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Simple coculture system shows mutualism between anaerobic faecalibacteria and epithelial Caco-2 cells

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ABSTRACT

Most gut bacteria are obligate anaerobes and are important for human health. We developed a simple coculture system for oxygen-requiring intestinal Caco-2 cells and the anaerobic symbiont *Faecalibacterium prausnitzii*. In 18-36 h-cocultures, coverslip-attached Caco-2 cells promoted growth and metabolism of *F. prausnitzii* present in agar broth, while *F. prausnitzii* suppressed inflammation and oxidative stress in Caco-2 cells. This uniquely establishes host-microbe mutualism of a beneficial gut microbe *in vitro*. 
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**INTRODUCTION**

The human gut microbiome is increasingly recognized as an important determinant for human health, affecting a variety of gut, metabolic, neurological and psychological disorders. Gut microbiota provide essential nutrients and anti-inflammatory compounds to the host and confine expansion of pathogens. High-throughput sequencing techniques have uncovered the high complexity of the gut microbiome and the composition changes during ageing and disease. The healthy gut microbiome contains 500-1,000 different bacterial species and their collective genomes (metagenome) encode at least a 100-fold more genes compared to the human genome. Only a small fraction of these bacterial species are cultured in vitro and even more challenging is to coculture gut bacteria and human cells. The main obstacle in a host-microbiome coculture system is that most (>90%) gut bacteria are obligate anaerobes that die instantly when exposed to atmospheric conditions (21% O₂), while human cells depend on oxygen.

*Faecalibacterium prausnitzii* is an obligate anaerobe that may represent up to 25% of the bacteria in the healthy gut. *F. prausnitzii*-excreted products and cell extracts suppress inflammatory signaling in intestinal epithelial (Caco-2) cells in vitro, as well as in 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis in mice in vivo. Moreover, human intestinal inflammation is associated with decreased numbers of *F. prausnitzii* and predisposes for post-operative ileal recurrence of Crohn’s disease. Among anaerobes, *F. prausnitzii* has the unique ability to grow close to the intestinal epithelium in the oxic-anoxic interphase of the gut. However, it remains elusive whether direct mutualism exists between this gut microbe and intestinal epithelial cells.

Thus, we set out to develop a coculture system for oxygen-requiring human gut epithelial (Caco-2) cells and an anaerobic gut bacterium (*F. prausnitzii*). This “Human oxygen-Bacteria anaerobic” (HoxBan) system establishes coculturing of glass-adherent human cells in liquid medium and anaerobic bacteria in solid agar broth for over 24 h and allows the analysis of cell growth, transcriptome and exo-metabolome of cocultured cells. A detailed protocol is given in the supplementary/online section. In short, hand warm (~40°C) agar broth was inoculated with *F. prausnitzii* starter cultures and aliquots of 40 ml were allowed to solidify in 50 ml Falcon centrifugation tubes. Caco-2 cells grown on coverslips were placed up-side-down on top of the *F. prausnitzii*-containing agar broth and overlaid with DMEM medium. The HoxBan coculture tubes (schematically drawn in Figure 1A) were placed either with a loose (air-open) or tightly-closed cap (air-closed) in a standard humidified incubator at 37°C, 5% CO₂ and atmospheric O₂. *F. prausnitzii* colony formation, transcriptional adaptations of Caco-2 cells and excreted metabolites in the liquid medium were analyzed after 18-36 h of coculture.

Within 18 h, *F. prausnitzii* formed colonies throughout the 40 ml YCFAG-agar. In the absence of Caco-2 cells, no *F. prausnitzii* colonies formed in the top agar layer, most likely
due to penetration of intolerable amounts of oxygen (Figure 1B, left 2 panels). In contrast, clear and bigger *F. prausnitzii* colonies appeared close to the coverslip-attached Caco-2 cells, both in air-closed and air-open tubes (Figure 1B, 2 panels on the right). Notably, *F. prausnitzii* continued to expand over a total coculture time of 36 h (Figure 1B-bottom panels). In contrast to Caco-2 cells, HepG2 (human hepatoma) cells did not stimulate *F. prausnitzii* growth, indicating that this effect is cell type-specific (Figure 1C). Caco-2 cells were viable and actively dividing even after 24 h of (co)culturing in the HoxBan system (Figure 1D).

Caco-2 cells harvested after 18 h culture with and without *F. prausnitzii* revealed that both IL-1β and iNOS mRNA levels were significantly reduced (p<0.05) in Caco-2-*F. prausnitzii* cocultures compared to Caco-2 mono-cultures (Figure 1E). A similar effect was observed for the oxidative stress marker heme oxygenase 1 (HO-1; Fig. 1E), indicating that expansion of *F. prausnitzii* close to the Caco-2 cells has both anti-inflammatory and anti-oxidant effects.

**Figure 1.** HoxBan coculturing of Caco-2 cells and *F. prausnitzii*. A) Schematic drawing of the HoxBan coculture system with *F. prausnitzii* growing in solid agar broth overlaid with liquid DMEM medium and Caco-2 cells on coverslips facing the agar. B) Pictures documenting *F. prausnitzii* colony formation in the absence and presence of Caco-2 cells after 18 h (top panel) and 36 h (bottom panel) in air-open and air-tight culture tubes. C) *F. prausnitzii* colony formation after 18 h coculture with HepG2 cells (left) or Caco-2 cells (right). D) Ki-67 staining of the Caco-2 cells after 24 h monoculture (top panel) or HoxBan coculture with *F. prausnitzii* (bottom panel). E) Comparison of mRNA levels of IL-1β, iNOS and HO-1 in Caco-2 monocultures and Caco-2-*F. prausnitzii* cocultures after 18 h.
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To obtain a comprehensive overview of the mutual metabolic effects of *F. prausnitzii* and Caco-2 cells, we performed a metabolome analysis on the liquid medium after 18 hours of (co)culturing Caco-2 cells and/or *F. prausnitzii*, which included short chain fatty acids (SCFAs), hydrocarbons, lipids and amino acids (Supplementary Figure 1A). Systematic exo-metabolic changes occurring in different culture condition were assessed using principal component analysis (PCA) (Fig. 2A). The first principal component (PC1) accounts for 49.7% of the total variance and separates the Liquid HoxBan culture medium and the Caco-2 monoculture from the *F. prausnitzii* monoculture and the two Caco-2-*F. prausnitzii* cocultures. This indicates that *F. prausnitzii* has the strongest effect on the level of metabolites in the culture medium. Main determinants of PC1 are the SCFAs butyrate and formate that are produced by *F. prausnitzii* (Fig. 2B and C). Other metabolites that associate with PC1 are the essential amino acid methionine and the amino acid-derivative N-acetyl aspartate. Concentrations of all these metabolites are enhanced in HoxBan cultures with *F. prausnitzii*. The second principal component (PC2) accounts for 16.8% of the variance in the data and separates the *F. prausnitzii* monoculture from the two cocultures. Metabolites that contribute to PC2 are formate, adenine and inosine (Fig. 2C, D and E). *F. prausnitzii* (strain A2-165 used in these studies) produces equimolar amounts of formate and butyrate under anaerobic conditions similar to those in the HoxBan system 11. Formate levels significantly increased after coculturing *F. prausnitzii* with Caco-2 cells, which is in line with the enhanced bacterial biomass under these conditions. However, butyrate levels did not change upon coculturing *F. prausnitzii* with Caco-2 cells. Butyrate is a preferred energy source of intestinal epithelial cells12 and our data suggests that Caco-2 cells consume part of the butyrate produced by *F. prausnitzii*. In turn, this may contribute to the suppression of inflammatory and oxidative stress markers in Caco-2 cells (Figure 1E).

Together with adenine and inosine, also concentrations of xanthosine and 5-methylthioadenosine were strongly reduced in medium of Caco-2-*F. prausnitzii* cocultures compared to the two monocultures (Fig. 2D; Supplementary Fig 1B and C). These compounds of purine metabolism are required for DNA (cell proliferation) and ATP (energy) synthesis. Their depletion from the medium is likely due to the stimulated growth of *F. prausnitzii*, although a pro-proliferative effect on Caco-2 cells cannot be excluded at this point. On PC2, a slight separation between the open and closed HoxBan Caco-2-*F. prausnitzii* cocultures was observed (Fig. 2A), however, this could not be attributed to significant changes in single metabolites. This indicates that even in the closed condition, 10 ml of DMEM medium carries sufficient oxygen to support the growth of the coverslip-attached Caco-2 cells, which is further supported by the suppressed expression of hypoxia-sensitive HO-1 in both coculture conditions (Figure 1E)13. These data show that coculturing Caco-2 cells with *F. prausnitzii* leads to a unique profile of excreted and consumed
metabolites that is not simply the cumulative result of the individual cell types, indicating that these cells modify each other’s metabolism.

Figure 2. Exo-metabolomic analysis of HoxBan mono- and coculture medium. A) Principle component analysis of the metabolites in the medium after 18 h culture. PC1 differentiates between cultures with or without F. prausnitzii. PC2 differentiates between F. prausnitzii monocultures and the two caco-2- F. prausnitzii cocultures. B-E) Normalized concentrations of butyrate (B), formate (C), adenine (D) and inosine (E) in media after the indicated culture conditions.

Thus, the HoxBan coculture system presented here demonstrates for the first time mutualism between oxygen-requiring intestinal epithelial cells (Caco-2) and an obligate anaerobic gut bacterium (F. prausnitzii) while both cell types are viable. Several other systems have been developed to study the interaction between human cells and F. prausnitzii and attributed anti-inflammatory features to this bacterium. However, these systems did not allow the analysis of the effect of human cells on F. prausnitzii, which were evidently observed in the HoxBan system. The simplicity of the HoxBan coculture system lies in the use of solid agar medium for growth of the anaerobic gut bacteria, overlaid with liquid medium exposed to air for human cells. The HoxBan is a robust system that can be implemented in almost any molecular biology research laboratory with access to an anaerobic facility and a tissue culture cabinet and incubator. As such, it has great potential to support research to understand the communication between gut microbes and their host. The HoxBan system is readily adaptable to coculture any other anaerobic gut bacterium, as well as complex mixtures of bacteria, with adherent and potentially also non-adherent- human cell lines or primary cells. Applications of the HoxBan coculture system for other cells than Caco-2 and/or F. prausnitzii may require specific optimization in
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culture conditions, but it holds the universal principle of coculturing oxygen-requiring human cells together with obligate anaerobic bacteria that will foster our understanding of the role of gut bacteria in human health and disease.

REFERENCES:
Supplementary Figure S1. A) Hierarchical clustering analysis (HCA) of the top 25 metabolites ranked by the Anova Test. B-D) Normalized concentrations of xanthosine (B) and 5-methylthioadenosine (5-MTA) (C) in media after the indicated culture conditions.

Supplementary Table S1

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SUPPLEMENTARY METHODS SECTION, including HoxBan coculture protocol for Caco-2 and *F. prausnitzii*.

MATERIAL AND METHODS

Protocol for “Human Oxygen - Bacteria anaerobic” (HoxBan) coculture of Caco-2 cells and *F. prausnitzii*

**Preculture of *F. prausnitzii***:

Frozen bacterial stocks were prepared by 1:4 mixing of glycerol (85%) with liquid cultures (optical density at 600nm (OD₆₀₀) between 1.0-1.5) of anaerobically-grown *F. prausnitzii* strain A2–165 (DSM 17677) at 37°C in yeast extract, casitone, fatty acids, acetate and glucose (YCFAG) medium¹,² and stored at -80°C. Five (5) µl of a *F. prausnitzii* glycerol stock was used to inoculate 5 ml liquid YCFAG medium and incubated for 14-16 hours at 37°C in an anaerobic incubator until an OD₆₀₀ of approximately 0.8.

YCFAG medium used in this study consisted of (all concentration per liter) casitone (10.0 g), glucose (4.52 g), NaHCO₃ (4 g), CH₃COONa (2.7 g), K₂HPO₄ (0.45 g), KH₂PO₄ (0.45 g), NaCl (0.9 g), MgSO₄·7H₂O (0.09 g), CaCl₂·2H₂O (0.12 g), resazurin (1 mg), hemin (10 mg), biotin (10 μg), cobalamin (10 μg), p-aminobenzoic acid (30 μg), folic acid (50 μg) and pyridoxamine (150 μg). Medium was boiled while flushing constantly with CO₂ and afterwards yeast extract (2.5 g) and cysteine (1 g) were added. Furthermore, short-chain fatty acids (SCFA) were added: propionate (90 mM); isobutyrate, isovalerate and valerate (10 mM each) (final concentrations). The pH of the YCFAG medium was adjusted to 6.5 – 7 using *MeterLab* pH meter (Radiometer Analytical-France). The medium was autoclaved and filter-sterilized solutions of heat labile thiamine and riboflavin were added afterwards to give the final concentrations of 0.05 μg ml⁻¹ of each.

**Preculture of Caco-2 cells**:

Human colon adenocarcinoma Caco-2 cells (~ passage 40) were cultured in a humidified incubator at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and Non-Essential Amino Acids (Gibco® MEM) (1 ml / 100ml) in T75 tissue culture flasks (Sigma). Caco-2 cells were seeded at ~50% confluency in 12-well plates containing coverslips and incubating for 48 hours to a confluence of 80-90%. Fresh pre-warmed DMEM medium without antibiotics was added 1 hour prior to transferring the Caco-2-containing coverslips to the HoxBan culture tubes.

**Protocol for the HoxBan coculture**:

*Starting in the anaerobic chamber*: One (1) mL of the overnight *F. prausnitzii* preculture was used to inoculate 1000 mL of freshly-autoclaved and cooled-down (-40°C) YCFAG broth.
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containing 1.5% agar. Aliquots of 40 ml of this inoculum were transferred to sterile 50 mL falcon tubes and allowed to solidify in 30 minutes. The F. prausnitzii-inoculated Falcon tube cultures were transferred to a tissue culture cabinet at ambient air and Caco-2 cells on coverslips were placed (up-side-down) on top of the agar and overlaid with 10 ml pre-warmed (37°C) DMEM medium (without antibiotics), after which the co-cultures were placed in a humidified incubator at 37°C and 5% CO₂ for 18-36 hours. The screw caps of the Falcon tubes were either tightly closed (to maintain maximum anaerobic conditions for F. prausnitzii) or kept loosely tightened (to allow oxygen exposure for Caco-2 cells). Control conditions were: 1) YCFAG-agar without F. prausnitzii inoculum; 2) F. prausnitzii-inoculated cultures with coverslips without Caco-2 cells and 3) F. prausnitzii-inoculated cultures with coverslips containing human HepG2 cells (growth conditions detailed below). Experiments were performed 3 times (N=3) in triplicate tubes for each condition, with a total of 9 tubes per condition.

F. prausnitzii growth rim visualization: Visualization of F. prausnitzii colony formation in the agar broth close to the Caco-2 containing coverslips was performed using a digital camera (Canon EOS 450D) and the obtained images were processed using Digital Photo Professional software (Canon) without any qualitative and quantitative changes to the raw images.

Harvesting of Caco-2 cells and analysis (Q-PCR and ki-67 staining):

At the end of the coculture experiment, Caco-2-containing coverslips were removed from the HoxBan coculture tubes and total RNA was isolated using Trizol according to the suppliers protocol (Sigma-Aldrich). RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription polymerase chain reaction (Rt PCR) was performed as described 3. Quantitative PCR (qPCR) for the inducible isoform of nitric oxide synthase (iNOS-NOS2), interleukin-1 beta (IL-1β) and heme oxygenase 1 (HO-1) were performed. Primers (Invitrogen) and probes (Eurogentec) were designed using Primer Express 2.0 software (Applied Biosystems). Details of primers and probes are given in Supplementary Table S1. Q-PCR conditions were as described by Blokzijl et al 3, except that 1 ng of undiluted complementary DNA was used. Fluorescence was measured using 7900 HT Fast Real-Time PCR system (Applied Biosystems). Each sample was analyzed in duplicate by ABI PRISM Sequence Detector software, version 2.1. Expression of the gene of interest was normalized to 18S 4. Cell proliferation was assessed by a nuclear Ki-67 staining using a rabbit polyclonal antibody directed against Ki-67 (dilution 1:1000 60 minutes at 25oC) based on manufacturers protocol (Monosan; Netherlands).
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**Metabolome analysis by Liquid Chromatography-Mass Spectrometry:**
At the end of the (co)culture experiments the liquid medium on top of the HoxBan cultures was collected (~10 ml) and polar metabolites were analyzed and quantified by ultra-performance liquid chromatography (UPLC Acquity, Waters, Manchester, UK) coupled in line with a quadrupole-time-of-flight hybrid mass spectrometer (Synapt G2, Waters, Manchester, UK) as previously reported\(^5\). All materials used in the UPLC-MS experiments were purchased from Sigma-Aldrich (Germany) and were of analytical grade or higher purity. For the analysis of targeted metabolites, data were processed using TargetLynx (Waters) while for untargeted analysis MarkerLynx (Waters) was used to integrate and align MS data points and convert them into exact mass retention time pairs. The identity of metabolites was established by comparison of accurate mass measurements and tandem mass spectrometry information against our in-house database and/or online databases\(^6,7\).

**Short Chain Fatty Acid analysis:**
Chromatography was used for short chain fatty acid (SCFA) analysis including lactate, formate, butyrate and acetate. A HPLC Ion Chromatography system (Metrohm AG, Herisau, Switzerland) with a conductivity detector was used as described before\(^8\).

**Statistics:**
Principal Component Analysis (PCA) were performed on all detected metabolites and SCFAs by using MetaboAnalyst\(^9\). Before PCA, data was normalized by the sum, log transformed and then scaled by using pareto scaling.

One-way Anova test was used to find metabolites that were significantly different between groups. Hierarchical Clustering Analysis (HCA) was then performed by using MetaboAnalyst\(^9\) on the top 25 metabolites ranked by the Anova Test. The similarity measure was obtained by applying the Spearman's rank correlation. The clustering algorithm used was the Ward's linkage. Differences in the gene expressions were assessed by using the Mann–Whitney U test, tests were two-tailed and p-values of 0.05 or lower were considered significant. Tests were performed with PASW Statistics 22 (SPSS, USA).

**REFERENCES:**
2. Lopez-Siles M, Khan TM, Duncan SH, Harmsen HJ, Garcia-Gil LJ, Flint HJ. Cultured representatives of two major phylogroups of human colonic Faecalibacterium prausnitzii
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