparation of a human fecal inoculum for *in vitro* studies. In experiments performed in rumen fluid, canine, and equine feces, freezing has been found to damage and disrupt the bacterial cell membrane, which causes the release of intracellular contents that subsequently led to loss of (members of the) communities (Bosch et al., 2013; Murray et al., 2012; Prates et al., 2010; Rose et al., 2010). Moreover, some groups of bacteria have been observed to be seriously damaged after freezing and thawing such as certain Gram-negative bacteria (Murray et al., 2012). Alterations in the kinetics of fermentation as well as production of gases have also been found to occur after manipulation (Murray et al., 2012; Pastorelli et al., 2014). However, these negative effects were not observed during the preparation of the human fecal inoculum by Rose et al. (2010) which, to our knowledge, is one of the few studies that have validated the use of fresh and frozen human microbiota. These authors observed that the viable cells in the microbiota stored for 44 weeks at −80 °C were not affected and the microbial diversity of this inoculum did not substantially differ from the fresh one, although in that previous study the direct comparisons between a frozen and fresh inoculum were not performed as the main goal. As explained before, there is a lack of information about an appropriate treatment to preserve human feces for *in vitro* fermentation experiments. Furthermore, these previous findings need to be expanded. Thus, the purpose of this study was to determine the optimal conditions to prepare a human fecal inoculum to be used in the TNO dynamic *in vitro* proximal colon model (TIM-2) (Venema et al., 2000). Four different treatments to prepare human fecal inocula were studied and their efficacy was evaluated by monitoring the composition and activity (in terms of SCFA and BCFA production) of the microbiota under standard fermentation experiments. A potential alternative to fresh feces was successfully found.

2. Material and methods

2.1. Fecal samples

Participants involved in this study were non-smokers and had not used antibiotics, prebiotics, probiotics or laxatives 3 weeks prior to the donation. Fresh fecal samples were directly collected in a closed box containing an anaerobic strip (AnaeroGen™, Oxoid, Cambridge, UK). Donations and treatment preparations were handled 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. A pool of feces was prepared from a group of 4 healthy volunteers (age range 29–62; 3 males, 1 female) recruited at TNO (Zeist, The Netherlands). Pooling has been previously shown to not drastically affect microbiota composition (Aguirre et al., 2014b).

The pooled fecal slurry was divided into four aliquots to prepare the different treatments as follows (Fig. 1): Treatment 1) fresh fecal slurry resuspended in dialysate solution + glycerol, this inoculum was tested right upon preparation; Treatment 2) fresh fecal slurry resuspended in dialysate solution + glycerol, frozen, stored and resuscitated prior testing; Treatment 3) fresh fecal slurry frozen with 1.5 g glycerol, stored, resuscitated and, prior testing, resuspended in dialysate solution + portion of glycerol; and Treatment 4) the fecal sample was frozen, stored, resuscitated and, prior testing, resuspended in dialysate solution + glycerol. All inocula (Treatments 2–4) that were stored (−80 °C, 1 week) were immediately snap frozen in liquid nitrogen (−196 °C) following their preparation and were resuscitated by immersion in a water bath (37 °C, 1 h) before parallel testing. Freezing in liquid nitrogen was performed in order to guarantee cell viability (Day et al., 1995; Malik, 1991). All the treatments contained 8.75 g of feces, 3.5 ml dialysate and 4.9 g glycerol when inoculated in TIM-2. Treatment 1 (fresh fecal preparation) was used as a reference. The dialysate preparation has been previously described by Maathuis et al. (2009). The dialysate content per liter was as follows: 2.5 g $K_2HPO_4 \cdot 3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MgSO_4 \cdot H_2O$, 0.45 g $CaCl_2 \cdot 2H_2O$, 0.05 g ox bile and 0.4 g cysteine hydrochloride. The dialysate preparation included bile salts in order to partly reproduce the environment that the gut microbiota is usually exposed in the colon (Ridlon et al., 2006). Therefore, bile salts were included in the preparation of the different treatments to keep the tolerance of the cells. The impact of bile in fresh and freeze-dried prepared axenic cultures has been tested before (Saarela et al., 2005) but not in fecal samples holding complex microbiota.

2.2. Standard ileal efflux medium (SIEM)

A growth medium prepared with complex, indigestible carbohydrates (pectin, xylan, arabinogalactan, amylopectin and starch), protein, vitamins, Tween 80 and bile (Tritium Microbiology; Veldhoven, The Netherlands) was used to feed the bacteria at a rate of 2.5 ml/h during fermentation experiments. Specific details about this SIEM preparation have been previously described (Maathuis et al., 2009; Van Nuenen et al., 2003).

2.3. In vitro fermentation

The TNO *in vitro* model of the proximal colon (TIM-2) has been described in detail before (Rose et al., 2010; Van Nuenen et al., 2003;

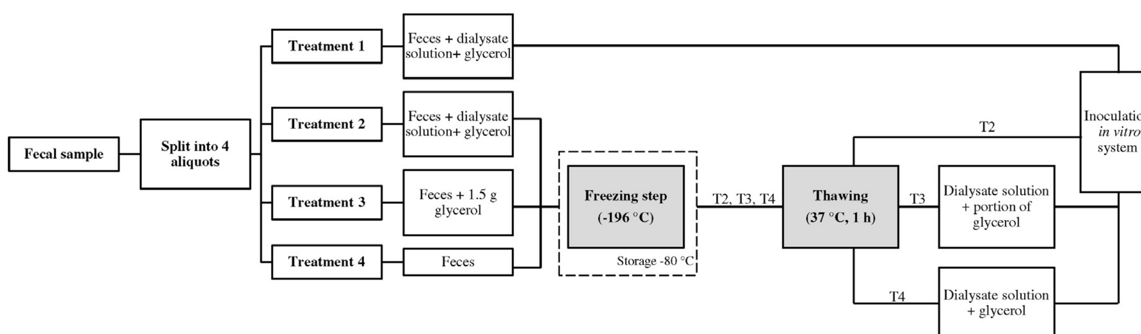

Fig. 1. Treatments for the different preparations of the inocula. Samples were frozen immediately, or mixed with glycerol and/or dialysate solution before freezing in liquid nitrogen. All the treatments contained 8.75 g of feces, 3.5 ml dialysate and 4.9 g glycerol when inoculated in TIM-2.

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