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J. Agric. Food Chem., Just Accepted Manuscript • DOI: 10.1021/jf402137r • Publication Date (Web): 05 Sep 2013
Downloaded from http://pubs.acs.org on September 11, 2013

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A dried yeast fermentate selectively modulates both the luminal and mucosal gut microbiota and protects against inflammation, as studied in an integrated in vitro approach\textsuperscript{1,2}

Possemiers Sam\textsuperscript{a}, Pinheiro Iris\textsuperscript{b}, Verhelst An\textsuperscript{b}, Van den Abbeele Pieter\textsuperscript{a}, Maignien Lois\textsuperscript{a}, Laukens Debby\textsuperscript{c}, Reeves Stuart G.\textsuperscript{d}, Robinson Larry E.\textsuperscript{d}, Raas Thomas\textsuperscript{e}, Schneider Yves-Jacques\textsuperscript{e}, Van de Wiele Tom\textsuperscript{a,*} and Marzorati Massimo\textsuperscript{a}

\textsuperscript{a} Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, B-9000 Ghent, Belgium

\textsuperscript{b} ProDigest BVBA, Technologiepark 3, B-9052 Ghent, Belgium

\textsuperscript{c} Department of Gastroenterology, Ghent University, 9000 Ghent, Belgium

\textsuperscript{d} Embria Health Sciences, 2105 SE Creelview Drive, Ankeny, IA 50021, USA

\textsuperscript{e} Institut des Sciences de la Vie, UCLouvain, B-1348, Louvain-la-Neuve, Belgium

*Corresponding author:

Tel: +32(0)92645976; Fax: +32(0)92646248; E-mail: tom.vandewiele@ugent.be

\textsuperscript{1} SP, PVDA, TVDW and MM benefit from a post-doctoral grant from the Research Foundation-Flanders (FWO-Vlaanderen).

\textsuperscript{2} SR and LR are employees of Embria Health Sciences, which markets the supplement discussed in this study. However, Embria Health Sciences had no role in the conception or design of the study and on collection, management, analysis and interpretation of the data. None of the other authors had appointments on any advisory board or financial or personal interests in any organization sponsoring this research at the time the research was done.
Abstract

EpiCor, derived from *Saccharomyces cerevisiae*, has been shown to have immunomodulating properties in human clinical trials and *in vitro*. However, the underlying mechanisms behind its immune-protection via the gut remain largely unknown. Therefore, the aim of this study was to use an integrated *in vitro* approach to evaluate the metabolism of EpiCor by the intestinal microflora, its modulating effect on the gut microbiota and its anti-inflammatory activity on human-derived cell lines. By using the SHIME® model, in combination with a mucus-adhesion assay, we have shown that low doses of EpiCor have a prebiotic-like modulatory effect on the luminal- and mucosa-associated microbiota. These include gradual changes in general community structure, reduction of potential pathogens, quantitative increase in lactobacilli and qualitative modulation of bifidobacteria. Moreover, by combining the SHIME with Caco-2 cells and Caco-2/THP1 co-cultures, we have observed a significant decrease in pro-inflammatory cytokines at the end of the treatment period.

Keywords

Yeast fermentate, SHIME, intestinal bacteria, prebiotic, immune response
Introduction

Due to its unique and continuous surveillance role, the gut associated lymphoid tissue (GALT) represents about 70% of the body's immune system. Actually, the gastrointestinal tract is the site where the need for host defense collides with the need for nutrient absorption, which requires a large contact surface and a thin epithelium. Such structure has the potential to compromise host defenses and therefore the need for extensive immune protection in the gut. Innate and adaptive immune responses to the vast number of dietary and microbial antigens are typically non-inflammatory, favoring a state of immune hypo-responsiveness known as oral tolerance. This is in fact crucial for the maintenance of immune homeostasis 1. However, epidemiological studies performed in industrialized countries show a dramatic increase in morbid conditions associated with chronic inflammation, such as inflammatory bowel diseases (IBD) 2 and metabolic disorders 3. An imbalanced and ongoing activation of the mucosal immune system in the gut is thought to contribute to the etiology of such disorders 4; in addition, is has been described that such patients have a dysfunctional intestinal microbiome 5. Therefore, there is an increasing interest in using specific nutritional interventions that can contribute to ameliorate the luminal microbial environment and to restore the balance on the intestinal immune system.

EpiCor is a natural fermentation product based on the culture of baker’s yeast (S. cerevisiae) under anaerobic conditions and stress in a proprietary medium (henceforth referred to as ‘fermentate’). After fermentation, the entire culture is dried, resulting in a product rich in yeast cell fragments, metabolites and medium components. First observations on immune-protecting properties of this fermentate were made at a fermentation facility in Cedar Rapids, Iowa, where unusually low incidences of influenza infectivity were noted among employees. This may presumably have been related to daily inhalation or ingestion of the yeast fermentate. Since then, a number of studies have been performed, indicating that regular
consumption of this fermentate has the potential to decrease cold/flu-like symptoms\(^6\) and allergic rhinitis-induced nasal congestion\(^7\), and to enhance erythrocyte health and mucosal immune protection\(^8\).

However, despite some previous *in vitro* work\(^9\), the mode-of-action behind this immune protective effect remains largely unknown. The observed increase in secretory immunoglobulin (Ig)A in humans\(^8\) indicates a role for the mucosal intestinal immune system, and these effects may be related to direct interactions with compounds present in the product.

In fact, some products derived from yeast cultures, such as beta-glucans, have been shown to possess immunomodulatory properties\(^10\). Moreover, yeast-based products have been shown to affect the intestinal microbiota\(^11,12\), which in turn is able to modulate host’s immunity\(^1,2\).

To further elucidate the underlying mechanisms involved in the observed immune effects of the fermentate, suitable study models must be used. Whereas human intervention trials and, to a lesser extent, animal studies suffer from a ‘black box’ effect (due to sampling difficulties), well-designed *in vitro* models may offer a useful alternative for mode-of-action studies\(^13\).

Moreover, cell culture models are interesting to study specific cell-compound interactions\(^14\), however, typical cell culture experiments are limited to the evaluation of direct effects of isolated test compounds or fractions. Still, the ultimate effects of food-derived products on the intestinal immune system are often the result of a complex network of processes, involving metabolism of the test compound, modulation of bacterial processes and interaction of the complete intestinal environment with immune pathways. Similarly, dynamic models of the intestine have proven to be useful on the evaluation of specific effects of nutritional interventions on the intestinal microbiota\(^15,16\). Nonetheless, typical gut models are limited to studying luminal intestinal processes, and do not include a host compartment, thereby hampering the study of host-bacteria interactions. Additionally, they do not simulate the mucosa-associated microbiota, a community with an important potential to modulate host
processes, such as immunity\textsuperscript{17}. To overcome this latter limitation, an integrated technology platform was developed for the combined study of intestinal processes, where effects on both luminal and mucus-associated microbiota and effects on immunity are evaluated. After a first batch screening on the fermentability of the yeast fermentate, the dynamic Simulator of the Human Intestinal Microbial Ecosystem (SHIME\textsuperscript{®})\textsuperscript{18,19} was used in combination with a mucus-adhesion assay\textsuperscript{20} and with cell cultures. This allowed studying the long-term \textit{in vitro} effect of the repeated daily intake (4 weeks) of a representative dose (0.63 g/d) of the fermentate on the intestinal microbiota and the resulting protection against inflammation.

\textbf{Materials and Methods}

\textbf{Test products}

The test product used in this study consisted of a dried, modified \textit{Saccharomyces cerevisiae} fermentation product (EpiCor\textsuperscript{®}, Embria Health Sciences, Iowa, USA). Inulin (Fibruline Instant) and oligofructose (Fibrulose F97) were provided by Cosucra (Warcoing, Belgium). Microcrystalline cellulose was purchased from Sigma-Aldrich (Schnelldorf, Germany).

\textbf{Short-term experiment}

Short-term experiments were performed in a standardized batch setup\textsuperscript{21}. Different doses of yeast fermentate (0, 0.5, 1.0, 5.0 and 10 g/L) were first submitted to stomach-simulating digestion at pH 2.0 and to enzymatic breakdown by pancreatic enzymes under small-intestine simulating conditions. Then, the entire digested content was added to 54 mL of sterile basal medium\textsuperscript{22} and transferred to penicillin bottles under sterile conditions. The bottles were sealed and anaerobiosis was obtained by flushing the bottles with N\textsubscript{2} during 20 cycles of 2 min at 700 mbar overpressure and 900 mbar under pressure. Next, a 6 mL inoculum was withdrawn from the ascending colon compartment of the SHIME, prior to its use in the
long-term experiment, and added to the bottles. This inoculum contains all the representative microbial groups present in the human intestine (reviewed in 13). The bottles were then incubated at 37 °C with shaking at 120 rpm for the total duration of the experiment (48h). At predefined time points samples were collected using syringes and 23G needles under sterile conditions. Inulin and oligofructose (FOS), two well-known fermentable dietary fibers and cellulose, a poorly fermentable fiber, were also used for comparison (dosed at 5.0 g/L). All experiments were performed in triplicate.

**Long-term experiment**

Long-term repeated oral intake of the yeast fermentate was studied using the dynamic SHIME® (ProDigest-Ghent University, Ghent, Belgium). The SHIME mimics the different regions of the human gut in a succession of five reactors 18,19. The first two have a fill-and-draw principle that simulates the different steps of food uptake and digestion, with pumps adding a defined amount of a carbohydrate-based nutritional medium (140 mL 3x/d) and pancreatic and bile solutions (60 mL 3x/d), respectively to the stomach and duodenum compartments and emptying the reactors after specified intervals. The last three are continuously stirred reactors filled with a constant volume and having specific pH control. Upon inoculation with fecal microbiota, these reactors simulate the ascending (AC), transverse (TC) and descending (DC) colon. Inoculum preparation, retention time, pH, temperature settings and nutritional medium composition have been described elsewhere 19. The fecal inoculum to start the SHIME run was derived from a healthy individual with no history of antibiotic treatment in the last year. Upon reactor start up, the system was allowed to stabilize for 3 weeks (wks) 19.

The long-term experiment consisted of a 2 wks control period in which the standard nutritional medium was administered (3 times/day): starch (3.0 g L⁻¹), arabinogalactan (1.0 g
L⁻¹), pectin (2.0 g L⁻¹), xylan (1.0 g L⁻¹), glucose (0.4 g L⁻¹), yeast extract (3.0 g L⁻¹), peptone (1.0 g L⁻¹), mucin (4.0 g L⁻¹), and cysteine (0.5 g L⁻¹) (Sigma-Aldrich). After this, a 4 wk treatment period was performed in which the nutritional medium was supplemented with 1.5 g/L of yeast fermentate. To compensate for the additional administration of carbon sources, a corresponding amount of 1.5 g/L starch was removed. Based on the volume of administered medium to the system (140 mL 3x/d), the daily dose of the yeast fermentate was set at 0.63 g/d.

To analyze the effect of the yeast fermentate on microbial community composition and activity and on immune parameters, liquid samples were collected 3 times/wk from the three colon reactors for evaluation of metabolic activity and once per week for microbiological analysis (see below for further details). Samples were stored at -20 °C until further use.

**Mucus adhesion assay**

Bacterial suspensions from the different colon compartments of the SHIME were collected once a week and used in the mucin-adhesion assay, which was performed as previously described. At the start of the experiments, 1 mL of bacterial suspensions was added to the respective wells and bacteria were allowed to adhere to the mucin layer under anaerobic conditions, at 37 °C and under gentle agitation. After 80 min of incubation, non-adhered bacteria were removed by rinsing each well twice with PBS 1X and the remaining adhered bacteria were detached using Triton X-100. The amount of adhered bacteria was quantified using specific plate count media for bifidobacteria, lactobacilli, clostridia, fecal coliforms and facultative anaerobes.

**Metabolic activity analysis**
Short-chain fatty acid (SCFA) and ammonium levels were determined as previously described. Phenol and p-cresol concentrations were determined as described elsewhere. D-lactic acid and L-lactic acid quantification were conducted using a commercial detection kit (Bioline, Brussels, Belgium) according to the manufacturer’s instructions.

**Culture-based microbiological analysis**

Decimal dilutions of the samples were performed in saline solution (NaCl, 8.5%) and subsequently plated and incubated at 37 °C. Anaerobic incubation was performed in jars with gas atmosphere (84% N₂, 8% CO₂, and 8% H₂) adjusted by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium). Counts were performed on McConkey-agar (Oxoid, Basingstoke, UK) for coliforms, LAMVAB for lactobacilli, Enterococcus-agar (Difco, Sparks, MD, USA) for enterococci, TSC-agar (Merck, Darmstadt, Germany) for clostridia, RB-agar for bifidobacteria, MSA-agar (Oxoid) for staphylococci and BHI-agar (Oxoid) for facultative anaerobes and anaerobes.

**Molecular microbiological analyses**

DNA was extracted from the samples as previously described. Denaturing Gradient Gel Electrophoresis (DGGE) on total bacteria, bifidobacteria and lactobacilli was performed to study the qualitative effect on the structure of the intestinal microbiota, as previously described. Analysis of the DGGE patterns was performed using Bionumerics software version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Briefly, calculation of similarities was based on the Pearson correlation coefficient. Clustering analysis was performed using the unweighted pair group method with arithmetic mean clustering algorithm (UPGMA) to calculate the dendrograms of each DGGE gel and a combination of all gels (composite dataset). Principal coordinate analysis (PCoA) was used to reduce the different
data of the complex DGGE patterns of one sample to one point in a three-dimensional space.

PCoA was based on the combined information from the distance matrices of each DGGE, obtained using similarity coefficients (Pearson correlation) \(^{27}\).

Quantitative PCR (Q-PCR) was performed to study the quantitative effect of the treatment on the composition of the intestinal microbial community. The different used protocols are described in Table 1.

**Pyrosequencing analysis and phylogenetic classification**

The V5 and V6 regions of the 16S rRNA genes were amplified using a primer set corresponding to primers 784F and 1061R described by Andersson and colleagues \(^{28}\). The specific PCR conditions and sample preparation are those reported by De Filippo at al. \(^{29}\). Pyrosequencing was carried out on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Mannheim, Germany), following titanium chemistry by DNAVision (Charleroi, Belgium). Pyrosequencing of the 6 amplicon libraries produced a total of 49,565 16S rDNA reads. Processing, analysis, filtering and trimming of these sequences was carried out as previously described \(^{30}\). The dataset was dereplicated for downstream analysis and the resulting unique sequences were aligned using the SILVA core alignment as template \(^{31}\).

Further filtering was applied to: (i) sequences not starting at the correct alignment position (forward primer annealing site upstream of the V5 region), (ii) rare OTU’s \(^{32}\), and (iii) putative chimeric sequences \(^{31,33}\). This preprocessing resulted in a high quality dataset of filtered and aligned sequences with a mean average of 1,669 sequences per library. In order to avoid possible biases due to library size differences \(^{34}\), these were subsampled to the smallest library size, *i.e.* 979 reads using the Mothur package.

The high-quality dataset was analyzed using a “phylogenetic-based” approach. A neighbor-joining phylogenetic tree of the filtered sequences, constructed with the Clearcut
software \textsuperscript{35,36}, implemented in Mothur, was used to compare the different samples using weighted and unweighted UNIFRAC \textsuperscript{37,38}. An OTU-based approach was used to cluster sequences into OTU (97% similarity threshold). OTU composition was used to evaluate the alpha diversity (observed richness, Chao 1 index, Shannon index and rarefaction curves) within each sample.

\textbf{Caco-2 cells preliminary experiments}

\textbf{Cell cultures}

Caco-2 cells (HTB-37) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells at passage 18 were seeded at a density of 10,000 cells/cm\textsuperscript{2} and grown with 7-day passage frequency in Dulbecco’s modified Eagle medium (DMEM) containing 25 mM glucose and 4 mM glutamine and supplemented with 0.1% (v/v) non-essential amino acids (Invitrogen, Belgium) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Perbio Science, Erembodegem, Belgium). Cells were incubated at 37 °C in a humidified atmosphere of air/CO\textsubscript{2} (95:5, v/v) and medium was changed 3 times/week.

\textbf{Cytotoxicity evaluation}

To evaluate if the SHIME suspensions were toxic to the cells a cytotoxicity test was performed. For that, Caco-2 cells were grown in 96-well plates (Corning\textsuperscript{®} CellBIND, Badhoevedorp, Netherlands) at a density of 32,000 cells/well for 7 days. Then, the cells were exposed to different concentrations of sterile-filtered (0.22 \textmu m) colonic SHIME suspensions diluted in culture medium (50, 20, 10 and 5%, v/v). After 24h exposure at 37 °C, the supernatant was collected and immediately assessed for cytotoxicity (Cytotoxicity detection kit, Roche), which is based on the measurement of lactate dehydrogenase (LDH) activity.
Cytotoxicity was expressed as percentage of the positive control (100% of LDH activity) generated with control cells incubated with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 5 to 10 min. This test was intended to determine the concentrations of SHIME suspensions to be used on further experiments. Cytotoxicity was checked for each experiment with the same LDH colorimetric assay to ensure the validity of the results.

**Interleukin-8 evaluation**

To determine if the yeast fermentate-derived metabolites collected from the SHIME may have an immunomodulatory effect on intestinal-like epithelial cells, samples from the 3 colon reactors of the SHIME were given to Caco-2 cells. For this purpose, Caco-2 cells were seeded on 12-well semi-permeable inserts (0.4 µm Thincerts, Greiner bio-ne, Wemmel, Belgium) at a density of 200,000 cells/well and cultured for 21 days with 3 medium changes/week. After this period, the integrity of the monolayer was evaluated by measuring the transepithelial electric resistance (TEER) using an Epithelial Volt-Ohm meter and two electrodes ENDHOM-12 (World Precision Instruments, Sarasota, FL) to ensure that a monolayer with more than 1000 Ω.cm² was obtained. Caco-2 cells cultured in transwell inserts spontaneously differentiate into a functional polarized epithelial monolayer that expresses several morphological and functional characteristics of mature enterocytes lining the small intestine. Then, sterile-filtered (0.22 µm) SHIME colonic suspensions were diluted 1:10 (v/v) in Caco-2 culture media and cells incubated at 37 °C/5% CO₂ for 24h, in the presence or absence of a pro-inflammatory cocktail composed of 25 ng/mL tumor necrosis factor (TNF)-α (Sigma-Aldrich), 25 ng/mL interleukin (IL)-1β (Sigma–Aldrich), 50 ng/mL interferon (IFN)-γ (Calbiochem, Darmstadt, Germany) and 1 µg/mL lipopolysaccharides (LPS; Sigma-Aldrich). After incubation, the supernatants from the upper and lower compartments were collected, pooled and IL-8 measured by using an enzyme-linked immunosorbent assay (ELISA) (BD).
Biosciences, San Diego, CA) according to the manufacturer’s instructions. Values of pg IL-8/mg protein were calculated after protein quantification (Bicinchoninic assay; Sigma-Aldrich).

Caco-2/THP1 co-cultures

Cell cultures
To evaluate the immune-protective effect of the yeast fermentate-derived metabolites in a more relevant *in vitro* system, where intestinal epithelial cells are cultured in the presence of immune cells, a co-culture model of Caco-2 cells and THP1 cells was used. Caco-2 cells (HTB-37) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells were seeded at a density of 10,000 cells/cm² and grown with 7-day passage frequency in DMEM containing 25 mM glucose and 4 mM glutamine and supplemented with 10 mM HEPES and 10% (v/v) heat-inactivated FBS. THP1-XBlue™ cells were purchased from InvivoGen (Toulouse, France). These are THP1 human monocytes stably transfected with a reporter construct expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by the transcription factors nuclear factor kappa-B (NF-κB) and activator protein (AP)-1. Upon toll-like receptor (TLR) stimulation, NF-κB and AP-1 become activated and subsequently induce the production and secretion of SEAP. The reporter protein is detectable and measurable by using the QUANTI-Blue™ assay (InvivoGen, Toulouse, France). THP1-XBlue cells were maintained at a density of 300,000 cells/mL in Roswell Park Memorial Institute (RPMI) 1640 medium containing 11 mM glucose and 2 mM glutamine and supplemented with 10 mM HEPES, 1 mM Sodium pyruvate and 10% (v/v) heat-inactivated FBS. All media and
supplements were purchased from Invitrogen (Gent, Belgium). All cells were incubated at 37 °C in a humidified atmosphere of air/CO\(_2\) (95:5, v/v) and medium was changed 3 times/week.

**Co-cultures**

For the co-culture experiments Caco-2 cells at passage 29 were seeded in 24-well semi-permeable inserts (0.4 µm Thincerts, Greiner bio-one, Wemmel, Belgium) at a density of 100,000 cells/insert. Caco-2 cell monolayers were cultured for 14 days, with 3 medium changes/week, until a functional cell monolayer with a TEER of more than 1000 Ω.cm\(^2\) was obtained. THP1-XBlue cells at passage 18 were seeded in 24-well plates at a density of 500,000 cells/well and treated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48h. PMA treatment has been shown to induce the differentiation of THP1 cells into macrophage-like cells\(^{40,41}\) and to trigger TLR up-regulation on these cells, thereby priming them for pathogen associated molecular pattern (PAMP) recognition, such as LPS\(^{42}\). After 48h of PMA treatment, cells were washed once with PBS 1X, and the wells filled with Caco-2-culture media. Then, the Caco-2 inserts were placed on top of the PMA-differentiated THP1-XBlue cells for further experiments. As previously described\(^{43,44}\), PMA-activated THP-1 macrophages induce damage on Caco-2 cells as measured by a decrease in TEER of the monolayer.

**Transepithelial electrical resistance (TEER) measurement**

Transepithelial electrical resistance is a measure of barrier function and monolayer integrity. Exposure of cells to toxic compounds may induce cell damage and lead to barrier disruption, thereby resulting in a decrease in TEER. Hence, the integrity of the Caco-2 monolayer was monitored before placing the inserts on top of the THP1-XBlue cells (0h time point) and 24h after treatment of the co-cultures with the SHIME-collected samples by using an Epithelial
Volt-Ohm meter Millicell ERS-2 from Millipore. The TEER of an empty insert was subtracted from the treated wells (residual electrical resistance of the insert) and all values were multiplied by the surface of the inserts. Then, the 24h values (after treatment) were normalized to its 0h time point (before treatment) and are expressed as percentage from the initial value.

**Co-cultures treatment with SHIME-collected samples**

Caco-2 cells placed on top of PMA-differentiated THP1-XBlue cells (co-cultures) were treated apically with sterile-filtered (0.22 µm) colonic SHIME suspensions collected from the transverse colon vessels at the end of the control period (Yeast C) and at the end of the treatment period (Yeast T). The SHIME suspensions were diluted 1:5 (v/v) in Caco-2-culture media and cells incubated at 37 °C/5% CO₂ for 24h. Cells were also exposed to culture media alone (on both chambers) as control. After this treatment period, the TEER was measured and cells were stimulated basolaterally with 100 ng/mL of LPS (E. coli K12 LPS, InvivoGen, Toulouse, France) or with DMEM (control) for 6h, after which the basolateral media was collected for cytokines measurement and determination of NF-κB/AP-1 activity. Cellular proliferation was also measured on the apical compartment as further described. All treatments were done in triplicate.

**Measurement of cytokines secretion and NF-κB/AP-1 activity**

Human IL-8, IL-6 and TNF-α were measured on the basolateral supernatant after LPS stimulation by using an ELISA assay (R&D Systems and eBioscience) according to the manufacturer’s instructions. The QUANTI-Blue™ assay (InvivoGen, Toulouse, France) was used to measure NF-κB/AP-1 activity of THP1-XBlue cells on the basolateral supernatant after LPS stimulation according to the manufacturer’s instructions. Briefly, 20 µL of each
sample were plated on a 96-well microtiter plate and 200 µL/well of QUANTI-Blue were added. The plate was incubated for 24h at 37 °C, after which optical density was read at 630 nm. An aliquot of culture media alone was added as a negative control to determine the basal levels of alkaline phosphatases possibly present on the FBS. Cells stimulated only with DMEM were used as control.

**Determination of cell proliferation**

Caco-2 cell proliferation was measured on the apical compartment using the cell proliferation reagent WST-1 from ROCHE. This assay is based on the reduction of WST-1 by viable cells. The reaction produces a soluble formazan salt which is then quantified by using a microplate reader. The absorbance directly correlates with cell number. Briefly, 10 µL of WST-1 were added to 100 µL of culture media present on the apical compartment. Cells were incubated at 37 °C/5% CO₂ for 30 min, after which the supernatant was collected onto a 96-well microtiter plate and read at 450 nm. A reference wavelength of 690 nm was used.

**Statistical analysis**

Normality of the data and equality of the variances were assessed using the Kolmogorov-Smirnov test and Levene’s test, respectively. Comparison of means on non-normally distributed data was evaluated with the non-parametric Kruskal-Wallis test. Comparison of normally distributed data was performed with the Student’s t-test for pairwise comparisons or with one-way ANOVA for multiple comparisons; when ANOVA indicated significant differences, means were compared using the Tukey, Bonferroni (equal variances) or Dunnett’s T3 multiple comparisons test. Linear regression was used to predict change over time by the Least-squares method. Statistical significance was set at p<0.05. Calculations were performed using the SPSS software (version 16.0, SPSS Inc., Chicago, IL) and the
Results

Short-term experiment

The yeast fermentate is well fermented in a dose-response manner

To evaluate the dose-response fermentation of the yeast fermentate, EpiCor was incubated in a short-term batch screening setup at 0, 0.5, 1.0, 5.0 and 10 g/L. The observed linear increase of both individual and total SCFA at increasing concentrations of EpiCor (Fig. 1B), indicates on the one hand, that this product is well fermented in a saccharolytic manner; on the other hand, the observed linear increase in ammonium production at increasing concentrations of the yeast fermentate (Fig. 1D) is related to the presence of a protein fraction in this product. Finally, a strong dose-dependent stimulation of lactobacilli was observed (Fig. 1F). At a lower extent, a similar stimulation of bifidobacteria was observed up to 5.0 g/L of yeast fermentate (Fig. 1F); however this increase was not observed at the highest concentration.

The yeast fermentate has a significant prebiotic potential

To compare the fermentation profile of the yeast product with that of well-established prebiotics, the fermentate was dosed at the same concentration as inulin and FOS (5.0 g/L). Cellulose (5.0 g/L) was used as negative control. After 48h, significantly higher total SCFA concentrations were detected for the yeast fermentate, inulin and FOS (35 to 40 mmol/L) as compared to cellulose (21 mmol/L), indicating good fermentability of the fermentate (Fig. 1A). However, despite similar total SCFA, a specific fermentation profile was observed for
the yeast fermentate, with lower acetate, similar propionate and higher butyrate production as compared to inulin and FOS (7.0, 6.0 and 3.2 mmol/L butyrate, respectively). The higher ammonium production observed for the fermentate again indicated the presence of a protein fraction in the product (Fig. 1C). Finally, the highest increase in bifidobacteria and lactobacilli was observed for the FOS incubation (Fig. 1E). Similar levels of bifidobacteria and higher levels of lactobacilli were detected for the yeast fermentate as compared to inulin (1.3 log increase against a 0.7 log increase, respectively).

**Long-term SHIME experiment with the yeast fermentate**

**The yeast fermentate modulates the colonic microbiota**

A combination of culture-based and molecular methods was used to characterize the microbial community in the different colon-simulating compartments (referred to as ‘lumen’) (Table 2). The use of selective culture-plate media showed that the yeast fermentate decreased the concentration of coliforms, clostridia, staphylococci and facultative anaerobes in the simulated ascending and transverse colon vessels. In contrast, a significant increase in enterococci was noted in the simulated transverse and descending colon.

Q-PCR analysis (Table 2) showed that administration of the fermentate did not drastically alter the community composition, with no changes in total bacterial counts and the counts of Bacteroidetes and Firmicutes. However, at lower phylogenetic level, a significant increase in lactobacilli was observed, with an increase of 0.7, 0.9 and 0.6 log units for the ascending, transverse and descending colon compartments, respectively. Additionally, the counts of Clostridia cluster I significantly increased in all colon compartments (1.6, 1.2 and 0.2 log units, respectively). This cluster contains potentially beneficial bacteria such as *Clostridium butyricum*, although it also includes the pathogenic *Clostridium perfringens*. To rule out that
the fermentate would increase the counts of this pathogen, a specific Q-PCR, targeting the alpha toxin gene (cpa) of *C. perfringens*, was performed, showing no increase of this pathogen in the different colon-simulating compartments (Table 2).

Changes in the microbial community were also observed upon DGGE analysis of SHIME samples (Fig 2A-C). Clustering of the DGGE fingerprints for total bacteria, bifidobacteria and lactobacilli displayed separate clusters for samples from the control period and for the treatment period. Additionally, separate clustering was observed for the different treatment weeks, in which the similarity with the control period was highest in the beginning of the treatment period. This indicates that long-term administration of the yeast fermentate induces gradual changes in the microbial community composition *in vitro*. This gradual microbiota modulation is also evident in the PCoA analysis (Fig. 2D), performed on the composite data set derived from the combination of all the DGGE.

Finally, a pyrosequencing approach was used to compare the microbial community composition of the different samples with higher resolution. The weighted and unweighted UNIFRAC metrics were used to perform similarity analysis on samples collected from the colon compartments at the end of the control and treatment periods (Fig. 3). In both cases, the samples collected from the simulated proximal colon (*i.e.*, the main area of fermentation of the test product) at the end of the treatment period formed separated clusters, further confirming that the yeast fermentate affects the colonic microbiota *in vitro*. The clustering was more defined in the weighted as compared to the unweighted UNIFRAC. This means that the gradual microbiota changes observed, involved essentially the relative abundance of the different phylotypes. The higher efficacy of the fermentate in modulating the composition of the microbial community in the simulated proximal colon (ascending and transverse colon) was also confirmed by analysis of the rarefaction curves and the calculation of the Chao 1 and Shannon indexes (Fig. S1). These results show that the diversity increased in the area of the
The mucus-associated microbial community composition is affected by the yeast fermentate

Combination of the SHIME with mucus-adhesion assays allowed evaluating the effect of the yeast fermentate on the adherence of intestinal microbiota to the mucus. Again, no strong changes were observed in the general microbial community composition (Table 2); however, effects were noted at specific groups’ level, with a 1.7 log increase in adhered lactobacilli in the simulated descending colon and a 0.9 log decrease in the adherence of clostridia.

The yeast fermentate induces butyrate production in the simulated colon

Replacement of part of the starch by the yeast fermentate in the SHIME nutritional medium induced SCFA profiles with lower acetate concentration in the transverse colon and higher butyrate levels in the transverse (increase both in absolute and relative numbers) and descending colon (relative increase only) (Table 3). Furthermore, increased lactate concentrations were observed in the simulated ascending colon. Similarly to what was observed in the short-term experiments, increased ammonium levels were detected, indicating proteolysis of the protein content of the yeast fermentate. This was confirmed by the increased phenol concentrations (Table 3).

Caco-2 cells preliminary experiments
The SHIME matrix is not cytotoxic and the yeast fermentate inhibits in vitro IL-8 secretion by Caco-2 cells

To evaluate the potential negative effects of the SHIME matrix on the Caco-2 cells, cytotoxicity was evaluated upon administration of different concentrations of sterile-filtered colonic suspensions to the cells. No cytotoxic effects of the SHIME matrix were observed at 5, 10 and 20% (results not shown). In addition, the integrity of the monolayer was monitored during each experiment by measuring the TEER, and no significant changes were observed (results not shown).

Potential immune-protective effects of the SHIME colonic suspensions collected from the control and treatment periods were evaluated by measuring IL-8 secretion upon pre-incubation of either non-stimulated or stimulated Caco-2 cells with sterile-filtered intestinal suspensions. In non-stimulated cells, a small increase in IL-8 secretion was observed for all SHIME samples as compared to the blank; however, levels remained very low and no differences were observed between samples (data not shown). Therefore, a pro-inflammatory cocktail was used to stimulate IL-8 production. This resulted in a strong increase in IL-8 production for all experiments (in the range of 150-fold increase as compared to the non-stimulated blank control). For all SHIME reactors, it is possible to see a significant trend towards decreasing in vitro IL-8 levels during the course of the experiment, from week 1 (TR1) to week 4 (TR4) (Fig. S2).

Caco-2/THP1 co-culture experiments

The yeast fermentate does not affect the intestinal-epithelial barrier in vitro

It was clear from the preliminary experiments using Caco-2 cells that the yeast fermentate-derived metabolites have a potential anti-inflammatory effect, which is more
pronounced in the last two weeks of the treatment. However, Caco-2 cells are not immune cells, and therefore we have made use of a more relevant *in vitro* model that mimics the intestinal mucosa $^{43,44}$ to assess the immunomodulatory properties of this product. Because the long-term SHIME experiment showed a significant metabolic activity induced by the yeast fermentate in the simulated transverse colon (Table 3), with increased butyrate production, we have used the sterile-filtered suspensions collected from this vessel at the end of the control (Yeast C) and treatment (Yeast T) periods to treat the Caco-2/THP1 co-cultures. As previously described, PMA-treated THP1 cells induce damage in Caco-2 cells $^{43,44}$. This damage can be evaluated by measuring the decrease in TEER of the Caco-2 monolayer, an indication that the epithelial barrier has been compromised. To determine if the SHIME suspensions affect the integrity of the monolayer and if the yeast fermentate has a beneficial effect on the epithelial barrier as compared to the control, the TEER was measured before and after 24h of apical treatment of the co-cultures with the transverse colon-collected samples (Fig. 4). As expected, the wells treated with growth media alone (DMEM) show a 40% decrease in TEER (damage induced by PMA-treated THP1 cells). Surprisingly, the SHIME suspensions show a very pronounced protective effect on the epithelial monolayer integrity, as seen by a 50% increase in the TEER after treatment of the Caco-2 cells. However, no difference is seen between the control and the yeast fermentate SHIME suspensions (Fig. 4). This is possibly a result from the starch fermentation-derived metabolites (control) which support microbial activity and seem to have some protective effects, at least at the level of barrier function of Caco-2 cells.

*The yeast fermentation-derived metabolites lead to a decrease in TNF-α secretion by Caco-2/THP1 co-cultures*
To determine if the yeast fermentate-derived metabolites collected from the SHIME have a potential anti-inflammatory effect \textit{in vitro}, after an initial apical pre-treatment (24h) of the co-cultures with SHIME-collected samples, cells were treated basolaterally with LPS or with DMEM for 6h, after which the supernatant was collected to measure pro-inflammatory cytokines (Fig. 5). LPS, derived from the outer membrane of Gram-negative bacteria, is a potent pro-inflammatory agent, which induces NF-κB-dependent cytokines production, such as IL-8, IL-6 and TNF-α upon TLR4 binding\textsuperscript{45}. Note however, that IL-8 is also slightly induced by PMA, and so this cytokine was also detected in non-LPS stimulated cells (Fig. 5A). In contrast, IL-6 and TNF-α were only detected in LPS-stimulated cells (Fig. 5B and C). The sample collected from the SHIME treated with yeast fermentate (Yeast T) is able to significantly reduce PMA-induced IL-8 secretion \textit{in vitro}, an effect which is no longer observed after LPS stimulation (Fig. 5A). This is in contrast with the results obtained by the preliminary experiments (Fig. S2), but the co-culture nature of this experiment \textit{vs.} the single Caco-2 experiment and the different pro-inflammatory stimulants used, may account for the differences observed. In addition, the yeast fermentate-derived metabolites (Yeast T) are able to drastically reduce \textit{in vitro} TNF-α production as compared to the SHIME control (Yeast C) (Fig. 5C). However, the same effect is not observed for \textit{in vitro} IL-6 secretion (Fig. 5B).

Although TNF-α is known to be induced by NF-κB, no differences in NF-κB/AP-1 activity of THP1-XBlues cells were seen between the control SHIME and the yeast fermentate (results not shown). By the fact that the reporter gene measures the activity of both transcription factors (NF-κB and AP-1), it is not possible to confirm that NF-κB activity is lower in the yeast fermentate-treated cells. No differences in Caco-2 cellular proliferation, as measured by the WST-1 assay on the apical compartment, were observed between the different treatments both with and without LPS stimulation (results not shown). This suggests that the SHIME-collected samples do not affect cell number.
Discussion

In this manuscript, an innovative in vitro approach was used to study the specific fermentation pattern of a yeast fermentate and its subsequent effect on both luminal and mucus-associated microbiota and the resulting immunomodulatory effects on the gut mucosa. After having obtained a first indication on the (selective) fermentability of the product, showing specific butyrate production and stimulation of lactobacilli growth, repeated administration (4 wks) to the SHIME provided insights into the mode-of-action behind the immune-protective properties of this fermentate. Gradual changes, indicative for an adaptation of the microbiota over time, were observed in both colonic microbial community activity (e.g. butyrate production) and composition in the gut lumen (decrease of potential pathogens and increased lactobacilli concentrations) and mucus layer (e.g. increased adhesion of lactobacilli). In parallel, a decrease in inflammation-induced IL-8 production in Caco-2 cells was noted throughout the course of treatment, suggesting that long-term consumption of this product may have positive health effects. In addition, an in vitro co-culture model which mimics the intestinal mucosal interface \(^{43,44}\), with immune cells lining the intestinal epithelia, showed that the yeast fermentate is also able to decrease LPS-induced TNF-\(\alpha\) production, confirming that the metabolites produced upon bacterial fermentation of EpiCor have an anti-inflammatory effect. This effect, observed in the basolateral compartment of the co-cultures upon apical treatment of the intestinal epithelial-like Caco-2 cells indicates an important indirect mode-of-action. Altogether the results suggest that prebiotic modulation of the intestinal microbiota can be an important factor determining the biological activity of this product.

The different analytical techniques used here allowed to investigate the similarity between the different colonic samples and showed a clear treatment effect on the microbial community in vitro, particularly in the simulated proximal colon, as shown by pyrosequencing analysis. In
addition, DGGE fingerprinting showed a gradual change on the microbiota, separating the first and second halves of the treatment periods. Plate counts showed a significant decrease in coliforms and staphylococci in the gut lumen, groups typically associated with the presence of potential pathogens. Clostridia also decreased, although it is difficult to conclude on the negative aspects, as the broad phylogenetic group of clostridia contains both pathogens and beneficial bacteria. At the mucus layer, increased lactobacilli and decreased clostridia levels were observed in the descending and ascending colon, respectively. QPCR allowed detecting specific stimulation of lactobacilli and members of Clostridium cluster I, but not the pathogenic *C. perfringens*. In parallel with gradual changes in community composition, selective modulation of microbial activity was observed, such as increased lactate production in the simulated ascending colon and increased butyrate levels in the transverse and descending colon. Lactate has antibacterial properties, which may account for the decrease in coliforms and staphylococci observed, and is an important precursor for butyrate production. Increased butyrate levels were already observed upon fermentation of yeast products, beta-glucans from cereals and mannan-oligosaccharides. SCFA are the main metabolic products of anaerobic bacteria fermentation, are suggested to be the link between microbiota and host tissues, and are known to prevent pathological conditions such as inflammatory bowel disease (IBD), diabetes and cancer. Actually, SCFA and butyrate enemas have been successfully used to treat acute inflammation in patients with ulcerative colitis. SCFA, particularly butyrate, are able to modulate the production and release of chemotactic and adhesion molecules in neutrophils and the expression of cytokines in intestinal epithelial cells and immune cells (reviewed in). Therefore, the metabolic activity induced by the yeast fermentate towards butyrate production may contribute to its immune-protective effects.

The effect of yeast-derived products on the intestinal microbiota has been previously described. In some studies, the effect of the yeast food additive (Diamond V XP Yeast...
Culture) made by the same process as the product here studied, was shown in vivo\(^\text{12}\) and in vitro\(^\text{53}\) to increase lactobacilli and to decrease \(E.\ coli\) numbers. Several components of the yeast fermentate may be responsible for such changes. For instance, yeast cell mannan-oligosaccharides can prevent intestinal bacteria to attach to mannose residues on the gut epithelium\(^\text{54}\) and selective modulation of the microbiota in combination with improved gut morphology has been observed in fish\(^\text{55}\). Moreover, these compounds are also known to support probiotic lactobacillus growth\(^\text{56}\). EpiCor contains different elements which were previously shown to affect immune parameters by direct interactions. For instance, the yeast cell wall fragments beta-glucans and mannan-oligosaccharides are well documented for their positive effects on the immune system\(^\text{57}\). However, in addition to these potential direct immune effects, our results suggest an important role for indirect immune effects related to the regular consumption of the yeast fermentate and the metabolites resulting from its fermentation. The application of the SHIME matrix containing the yeast fermentate-derived metabolites to our in vitro co-culture model is unique because it allows testing the indirect effects of the fermented product rather than the intact product, which is surely more relevant when the ultimate goal is to evaluate the beneficial effects of its oral consumption\(^\text{30}\). The Caco-2/THP1 co-culture model used in the present study is based on the model proposed by Satsu and colleagues\(^\text{43}\) and allows testing for putative beneficial effects both on the intestinal epithelia and on the immune system in vitro. It has also been suggested to mimic the damage observed in IBD, and therefore it represents a good model to screen for drugs or food substances that can be used to treat or prevent IBD-like symptoms\(^\text{43}\). This is interesting because we have observed that the metabolites derived from the fermentation of EpiCor are able to decrease TNF-\(\alpha\) release by cells in vitro. A continuous and inappropriate immune response of the immune system to the commensal microflora of the human gut is a hallmark of IBD, such as Crohn’s disease and ulcerative colitis\(^\text{4}\), and the currently accepted therapies
are aimed to manage this chronic inflammation by using TNF-α inhibitors, such as Infliximab 
Moreover, butyrate was shown to inhibit TNF-α production in human peripheral 
monocytes and murine macrophages. Therefore, and albeit being speculative, we believe 
that the enhanced butyrate production upon fermentation of EpiCor may contribute to the 
decrease in TNF-α production observed in our in vitro co-cultures. Hence, the consumption of 
this product may be beneficial to prevent IBD and to help patients to manage their symptoms. 
Health effects from nutritional interventions, such as immune protection by prebiotics, are the 
result of a complex interplay between processes occurring in the gut lumen, at the mucosal 
surface and intestinal epithelium. Thus, the yeast fermentate may contain products which 
directly interact with the mucosal immune system, but they may also be digested in the upper 
intestine and fermented in the lower intestine, resulting into breakdown products which in 
turn interact with the immune system. In addition, selective fermentation of the product can 
affect the composition of both the luminal- and mucosa-associated microbiota, which will 
modulate specific host-bacteria interactions involved in immune responses. Finally, bacterial 
metabolites produced in response to the administered product, such as butyrate and lactate, 
can affect other bacteria, including pathogens, and thereby improve immune function.

As already mentioned, human and animal studies are essential in order to study the final 
immune response to nutritional interventions. However, they do not allow investigating each 
of the intestinal processes involved in the complex interplay between the intestine and the 
immune system. In vitro models offer therefore a valuable alternative. The SHIME® has been 
extensively validated as a valuable in vitro tool to study intestinal digestion and fermentation 
under representative conditions in a long-term study setting. The new integrated SHIME 
platform which was presented here has the unique capacity to combine the study of both 
direct and indirect effects of food ingredients on the immune system. Because butyrate has 
received considerable attention due to its immune-protective properties, the enhanced
butyrate production observed here may help explaining the underlying mechanism behind EpiCor’s immunomodulatory effects. However, a reduced IL-8 production by Caco-2 cells was observed for the ascending colon already from the first week of treatment, where butyrate production was not yet stimulated. Thus, other aspects related to the intestinal ecosystem are expected to be involved. Hence, further research is needed to elucidate the complex mechanism of interaction of the yeast fermentate with the immune system. Despite these promising results, *in vitro* studies should be corroborated by *in vivo* experimental data. Therefore, such *in vitro* findings may be useful when designing targeted clinical studies aimed to confirm the mode-of-action and the relevance of the observed effects.

### Abbreviations used

AC: ascending colon; ANOVA: analysis of variance; AP-1: activator protein 1; DC: descending colon; DGGE: denaturing Gradient Gel Electrophoresis; DMEM: Dulbecco’s modified Eagle medium; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; FOS: oligofructose; GALT: gut associated lymphoid tissue; IBD: inflammatory bowel diseases; IFN: interferon; Ig: immunoglobulin; IL: interleukin; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; NF-κB: nuclear factor kappa-B; OTU: operational taxonomic units; PAMP: pathogen associated molecular pattern; PBS: phosphate buffered saline; PCoA: principal coordinate analysis; PMA: phorbol 12-myristate 13-acetate; QPCR: quantitative PCR; RPMI: Roswell Park Memorial Institute; rRNA: ribosomal ribonucleic acid; SCFA: short chain fatty acids; SEAP: secreted embryonic alkaline phosphatase; SHIME: simulator of the Human Intestinal Microbial Ecosystem; TC: transverse colon; TEER: transepithelial electric resistance; TLR: toll-like receptor; TNF-α: tumor necrosis factor alpha; Wks: weeks; Yeast C: yeast control (SHIME); Yeast T: yeast treatment (SHIME).
Supporting Information Available:

Supplementary Figure 1: Rarefaction curves and diversity indexes. Operational taxonomic units (OUT) composition was used to evaluate the alpha diversity for rarefaction curves (panel A) and observed richness (Sobs), Chao 1 index, Shannon index (panel B). Values have been calculated for samples collected from ascending (AC), transverse (TC) and descending colon (DC) compartments at the end of the control (CT) and treatment (TR) periods. Supplementary Figure 2: Effect of SHIME samples on the secretion of IL-8 by Caco-2 cells upon stimulation with a pro-inflammatory cocktail. Samples were collected from the simulated ascending (AC), transverse (TC) and descending (DC) colon compartments, at the end of the control period (CT) and at the end of each week of treatment (TR1-4) of the SHIME experiment. The sterile-filtered samples were added (1:10 v/v) in combination with a pro-inflammatory cocktail (TNF-α, IL-1β, IFN-γ, LPS) to the cell culture medium. After 24h, IL-8 secretion was quantified by ELISA on the pooled samples collected from the apical and basolateral compartments. Results are presented as the average secretion of IL-8/mg of secreted protein of three independent experiments. The plotted lines represent the best lines that fit the data (Linear regression, Least-squares method). (*) Slope significantly different from zero, p<0.05. This material is available free of charge via the Internet at http://pubs.acs.org.

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producing bacteria and butyrate fermentation in weaned pigs. *FEMS Microbiol. Ecol.* 2011,
75, 402-413.


**Figure captions**

**Figure 1:** Fermentation profile of the yeast fermentate after the short-term screening setup. (A) SCFA concentrations, (C) Ammonium concentrations and (E) Total bacteria, bifidobacteria and lactobacilli. Panels A, C and E present the results from the comparison of the short-term colon incubations of the yeast fermentate (Yeast), cellulose, inulin and fructo-oligosaccharides (FOS). Panels B, D and F show the results from the same analyses, but of a dose-response experiment with the yeast fermentate alone.

**Figure 2:** Microbial composition change upon long-term fermentation of the yeast fermentate. (A,B,C) Denaturing gel electrophoresis (DGGE) fingerprints and (D) principal coordinate analysis (PCoA) of samples from the control (CT) and treatment (TR) periods of the SHIME experiment. DGGE fingerprints of the microbial community from the simulated ascending (AC), transverse (TC) and descending (DC) colon compartments were constructed for total bacteria (panel A), bifidobacteria (panel B) and lactobacilli (panel C). Numbering and color codes refer to the different experimental weeks. PCoA was used to explore the similarity within a composite data set consisting of DGGE fingerprints of total bacteria, bifidobacteria and lactobacilli (panel D). The arrow indicates time evolution.
Figure 3: Neighbor-joining tree of the different intestinal regions obtained upon microbial sequencing. High-throughput sequencing analysis of the bacterial community of samples from the simulated ascending (AC), transverse (TC) and descending (DC) colon compartments, collected at the end of the control (CT) and treatment (TR) periods of the long-term SHIME experiment. Similarity analysis was performed using the unweighted (panel A) and weighted (panel B) UNIFRAC metrics.

Figure 4: Effect of the SHIME samples on the transepithelial electrical resistance (TEER) of Caco-2 cells cultured in the presence of THP1 cells. Samples collected from the transverse colon at the end of the control (Yeast C) and treatment (Yeast T) periods were sterile-filtered and added (1:5 v/v) for 24h to the apical compartment of Caco-2 cells grown for 14 days on semi-permeable inserts and placed on top of PMA-stimulated THP1-derived macrophages (co-cultures). Growth media alone (DMEM) was used as control. THP1 cells cultured in the presence of PMA for 48h induce damage on the Caco-2 cells as measured by a decrease in TEER in the DMEM control. All treatments were done in triplicate. TEER values have been normalized to the values measured before co-culture (0h) and are expressed as percentage from the initial value. Results are presented as Mean±SEM. (*** Represents significantly different from the DMEM, p<0.001.

Figure 5: Effect of the SHIME samples on the basolateral secretion of pro-inflammatory cytokines upon LPS stimulation of the Caco-2/THP1 co-cultures. Samples collected from the simulated transverse colon at the end of the control (Yeast C) and treatment (Yeast T) periods were sterile-filtered and added (1:5 v/v) for 24h to the apical compartment of Caco-2 cells grown for 14 days on semi-permeable inserts and placed on top of PMA-stimulated
THP1-derived macrophages (co-cultures). Then, the co-cultures were stimulated with 100 ng/mL of LPS or DMEM only on the basolateral compartment for 6h. After this period, the basolateral supernatant was collected and cytokines (IL-8 panel A, IL-6 panel B and TNF-α panel C) were measured by ELISA. All treatments were done in triplicate. Results are presented as Mean±SEM. (*) Represents significantly different from Yeast C, p<0.05; ns, not significant.
### Tables

**Table 1:** Group-specific 16S rRNA and Functional Gene-targeted Primers Used in This Study for Quantitative PCR Assessment. QPCR protocols were performed using either the Power SYBR Green PCR Master kit (Applied Biosystems, Foster City, Ca) (1), qPCRT core kit for Sybr Green I (Eurogentec, Seraing, Belgium) (2) or the Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA) (3).

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<th>Kit</th>
<th>Reference</th>
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<td>P518r</td>
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Table 2: Microbial Counts in the Colon Compartments of the SHIME Reactor During the Control Period (n=3) and During the Treatment Period (n=4) in Which the Yeast Fermentate was Administered to the SHIME at a Daily Dose of 0.63 g/d. 

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<th>Control period</th>
<th>Treatment period</th>
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<tbody>
<tr>
<td></td>
<td>Ascending</td>
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<tr>
<td>LUMINAL MICROBIOTA</td>
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<tr>
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<tr>
<td>Total anaerobes</td>
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<td>8.06 ± 0.21</td>
<td>7.51 ± 0.37</td>
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<td>8.18 ± 0.32</td>
<td>7.84 ± 0.38</td>
<td>7.64 ± 0.44</td>
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<td>Facultative anaerobes</td>
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<td>4.53 ± 0.51 b</td>
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<td></td>
<td>7.07 ± 0.35 b</td>
<td>6.57 ± 0.36 b</td>
<td>6.08 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>Based on quantitative PCR (16S rDNA copies/mL)</td>
<td></td>
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<tr>
<td>Total bacteria</td>
<td>11.21 ± 0.18</td>
<td>10.66 ± 0.67</td>
<td>10.92 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>11.10 ± 0.49</td>
<td>11.11 ± 0.12</td>
<td>10.92 ± 0.21</td>
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<tr>
<td>Firmicutes</td>
<td>8.85 ± 0.13</td>
<td>8.65 ± 0.24</td>
<td>8.83 ± 0.06</td>
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<tr>
<td></td>
<td>8.75 ± 0.13</td>
<td>8.82 ± 0.10</td>
<td>8.62 ± 0.15</td>
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<tr>
<td>Bacteroidetes</td>
<td>9.94 ± 0.10</td>
<td>9.82 ± 0.16</td>
<td>9.24 ± 0.46</td>
</tr>
<tr>
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<td>9.90 ± 0.16</td>
<td>9.75 ± 0.09</td>
<td>9.45 ± 0.19</td>
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<tr>
<td>Bifidobacteria</td>
<td>8.21 ± 0.09</td>
<td>8.08 ± 0.11</td>
<td>7.81 ± 0.36</td>
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<tr>
<td></td>
<td>8.23 ± 0.11</td>
<td>8.14 ± 0.11</td>
<td>8.13 ± 0.09</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>4.55 ± 0.18</td>
<td>4.16 ± 0.56</td>
<td>4.07 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>5.22 ± 0.47 b</td>
<td>5.10 ± 0.24 b</td>
<td>4.71 ± 0.20 b</td>
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<tr>
<td>Roseburia spp.</td>
<td>6.64 ± 0.27</td>
<td>6.51 ± 0.22</td>
<td>6.51 ± 0.14</td>
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<td>6.48 ± 0.07 b</td>
<td>6.56 ± 0.09</td>
<td>6.49 ± 0.12</td>
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<tr>
<td>Clostridia cluster I</td>
<td>6.58 ± 0.22</td>
<td>6.60 ± 0.08</td>
<td>7.22 ± 0.04</td>
</tr>
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<td>8.13 ± 0.31 b</td>
<td>7.76 ± 0.13 b</td>
<td>7.42 ± 0.11 b</td>
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<tr>
<td>C. perfringens</td>
<td>6.61 ± 0.09</td>
<td>6.60 ± 0.11</td>
<td>6.57 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>6.56 ± 0.14</td>
<td>6.64 ± 0.14</td>
<td>6.55 ± 0.08</td>
</tr>
<tr>
<td>MUCUS-ASSOCIATED MICROBIOTA (plate counts (CFU/mL))</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total anaerobes</td>
<td>6.71 ± 0.49</td>
<td>6.17 ± 0.59</td>
<td>6.06 ± 0.34</td>
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<tr>
<td></td>
<td>6.04 ± 0.40 b</td>
<td>6.10 ± 0.28</td>
<td>5.96 ± 0.28</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>2.98 ± 1.01</td>
<td>2.86 ± 0.83</td>
<td>1.59 ± 0.14</td>
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<tr>
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<td>2.64 ± 0.63</td>
<td>3.20 ± 0.71</td>
<td>3.24 ± 1.02 b</td>
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<tr>
<td>Bifidobacteria</td>
<td>6.57 ± 0.37</td>
<td>6.16 ± 0.30</td>
<td>5.99 ± 0.19</td>
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<td>6.04 ± 0.75</td>
<td>5.86 ± 0.43</td>
<td>5.51 ± 0.55</td>
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<tr>
<td>Clostridia</td>
<td>6.70 ± 0.18</td>
<td>5.64 ± 0.31</td>
<td>5.37 ± 0.49</td>
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<tr>
<td></td>
<td>5.82 ± 0.64 b</td>
<td>5.46 ± 0.52</td>
<td>5.37 ± 0.32</td>
</tr>
<tr>
<td>Coliforms</td>
<td>6.85 ± 0.13</td>
<td>6.27 ± 0.33</td>
<td>6.20 ± 0.42</td>
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<tr>
<td></td>
<td>7.01 ± 0.29</td>
<td>6.66 ± 0.34</td>
<td>6.55 ± 0.45</td>
</tr>
</tbody>
</table>

* Results are presented as Mean±SD. b Represents significantly different from the control period, p<0.05.
Table 3: Short-Chain Fatty Acid (SCFA) Levels (Presented as Concentrations and as the Ratio of Respectively Acetate (A), Propionate (P) or Butyrate (B) to the Sum of the Latter 3 acids) as well as Lactate, Ammonium, Phenol and P-cresol Concentrations in the Colon Compartments of the SHIME Reactor During the Control Period (n=6) and During the Treatment Period (n=12) in Which the Yeast Fermentate was Administered to the SHIME at a Daily Dose of 0.63 g/d.  

<table>
<thead>
<tr>
<th>SCFA (mmol/L)</th>
<th>Control period</th>
<th>Treatment period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascending</td>
<td>Transverse</td>
</tr>
<tr>
<td>Acetate</td>
<td>25.79 ± 4.63</td>
<td>34.05 ± 3.92</td>
</tr>
<tr>
<td>Propionate</td>
<td>13.30 ± 1.22</td>
<td>20.19 ± 2.34</td>
</tr>
<tr>
<td>Butyrate</td>
<td>9.41 ± 0.60</td>
<td>10.38 ± 0.62</td>
</tr>
<tr>
<td>Other&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.60 ± 0.64</td>
<td>4.40 ± 2.58</td>
</tr>
<tr>
<td>Total</td>
<td>51.10 ± 3.72</td>
<td>69.02 ± 3.23</td>
</tr>
<tr>
<td>A/(A+P+B)</td>
<td>0.53</td>
<td>0.52</td>
</tr>
<tr>
<td>P/(A+P+B)</td>
<td>0.28</td>
<td>0.32</td>
</tr>
<tr>
<td>B/(A+P+B)</td>
<td>0.19</td>
<td>0.16</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>2.16 ± 0.70</td>
<td>4.03 ± 0.96</td>
</tr>
<tr>
<td>Ammonium (mg NH₄⁺/L)</td>
<td>293.1 ± 52.6</td>
<td>362.4 ± 68.0</td>
</tr>
<tr>
<td>Phenol (mg/L)</td>
<td>0.01 ± 0.09</td>
<td>0.06 ± 0.05</td>
</tr>
<tr>
<td>p-Cresol (mg/L)</td>
<td>0.04 ± 0.0002</td>
<td>0.07 ± 0.005</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are presented as Mean±SD.
<sup>b</sup> Represents significantly different from the control period, p<0.05.
<sup>c</sup> Represents significantly different from the control period when only the second half of the treatment period is included in the analysis (n=6), p<0.05.
<sup>d</sup> Other SCFA include: isobutyrate, valerate, isovalerate and caproate.
Figure graphics

Figure 1

A

<table>
<thead>
<tr>
<th>Concentration (mM/L)</th>
</tr>
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<tbody>
<tr>
<td>Acetate</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Concentration (mM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>NH₄-N (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
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D

<table>
<thead>
<tr>
<th>NH₄-N (mg/L)</th>
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<tr>
<td>Cellulose</td>
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</table>

E

<table>
<thead>
<tr>
<th>Log increase (CFU/mL)</th>
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<tbody>
<tr>
<td>Anaerobes</td>
</tr>
</tbody>
</table>

F

<table>
<thead>
<tr>
<th>Log increase (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobes</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 4

![Bar chart showing normalized TEER (%)](chart)

- DMEM
- Yeast C
- Yeast T

*** indicates statistically significant difference.
Figure 5

A

B

C

IL-8 (ng/ml)

IL-6 (pg/ml)

TNF-α (pg/ml)

- LPS  + LPS  - LPS  + LPS

- LPS  + LPS  - LPS  + LPS

- LPS  + LPS  - LPS  + LPS

Yeast C  Yeast T  Yeast C  Yeast T  Yeast C  Yeast T

* p < 0.05

ACS Paragon Plus Environment
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