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Three-stage continuous culture system with a self-generated anaerobia to study the regionalized metabolism of the human gut microbiota

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ABSTRACT

The technical and ethical difficulties in studying the gut microbiota in vivo warrant the development and improvement of in vitro systems able to simulate and control the physicochemical factors of the gut biology. Moreover, the functional regionalization of this organ implies a model simulating these differences. Here we propose an improved and alternative three-stage continuous bioreactor called 3S-ECSIM (three-stage Environmental Control System for Intestinal Microbiota) to study the human large intestine. Its main feature compared with other in vitro systems is the anaerobic atmosphere originating directly from the microbiota metabolism, leading to different gas ratios of CO_2 and H_2 in each compartment. Analyses of the metabolic and microbiological profiles (LC–MS and a phylogenetic microarray) show different profiles together with a maintenance of this differentiation between the three compartments, simulating respectively a proximal, a transversal and a distal colon. Moreover, the last reactor presents a high similarity with the initial fecal sample, at the microbiological diversity level. Based on our results, this in-vitro process improvement is a valuable alternative tool to dynamically study the structure and metabolism of gut microbiota, and its response to nutrients, prebiotics, probiotics, drugs or xenobiotics.

1. Introduction

The human gut microbiota is constituted of thousands of different microbes. It reaches 10^{12} microbes per g of fecal content and has been labeled as the "forgotten organ" (O'Hara and Shanahan, 2006). This community presents many properties, some of which are metabolic, and not only contributes to the digestion of nutritional compounds but is also relevant to the host's health and well-being. This importance led to an increase study of this ecosystem in recent years to gain a better knowledge of its structure and function. However, until the recent development of molecular methods (Brugère et al., 2009; Fraher et al., 2012), it has been difficult to study the intestinal microbiota as less than 20% of the microbiota communities were estimated cultivable (Eckburg et al., 2005). Recent advances in high throughput techniques helped to renew our vision of its diversity and dynamics towards its hosts (Arumugam et al., 2011; Eckburg et al., 2005; Qin et al., 2010; Tap et al., 2009). Data also indicated important qualitative and quantitative differences among the bacteria recovered from different segments of the digestive tract and the fecal matter, and along the colon itself (Eckburg et al., 2005; Stearns et al., 2011). Genomic and metagenomic data have already helped understanding potent metabolic interactions and functionalities (Qin et al., 2010) but ethical as well; technical questions limit in vivo functional studies in human. Several in-vivo/in-vitro approaches have been developed to overcome these limitations: In vivo studies on humanized gnotobiotic mice are one of them. The animals can be associated with either a partial (Samuel and Gordon, 2006) or complete human microbiota (Goodman et al., 2011; Turnbaugh et al., 2009). However, monitoring of the important number of required animals or of all the different parameters involved in the studies, due to individual variations, can be difficult and labor intensive. A second approach to those limitations is the development and use of in-vitro simulations of the human gut. Several contributions have already been made from simple batch culture conditions (Kim et al., in press) to more complex apparatus, for example the TNO in vitro models (TIM 2), replicating the proximal colon in a single compartment fermenter (Minekus et al., 1999; Rajilić-Stojanović et al., 2010), a 3-stage continuous culture system (Macfarlane and Macfarlane, 2007; Payne et al., 2012) and the SHIME system which replicates the whole gastro-intestinal tract (Molly et al., 1993; Van den Abbeele et al., 2010). One of the major disadvantages of these systems is the potent variability of the inoculums with time, and therefore the difficulty in obtaining reproducibility between experiments and laboratories. The LabMET of the Ghent University recently developed the TWINSHIME, an in vitro system permitting two experiments to be performed at the same time,

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for example a control versus a test (Eeckhaut et al., 2008; Van den Abbeele et al., 2010). Other possibilities rely on the use of a mix of several fresh fecal matters (but this hides individual microbial variability) or of an in-vitro mix of several bacterial representatives of the gut microbiota. We recently proposed an alternative applying an initial amplification of a gut microbiota originating from fresh fecal matter using a batch fermentation process (Feria-Gervasio et al., 2011). The resulting microbiota were sampled and stored, in order to get a stock of similar inoculums. Even if freezing is controversial as it may artificially select micro-organisms, this approach provided similar results with a ten-months delay in experiments performed in a single continuous fermentation system called P-ECSIM (Proximal-Environmental Control System for Intestinal Microbiota) simulating the environmental conditions of proximal human colon (Feria-Gervasio et al., 2011). This system can also be used with three reactors working in parallel (Brugère et al., 2011). In vitro models mimicking the colon microbiota, which have been developed and validated for more than 20 years (Gibson et al., 1988), are valuable tools to study the ecology and metabolism of the gut bacteria when applying probiotics, prebiotics, chemicals or pathogens (Macfarlane et al., 2005; Tzortzis et al., 2005).

However, the in-vitro systems developed to date are all under anaerobia obtained and maintained throughout the experiments by a continuous flush of N_2 or CO_2. These gases are known to have an influence on the bacterial metabolism and fermentation yields, while other generated by some population of a typical gut microbiota impact the disposal of some metabolites for other populations: for example, hydrogenotrophic organisms (encompassing acetogens, sulfate-reducing bacteria (SRB) and methanogens in the human colon) limit H_2 partial pressure originating from the bacterial fermentations that would otherwise restrict fermentations due to thermodynamically unfavorable conditions (review in Nakamura et al., 2010). Also, at least two different hydrogenotrophic gut populations are dependent on CO_2 (acetogens, methanogens). These mutual benefits lead to higher fermentation yields in the human gut. Our recently developed P-ECSIM provides the paradigm that anaerobia may be maintained in in-vitro systems by the gases produced by the microbiota itself, therefore leading to a one-reactor model believed to fit more closely to in vivo conditions (Feria-Gervasio et al., 2011).

Here, based on three-segment continuous fermentative systems, we propose an improvement, compared to other in vitro systems, with the development of a process to perform the fermentation under an anaerobia produced by the microbiota itself, admitting this would more closely mimic the colon microbiota environment. Evaluation of these objectives was achieved using a unique stored stool-derived inoculum by several techniques enabling to assess the microbial diversity (microbial culture, molecular fingerprint and phylogenic DNA microarray) and metabolic function (gas, SFCA, LC–MS based metabolic fingerprint).

2. Materials and methods

2.1. 3S-ECSIM procedure

The fermentation medium used was derived from other studies (Gibson et al., 1988; Molly et al., 1993) and has been described elsewhere (Feria-Gervasio et al., 2011). This fecal aliquot was initially obtained from a healthy female volunteer (29 years old), without recent treatment with antibiotics and not being a breath methane-producer. For 3S-ECSIM experiments, one 2-mL fecal aliquot with 30% (v/v) glycerol (Feria-Gervasio et al., 2011) was unfrozen on ice and used to inoculate a 5-mL preculture of fermentation medium, grown at 37 °C for 10 h. It was successively transferred for 15 h into a 1-L Erlenmeyer flask containing 95 mL of fermentation medium and into a 2-L bioreactor (GPC—Global Process Concept Inc., France) previously N_2 flushed containing 900 mL of fermentation medium for a 24 h batch culture at 37 °C, pH = 5.7 and 400 rpm. The same operations were used for each of the three-stage bioreactors R1 (proximal), R2 (transversal) and R3 (distal) with operating volume of 1 L for each (Fig. 1). After the 24 h batch condition, the 3S-ECSIM continuous culture was launched with operative conditions indicated in Table 1: Retention Time (RT) was calculated as the reciprocal of the dilution rate as follow: $R\ (h) = 1/D\ (h^{-1})$; where: R is the retention time in hours; D represents the dilution rate in h^{-1}. The system retention time constitutes the sum of individual R values in each bioreactor (Table 1), and corresponds to a typical human transit time of 48 h. Bioreactors R1 was continuously fed using a multi-head peristaltic pump (Watson Marlow, model 205U). Additional multi-head peristaltic pumps were used to transfer the medium from R1 to R2 and finally from R2 to R3. The fermentation volume was controlled by adjusting the height of an additional evacuation tube (particularly in R1). The temperature was controlled at 37 °C and the pH was controlled by an addition of NaOH 2M. Probes allowed to measure these parameters continuously and activated pumps to maintain the temperature and pH of the bioreactors. The culture pH in the three bioreactors was 5.7 (R1), 6.2 (R2) and 6.8 (R3). For each bioreactor the stirring rate was adjusted at 400 rpm. No gases were flushed during the course of the continuous culture, the anoxic environment being progressively and dynamically enriched by the gases originating directly from the microbiota metabolism. For each bioreactor, a stabilization phase corresponding to five RTs was performed in order to reach the steady state. Overall, the stabilization phase lasted 240 h. Samplings were performed in each reactor over a 48-h period, for microbial and biochemical analyses (total of 6 samples per condition). The acquisition and control software C-BIO was used for the batch and culture continuous conditions. The oxidation/reduction potential was monitored every 5 min using an Argenthal reference probe from Mettler Toledo (Impro® 3253i/SG/225 probe). Measured redox potentials E were corrected into an Eh value, at 37 °C, by adding + 198.32 mV.

2.2. Microbial analyses

2.2.1. Cultural method

Microbiota growth was evaluated by spectrophotometry (620 nm, Beckman® Coulter DU 640B spectrophotometer) and dry weight measurements. The biomass was measured on 5 mL of culture that was first centrifuged (13,000 ×g, 10 min), then the pellet was washed three times with distilled water before being deposited under vacuum and dried at 104 °C onto a pre-weighted membrane (Polyamid 0.45 μm, Sartorius). All other microbiological procedures were performed under strict anaerobic conditions as previously described (Bearne et al., 1990; Feria-Gervasio et al., 2011). Briefly, the solid bacterial media were the Wilkins–Chalgren agar for total anaerobes and total facultative anaerobes (Allison et al., 1989; Bearne et al., 1990), Bacteroides mineral salt for *Bacteroides* spp. (Allison et al., 1989), Beerens agar for bifidobacteria (Beerens, 1991), Reinforced Clostridial Medium for *Clostridium* spp., MacConkey agar for enterobacteria and Rogosa agar for lactobacilli (Mitsuoka and Hayakawa, 1973). For each counting, triplicate plates were inoculated with 0.1 mL of each dilution and incubated at 37 °C for 5 (facultative anaerobes) or 7 days (total anaerobes).

2.2.2. Fingerprinting method

Microbial community analyses were performed first by an original Ribosomal Intergenic Spacer Amplification (RISA) procedure: DNA was extracted from a 0.250 mL sample (Yu and Morrison, 2004). The molecular fingerprints due to polymorphic length of amplicons obtained using primers ITSF/ITSReub (Cardinale et al., 2004) were determined by capillary electrophoresis on the Bioanalyzer 2100 system using the DNA 12000 kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's instructions.

2.2.3. Phylogenetic microarray (HuGChip) analysis

An exhaustive community analysis was also performed at the phylum and family levels using the HuGChip, an explorative phylogenetic microarray designed to trigger 66 bacterial families usually recovered