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Real-time monitoring of HT29 epithelial cells as an *in vitro* model for assessing functional differences among intestinal microbiotas from different human population groups

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ABSTRACT

Several *in vitro* screening tests have been used for selecting probiotic strains; however they often show low predictive value and only a limited number of strains have demonstrated functionality *in vivo*. The most used *in vitro* tests represent a very simplified version of the gut environment, especially since they do not consider the accompanying microbiota. Therefore, there is a need to develop sensitive and discriminating *in vitro* models including the microbiota. Here we developed an *in vitro* model to discriminate among microbiotas/fecal waters from different population groups. To this end samples were obtained from seven healthy adults, five IBD-patients, ten full-term and ten preterm newborns. Fecal microbiotas were purified and their impact, as well as that of the fecal waters, on HT29 cells was continuously monitored for 22 h using a real-time cell analyzer (RTCA). The composition of the purified microbiotas was assessed by 16S rRNA gene profiling and qPCR and the levels of short chain fatty acids (SCFA) determined by gas chromatography. The microbiota fractions and SCFA concentrations obtained from IBD-patients, full-term and preterm babies, showed clear differences with regard to those of the control group (healthy adults). Moreover, the purified intestinal microbiotas and fecal waters also differed from the control group in the response induced on the HT29 cells assay developed. In short, we have developed a real-time, impedance-based *in vitro* model for assessing the functional response induced by purified microbiotas and fecal waters upon intestinal epithelial cells. The capability of the assay for discriminating the functional responses induced, by microbiotas or fecal waters from different human groups, promises to be of help on the search for compounds/strains to restore the functionality of the microbiota-host's interaction.

1. Introduction

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Probiotics have been included in functional food products for over three decades and several different *in vitro* screening tests have been used to select the strains with better potential (Vinderola et al., 2017). Among the most frequently used tests, the tolerance to the simulated gastrointestinal transit and the adhesion to epithelial cells and/or mucus have been used as predictors of survival and transient colonization of the intestine. The co-cultivation of probiotics with eukaryotic cells (epithelial and/or immune cells) has been applied to assess the response of the gastrointestinal and immune system of the host to the probiotic, whereas co-culture of probiotics with pathogens has been used for the selection of strains with anti-pathogenic properties. However, the *in vitro* tests available often display low predictive value of the actual *in vivo* performance of the probiotic, with several examples of strains performing well *in vitro* but showing poor *in vivo* performance (Vinderola et al., 2017). A reason for this limited predictivevalue of the *in vitro* functionality tests may reside on their excessively simplified version of the gut environment, especially regarding the lack of consideration about the influence of the accompanying microbiota. As a consequence, despite the large number of *in vitro* studies suggesting the functional potential of different

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microbial strains, only for a very limited number of them the functionality has been successfully demonstrated in clinical intervention studies. This lack of studies substantiating functionality together with the strict application of the European Regulation on Nutrition and Health Claims on Foods (EC 1924/2006) has resulted on rejection for most of the probiotic applications evaluated by the European Food Safety Authority (Van Loveren et al., 2012).

The effects exerted by intestinal microbes are likely to be dependent on the balance among the different microorganisms, rather than on any specific bacteria; nevertheless the influence of complex microbiotas on the response of the eukaryotic cells remains largely unknown. This has attracted the attention of researchers towards the development of *in vitro* models based on the co-cultivation of epithelial cells with the complex microbiota present in the human gut. To this regard, co-cultivation of primary human colonocytes with the fecal homogenate from a healthy volunteer has been recently used for assessing the transcriptional response of the epithelial cells (Richards et al., 2016). Moreover, the effect of the infant microbiota, co-cultivated or not with different probiotic strains, upon the production of cytokines by the intestinal HT29 cell line has also been evaluated (Arboleya et al., 2015a,b). Interestingly, in this work a high inter-individual variability on the response was observed, also being evident that co-cultivation of the HT29 cells with a pure culture of the probiotic induced much lower cytokine production than co-cultivation with the complex microbiota treated with the probiotic. Indeed, the marked inter-individual and inter-population variability on the composition of the gut microbiota may be an important factor explaining the limited correlation found between *in vivo* and *in vitro* studies (Vinderola et al., 2017). Moreover, the lack of consideration of this microbiota variability may further limit the predictive capability of the tests. Therefore, there is a need to explore more sensitive and discriminating *in vitro* models, considering the complexity of the microbiota, in order to develop screening tests with a higher ability to predict the *in vivo* response to probiotics and microbiotas.

In the present work we aimed at assessing the capability of an *in vitro* model, based on the co-cultivation of human intestinal epithelial cells with purified microbiotas and fecal waters from different human population groups, in order to discriminate among these groups' microbiotas on the basis of the functional response induced in HT29 cells.

2. Material and methods

2.1. Volunteers and fecal samples collection

Fecal samples were obtained from seven healthy adults (34–57 years-old) with unrestricted diet who had not taken antibiotics during the previous two months, and from five subjects (29–54 years-old) diagnosed with inflammatory bowel disease (IBD). Moreover, samples were taken from twenty 2–4 days-old infants, ten of them were healthy vaginally delivered full-term breastfed babies and ten were preterm newborns (gestational age $<$ 32 weeks). All subjects were recruited and the feces collected at the Asturias Central University Hospital (HUCA, Asturias, Spain). The study was approved by the Regional Ethical Committee of Asturias Public Health Service (SESPA) and an informed written consent was obtained from each adult volunteer or from the infant's parents.

Fresh samples were collected, immediately placed in an anaerobic jar filled with Anaerocult A (Merck, Darmstadt, Germany) and transported to the laboratory within 2 h of collection. A 1/10 w/v dilution in sterilized NaCl (0.9% w/v) solution was prepared and samples were homogenized in a Lab-Blender 400 stomacher (Seward Medical, London, UK) at full speed for 5 min. The homogenate was centrifuged at 16,000 $\times g$ at 4 °C, for 10 min and the resulting fecal supernatant was used to monitor the interaction with the HT29 cell line and for the determination of short chain fatty acids (SCFA) levels. For interaction with the cell line analyses 1 mL of the supernatant was filtered through a 0.25 μm pore-size filter and the filtrate, namedfecal water, was stored at −20 °C. Another 10 mL of the homogenate were used for microbiota purification using a Nycodenz® (PROGEN Biotechnik GmbH, Heidelberg, Denmark) gradient method (44%, w/v) as previously described (Hevia et al., 2015).

Aliquots from purified microbiotas were stored at −80 °C for microbiota analyses. In the remaining purified microbiota fraction the number of bacteria was determined using a Neubauer improved camera (Blau Brand, Germany) in an optical microscope (Olympus BH2, Olympus Iberia SA, Barcelona, Spain). The level of bacteria was then adjusted to 1×10^8/mL with PBS and microorganisms were then inactivated by exposing the suspensions to UV light (15 W, Selecta, Barcelona, Spain) by five consecutive times for 30 min each before confirming inactivation by plate counting in GAM medium (Nissui Pharma Co, Tokyo, Japan).

2.2. Quantification of intestinal microbial groups in purified fecal microbiotas

DNA was extracted from 100 μL of the purified microbiota fraction by using the QIAamp DNA stool kit (Qiagen GmbH, Hilden, Germany) as previously described (Arboleya et al., 2012) and was stored at −20 °C until use.

The extracted DNA was used for the assessment of the microbial populations by 16S rRNA Gene Sequence-based microbiota analysis. In brief, partial 16S rRNA gene sequences were PCR-amplified using previously described primers (Milani et al., 2013) and the amplicons were sequenced in a MiSeq (Illumina) platform by GenProbio srl (Parma, Italy). The individual sequence reads obtained were filtered, trimmed and processed as described by Nogacka et al. (2017). 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥97% sequence homology using uclust (Edgar, 2010). All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the SILVA database (Quast et al., 2013).

2.3. Analysis of fecal microbial groups by quantitative PCR

The levels of *Bacteroides-Prevotella-Porphyromonas* group, *Faecalibacterium*, *Bifidobacterium*, *Lactobacillus*-group, *Staphylococcus*, *Akkermansia*, *Enterobacteria*, *Clostridium* XIVa group as well as of total bacteria, were determined by quantitative PCR (qPCR) using previously described primers, standard strains and conditions (Arboleya et al., 2012; Valdes et al., 2017). All samples were analyzed by duplicate in two independent PCR runs.

2.4. Determination ofSCFA levels in fecal water

The analysis of SCFA was performed by gas chromatography in order to determine the concentrations of acetate, propionate, isobutyrate, butyrate and isovalerate. Cell free-supernatants (0.250 mL) from fecal dilutions prepared as indicated former were mixed with 0.3 mL methanol, 0.05 mL internal standard solution (2-ethylbutyric 1.05 mg/mL), and 0.05 mL 20% formic acid. This mixture was centrifuged and the supernatant obtained was used for quantification of SCFA by GC in a system composed of a 6890NGC injection module (Agilent Technologies Inc., Palo Alto, Ca, USA) connected to a flame injection detector (FID) and a mass spectrometry (MS) 5973N detector (Agilent), as described previously (Arboleya et al., 2016). Samples were analyzed by triplicate.

2.5. Intestinal epithelial cell line HT29 culture conditions

The intestinal cell line HT29 (ECACC 91072201) from human colon adenocarcinoma, was purchased from the “European Collection of Cell Cultures” (Salisbury, UK) and stored at IPLA-CSIC under liquid N_2. McCoy's Medium (MM) supplemented with 10% fetal bovine serum (FBS), 3 mM L-glutamine and a mixture of antibiotics (50 μg/mL