Colonic Transit Time Is a Driven Force of the Gut Microbiota Composition and Metabolism: In Vitro Evidence

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Background/Aims
Human gut microbiota harbors numerous metabolic properties essential for the host’s health. Increased intestinal transit time affects a part of the population and is notably observed with human aging, which also corresponds to modifications of the gut microbiota. Thus we tested the metabolic and compositional changes of a human gut microbiota induced by an increased transit time simulated in vitro.

Methods
The in vitro system, Environmental Control System for Intestinal Microbiota, was used to simulate the environmental conditions of 3 different anatomical parts of the human colon in a continuous process. The retention times of the chemostat conditions were established to correspond to a typical transit time of 48 hours next increased to 96 hours. The bacterial communities, short chain fatty acids and metabolite fingerprints were determined.

Results
Increase of transit time resulted in a decrease of biomass and of diversity in the more distal compartments. Short chain fatty acid analyses and metabolite fingerprinting revealed increased activity corresponding to carbohydrate fermentation in the proximal compartments while protein fermentations were increased in the lower parts.

Conclusions
This study provides the evidence that the increase of transit time, independently of other factors, affects the composition and metabolism of the gut microbiota. The transit time is one of the factors that explain some of the modifications seen in the gut microbiota of the elderly, as well as patients with slow transit time.

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Key Words
Aging; Colon; Constipation; Gut microbiome; Microbiological techniques
Introduction

The gut harbors a complex ecosystem composed of $1 \times 10^{14}$ microbial cells which present an important metabolic diversity and notably contributes to the host's health and well-being. Increases of transit time or even constipation is frequently observed among the human population. Recently, the colonic transit rate evaluated through stool consistency (categorized by the Bristol stool scale) has been strongly associated with gut microbiota composition, with

Figure 1. The 3-stage Environmental Control System for Intestinal Microbiota (3S-ECSIM) and overall design of the experiment. (A) The 3S-ECSIM system is depicted with its 3 reactors: proximal, transversal, and distal (P, T, and D), respectively, simulating the physicochemical parameters of the proximal, transversal, and distal parts of the colon. Each reactor is initially inoculated by the same regenerated fecal inoculum. The first one is fed with a growth medium simulating nutrients entering the proximal colon in a continuous fermentation process, using a retention time (RT) fitting with first a normal transit time (48 hours) and next a slow one (96 hours) (see Table 1 for parameters). Resulting medium from the P reactor is continuously used to feed the second reactor (T), and next the third reactor (D) in a similar process. Volumes of reaction are maintained by retrieving excess of medium to fit also with RT (reactors P and D, see Table 1). (B) After at least 5 RT of continuous feeding, the gut microbiota is considered to be in a steady-state (modeled at 96% stability) and the resulting metabolized medium considered to be stable. This time corresponds to $t_0$ in each reactor (see additional data, at time $t_0$ and 48 hours after, for metabolic comparisons at steady state). For a 48-hour gut transit simulation (orange, left part), the resulting medium from the P reactor is used to feed the second reactor during 90 hours more (in case of a gut normal transit time of 48 hours, Table 1) and the medium resulting from reactor T. The RT is next changed in each compartment in order to simulate a slow transit time (96 hours all over the gut, blue, right part) and similar principles are applied (see Table 1 for parameters). At each $t_0$ and 48 hours after, samplings are done and analyses performed (in grey boxes, lower part). This encompasses the determination of the microbial composition by the Human Gut Chip (HuGChip), a DNA phylogenetic microarray detecting 16S bacterial sequences, and its metabolic behavior by measuring the fermentative atmospheric gases, the production of short chain fatty acids (SCFAs) and by determining a high resolution metabolite fingerprint.
loose stool corresponding more frequently to the \textit{Prevotella} enterotype\textsuperscript{4} and with more abundant \textit{Bacteroides} spp.\textsuperscript{4}

This increase of transit time is also one of the gastrointestinal modifications encountered in elderly people either living in a community or in institutions\textsuperscript{5,6} and for whom human gut microbiota variations have been associated.\textsuperscript{7-10} To address the real part of the transit time influence, drugs have been administered to cohorts to change the patient transit time.\textsuperscript{11,12} These studies reported a negative correlation between the increase of transit time and fecal microbial biomass. Furthermore, the increase of transit time positively correlated with the increase of methanogens, of breath methane concentrations and pH, while being inversely correlated to the proportion of sulfate reducing bacteria and the concentration of short chain fatty acid (SCFA).\textsuperscript{12}

In vitro systems have been developed and used to study the human gut\textsuperscript{13} overcoming ethical questions.\textsuperscript{14} In previous experiments conducted by MacFarlane and collaborators,\textsuperscript{15} an in vitro system was used to simulate the passage from an average colon transit (66.7 hours) to a quick colon transit (27.1 hours) by a change of retention time. This modification induced variations on the microbial community structure and metabolic activity.\textsuperscript{15,16} The Environmental Control Systems for Intestinal Microbiota (ECSIM) models are inspired by those developed by MacFarlane and colleagues.\textsuperscript{17,18} In its 3-stage configuration (Fig. 1), it simulates the proximal, transversal and distal parts of the human colon in which gut microbes are known to be different,\textsuperscript{19} mimicking different physicochemical parameters and substrates availability for gut microbes.\textsuperscript{18} This biogeoography simulation is obtained by differential development of microbes from a same fecal microbe repertoire in the 3 linked fermenters. A growth medium determined to be representative of nutrients entering the proximal part of the colon under standard diet\textsuperscript{15} is given at a dilution rate that can be modified, therefore mimicking various retention times in the proximal part. This medium, modified by the microbial activity of the proximal part, is next used to feed the second reactor/transversal part, also with a specified dilution rate. The same principles are next applied to feed the third reactor/distal part. One of the other main characteristics of the ECSIM systems relies in the genesis of anaerobia which is necessary for microbial development: instead of being maintained by a continuous flush of gases devoid of O\textsubscript{2} anaerobia is here generated by the fermentative metabolism of the microbes themselves\textsuperscript{17,18} supposedly simulating conditions more closely related to the one encountered in vivo.

The objective of our study was to evaluate the impact of the increase of the transit time on the gut microbiota in 3 anatomical parts of the colon. This question is addressable through the use of dedicated parameters on the 3-stage ECSIM (3S-ECSIM) systems: the effect of retention time was evaluated, from a normal duration to an increased one (overall transit time from 48 hours to 96 hours) on the gut microbiota composition and its metabolism. This study should help to determine the gut microbiota modifications that would result in people with slow transit times and for which very sparse data are available regarding gut microbes. This would also contribute to determining to what extent the reduced transit time could contribute to the distinctive gut microbiota of elderly people.

**Materials and Methods**

Three-stage Environmental Control System for Intestinal Microbiota Procedure

The fecal aliquot was obtained from a healthy female volunteer (29 years old), without recent treatment with antibiotics and who was not a breath methane-producer. Briefly, one 2-mL fecal aliquot with 30% (v/v) glycerol\textsuperscript{17} was unfrozen on ice and used to inoculate a 5-mL preculture of fermentation medium, grown at 37°C for 10 hours. It was transferred for 15 hours into a 1-L Erlenmeyer flask containing 95 mL of fermentation medium and into a 2-L bioreactor (Global Process Concept Inc, Perigny, France) previously N\textsubscript{2} flushed containing 900 mL of fermentation medium for a 24 hour batch culture at 37°C, pH 5.7, and 400 rpm. The same operations were used for each of the 3-stage bioreactors P (proximal), T (transversal), and D (distal) with working volume of 1 L for each. After the 24 hour batch condition, the procedure and medium used were as described previously (Table 1).\textsuperscript{18} Figure 1 illustrates the 3S-ECSIM and the overall experimental design. Briefly, an initial retention time of 48 hours was applied to the system and after 240 hours of stabilization; it was increased to 96 hours by a decrease of dilution rate and left to stabilize for 480 hours. Samplings were performed in each reactor over a 48-hour period for microbial and biochemical analyses (n = 6 samples per condition). No gases were flushed during the course of the continuous culture, the anoxic environment being progressively and dynamically enriched by the gases originating directly from the microbiota metabolism. The acquisition and control software C-BIO (Global Process Concept Inc) was used for the batch and culture continuous conditions.

**In Vitro Microbiota Growth**

Microbiota growth was evaluated by spectrophotometry (620 nm, Beckman Coulter DU 640B spectrophotometer; Beckman Coulter, Fullerton, CA, USA) and by dry weight measurements:
for this purpose, 5 mL of culture was first centrifuged (13,000 × g, 10 minutes), then the pellet was washed 3 times with distilled water before being deposited under vacuum and dried at 104 °C onto a pre-weighted membrane (Polyamid 0.45 µm; Sartorius, Dourdan, France). The biomass (as a dry weight) was next determined.

### Human Gut Chip Microarray Analysis

A community analysis was performed at the phylum and family level using the Human Gut Chip (HuGChip; GEO: GSE44752). The HuGChip is an explorative phylogenetic microarray designed to target the 66 bacterial families usually recovered from the human gastrointestinal tract, which provided positive correlations with data from pyrosequencing of amplicons and metagenomic analysis.\(^2^\) It consists of around 3 × 4500 probes established from a previous work and was synthesized by Agilent Technologies (Palo Alto, CA, USA) as a 8 × 15K microarray.\(^3^\) Bacterial DNA was extracted from a 0.25 mL sample of fermentation medium as described by Yu and Morrison\(^4\) and followed by the Qiagen’s DNA stool kit (Qiagen Ltd, West Sussex, UK). Small subunit RNA coding genes were then amplified by PCR and purified using the MinElute PCR purification kit (Qiagen Ltd). One microgram was then labeled using either Cy3 or Cy5 (Genomic DNA ULS labeling Kit; Agilent Technologies, Palo Alto, CA, USA) and hybridizations/washings were performed following the manufacturers recommendations. Microarray scanning was performed on a Surescan microarray scanner (Agilent Technologie, USA).

### Short Chain Fatty Acid Analysis

SCFAs were quantified from a bioreactor volume of about 200 µL. It was quickly sampled, deproteinized using 400 µL of cold methanol and centrifuged (8000 × g, 10 minutes). The supernatant was stored at -20 °C until the analyses. It was used to determine the level of acetate, propionate, butyrate, isobutyrate, isovalerate, valerate, caproate, isocaprate, and heptanoate using gas chromatography (HP 6890 series; Agilent Technologies, Les Ulis, France). The chromatography was carried out using HP-INNOVAX column (30 m × 250 µm × 0.25 µm, split ratio = 25:1, Agilent Technologies, France). 2-Ethyl-butyrate was used as an internal standard.

### Metabolomic Fingerprint

A deproteinized sample (same procedure as for SCFA analysis) was evaporated under nitrogen and the dry residue was dissolved in the injection solvent (50/50 water/acetonitrile with 0.1% formic acid). Six microliters of the solution were then injected into a chromatographic system (Waters Acquity UPLC module, Saint Quentin en Yvelines, France). Separations were carried out at 30 °C using a 2.1 × 50 mm Acquity UPLC HSS T3 column (Waters), with a particle size of 1.8 µm at a flow rate of 0.4 mL/min. Plasma was eluted from the LC column using the following linear gradient (curve number 6): 0-2 minutes: 100% A; 2-15 minutes: 0-100% B; 15-22 minutes: 100% B; 22-26 minutes 100% A for re-equilibration. Solvent A was water and solvent B was acetonitrile, both solvents containing 0.1% formic acid. The UPLC system was coupled to a Waters Qtof-Micro equipped with an electrospray source and a lockmass sprayer to ensure accuracy. Experiments were carried out

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**Table 1. Fermentation Conditions Used in the Environmental Control System for Intestinal Microbiota System to Simulation a Normal and a Slow Transit Time by a Modification of the Retention Time**

| Reactor   | pH  | Dilution rate (hr⁻¹) | Retention time (hr) | Minimum doubling time (hr) |
|-----------|-----|----------------------|---------------------|---------------------------|
| Simulation of a 48 hr transit |     |                      |                     |                           |
| P48       | 5.8 | 0.080                | 12.48               | 8.65                      |
| T48       | 6.2 | 0.056                | 17.76               | 12.31                     |
| D48       | 6.8 | 0.056                | 17.76               | 12.31                     |
| Simulation of an 96 hr transit |     |                      |                     |                           |
| P96       | 5.8 | 0.040                | 24.96               | 17.3                      |
| T96       | 6.2 | 0.028                | 35.52               | 24.62                     |
| D96       | 6.8 | 0.028                | 35.32               | 24.62                     |

Minimum doubling time means the minimum doubling time of each bacterial species to avoid washing from the reactor and therefore the disappearance of the species when analyzed at steady-state.

P48, T48, and D48 indicate respectively the P (proximal), T (transversal), and D (distal) reactor simulating a 48-hour transit. P96, T96, and D96 indicate respectively the P (proximal), T (transversal), and D (distal) reactor simulating an 96-hour transit.
in positive ion mode with a scan range from 70 to 1000 m/z. Capillary voltage was set to 3 kV and cone voltage was optimized at 30 V. The scan time and the dwell time were fixed at 1 and 0.1 seconds, respectively. ESI needle and drying gas temperatures were set at 120 and 450°C, respectively. The drying and nebulizing gas flows (nitrogen) were set to 50 and 500 L/hr. All analyses were acquired by using the lockspray with a frequency of 5 seconds to ensure accuracy. Leucine enkephalin was used as the lock mass. To avoid possible differences between sample batches, a Latin square was carried out to obtain randomized analytical sequences. Raw data files were converted to NetCDF format using the Waters DataBridge software. All liquid chromatography – mass spectrometry (LC–MS) data were processed using XCMS to yield a data matrix containing retention times, accurate masses and normalized peak intensities. An identification of the molecules was performed on the basis of their exact masses which were compared to those registered in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as defined previously.

Statistical Methods

For microarray analyses, pixel intensity data was extracted using the Feature Extraction software (Agilent Technologies, USA). The retained intensity value for each probe was the spot’s median intensity signal. Specific scripts developed with the Delphi and C++ signal were used to automatically perform data extractions and loss of some uncultured Clostridium and of bacteria belonging to the family Desulfovibrionaceae. Furthermore, the apparition of bacteria from the family Enterobacteriaceae and the increase of Coriobacteriaceae were seen (Fig. 2A). In the T reactor simulating the transversal colon, an increase of the Clostridium cl. XIV with the higher retention time was observed associated with a decrease of the families Clostridium cl. I, Coriobacteriaceae, and Porphyromonadaceae, and a disappearance of Desulfovibrionaceae. Finally, the third reactor simulating the distal colon was characterized by the most important diversity and where the modifications were the most important: an increase of the relative abundance of Enterobacteriaceae, and in a lesser extent of Bacteroidaceae and Lactobacillaceae was observed. Inversely, there was a decrease of the relative abundance of Porphyromonadaceae and loss of some uncultured Clostridium and of bacteria belonging to the Rikenellaceae family. A Principal Component Analysis illustrates these changes induced by the retention time in each reactor (Fig. 2B): Microbial communities at the 2 different retention times coming from the same reactor shows a common clustering while microbial communities of the 3 reactors, whatever being the retention time applied, are more distantly related. Importantly, the first component (which accounts for more than half of the explanation of the differences among reactors, x-axis) indicates that the normal retention time induces a greater differentiation among colonic parts than the increased one. This is also particularly highlighted by the second most important principal component (y-axis), that supports 15.5% of the differences among samples (Fig. 2B).

Results

Retention Time Affects Overall and Specific Gut Microbiota Communities

The change of retention time passing from a total of 48 hours to 96 hours induced a significant decrease of biomass in the T96 and D96 reactors which simulate the transversal and distal colon respectively (Fig. 2A). The bacterial community was next determined at the bacterial family taxonomic level. Some diversity indexes were used to assess the richness, evenness, and species abundance in each compartment, and allowed underlining an overall effect of the treatment on the ecosystem. They indicated modifications, with Shannon diversity indexes in the T and D reactors decreased with the slower retention time compared to the quicker one (Fig. 2A). When considering the proportions of bacterial families in both conditions, the Clostridium cluster IX family (Clostridium cl. IX) was increased in the 3 reactors with the increased retention time. Other changes were dependent of each simulated region: the P reactor, simulating conditions similar to the proximal colon, was the least affected: the main modifications were the diminution of Clostridium cl. I and of the bacteria belonging to the family Desulfovibrionaceae. Furthermore, the apparition of bacteria from the family Enterobacteriaceae and the increase of Coriobacteriaceae were seen (Fig. 2A). In the T reactor simulating the transversal colon, an increase of the Clostridium cl. XIV with the higher retention time was observed associated with a decrease of the families Clostridium cl. I, Coriobacteriaceae, and Porphyromonadaceae, and a disappearance of Desulfovibrionaceae. Finally, the third reactor simulating the distal colon was characterized by the most important diversity and where the modifications were the most important: an increase of the relative abundance of Enterobacteriaceae, and in a lesser extent of Bacteroidaceae and Lactobacillaceae was observed. Inversely, there was a decrease of the relative abundance of Porphyromonadaceae and loss of some uncultured Clostridium and of bacteria belonging to the Rikenellaceae family. A Principal Component Analysis illustrates these changes induced by the retention time in each reactor (Fig. 2B): Microbial communities at the 2 different retention times coming from the same reactor shows a common clustering while microbial communities of the 3 reactors, whatever being the retention time applied, are more distantly related. Importantly, the first component (which accounts for more than half of the explanation of the differences among reactors, x-axis) indicates that the normal retention time induces a greater differentiation among colonic parts than the increased one. This is also particularly highlighted by the second most important principal component (y-axis), that supports 15.5% of the differences among samples (Fig. 2B).
in the first and second compartments. In the proximal-colon simulating reactor P96, this was principally due to the 3 major SCFAs (acetate, propionate, and butyrate) while in the transversal simulating part T96, a rise of longer, branched chains SCFAs and also of propionate and butyrate was observed (Table 2). In the third reactor D96, total SCFAs remains unaffected quantitatively, but a significant decrease of acetate was seen, partly compensated by a significant increase of some SCFAs with longer and branched chains. This reveals a change in fermentative activities indicative of a higher putrefaction activity with the increase of the retention time in last 2 reactors (T96 and D96).

Retention Time Affects Metabolic Behavior of Microbiota

A metabolomic analysis of the media of each reactor at both retention times was performed and revealed 554 different ions of over 100 Da (Supplementary Table). About half of them (259) were quantitatively affected, indicating important metabolic shifts in the system: the most affected compartment was the one simulating the proximal colon, with a majority of increases (167 of the 203 significantly modified ions, Fig. 3A). On the contrary, in the following compartments (T96 and D96) the majority of impacted ions decreased. This indicates likely a decrease of possible microbial dietary intakes in the lower parts of the 3S-ECSIM when retention time increases, due to better energy retrieval in the upper part. Globally these changes were region-specific with minor cases of shared changes throughout the compartments (5 ions, Fig. 3B). Overall qualitative changes with the increase of retention time were limited: only one ion appeared in P, and 13 and 6 metabolites disappeared in T and D, respectively. Using a Principal Component Analysis,
the first component (x-axis, accounting for about 38% of the differences between reactors) discriminates all 3 compartments under a normal transit time (P48, T48, and D48), while little differences are observed according to this component for the slow transit time. The P96, T96, and D96 compartments are inversely discriminated by the second component contributing also to a large part of the differences (about 32%, y-axis, Fig. 3C): the proximal and distal parts are metabolically different when comparing respectively each compartment at the 2 retention times highlighting a distinct metabolic behavior induced by a normal or slow transit time according to the gut-simulated section.

Discussion

The aim of this study was to determine to which extent the transit time, considered as an independent factor, could affect the

| Conc (mM) | P48      | T48      | D48      | P96       | T96      | D96      |
|-----------|----------|----------|----------|-----------|----------|----------|
| Acetate   | 50.2 ± 1.7 | 19.7 ± 1.5 | 26.1 ± 2.2 | 60.8 ± 1.5 \(^a\) | 19.9 ± 3.5 | 20.3 ± 1.4 \(^a\) |
| Propionate| 19.9 ± 0.6 | 5.4 ± 1.7 | 8.7 ± 1.1 | 22.7 ± 1.3 \(^a\) | 8.6 ± 0.9 \(^a\) | 8.6 ± 0.4 |
| Butyrate  | 14.1 ± 1.5 | 3.4 ± 0.1 | 9.0 ± 1.0 | 17.1 ± 0.4 \(^a\) | 9.5 ± 0.9 \(^a\) | 11.6 ± 2.5 |
| Total APB | 84.2 ± 0.7 | 28.5 ± 3.2 | 43.8 ± 4.2 | 100.6 ± 3.1 \(^a\) | 38.0 ± 4.8 \(^a\) | 40.5 ± 4.3 |
| Valerate  | 2.9 ± 0.1 | 0.8 ± 0.0 | 3.3 ± 0.6 | 2.8 ± 0.1 | 1.3 ± 0.2 \(^a\) | 1.7 ± 0.3 \(^a\) |
| Caproate  | 2.1 ± 0.1 | 0.7 ± 0.1 | 2.3 ± 0.1 | 2.0 ± 0.0 | 0.9 ± 0.0 \(^a\) | 1.8 ± 0.2 \(^a\) |
| Heptanoate| 0.7 ± 0.1 | 0.7 ± 0.1 | 1.6 ± 0.1 | 0.7 ± 0.0 | 0.7 ± 0.0 | 1.7 ± 0.3 |
| Isoheptanoate | 4.9 ± 0.3 | 1.3 ± 0.1 | 2.7 ± 0.4 | 4.7 ± 0.1 | 3.4 ± 0.0 \(^a\) | 2.8 ± 0.6 |
| Isovalerate| 4.2 ± 0.5 | 1.0 ± 0.1 | 2.5 ± 0.2 | 3.7 ± 0.1 | 3.5 ± 1.0 \(^a\) | 6.1 ± 1.0 \(^a\) |
| Isooctanoate| 2.5 ± 0.0 | 1.0 ± 0.1 | 2.3 ± 0.1 | 2.6 ± 0.1 | 1.4 ± 0.1 \(^a\) | 1.3 ± 0.1 \(^a\) |
| Total SCFA| 101.5 ± 0.4 | 33.8 ± 3.7 | 58.5 ± 5.7 | 117.0 ± 3.4 \(^a\) | 49.1 ± 5.9 \(^a\) | 55.7 ± 6.3 |

P48, T48, and D48 indicate respectively the P (proximal), T (transversal), and D (distal) reactor simulating a 48-hour transit while P96, T96, and D96 indicate respectively the P (proximal), T (transversal), and D (distal) reactor simulating an 96-hour transit. “Conc” stands for the concentration of short chain fatty acids (SCFAs) expressed in mM. Values reported in the table are corrected values (eg, the concentration in the reactor T48 corresponds to the concentration measured in the reactor to which the concentration of the reactor P48 was subtracted). "Total APB" indicates the total concentration of the 3 main SCFAs acetate, propionate and butyrate. "Indicates SCFA concentrations at the slow retention time which are significantly different (\(P < 0.05\)) to the values obtained in the same reactor at the normal simulated transit time.

Figure 3. Analysis of the metabolomic data obtained for each reactor at the 2 different simulated transit times. (A) Graphic representation of the proportion of ions which are significantly increased (blue) and decreased (red) in the 3 reactors with the simulated increase of transit time. (B) Venn diagram of the 259 ions which are significantly modified with the increase of the simulated transit time, showing shared ions among the different simulated anatomical parts. (C) Correlation matrix based Principal Component Analysis of metabolites profiles in each reactor (empty symbols, 48-hour transit time simulation; filled symbols, 96-hour transit time simulation; circles, proximal part; squares, transversal part; triangle, distal part). The arrows indicate the metabolic trajectory from the proximal part to the distal one at the 2 transit times.
microbiota and consequently its metabolic activity using a multi-compartmental in vitro continuous culture system (ECSIM) simulating the different physicochemical conditions encountered in the colon. Numerous systems have been developed and allowed to study the impact of prebiotics, probiotics, and antibiotics on the gut microbiota, or even the microbial composition at colon micro-environments.\textsuperscript{26-29} The system used here has the particularity to maintain anaerobia due to the metabolic activity of the microorganisms constituting the microbiota: therefore, it leads to atmospheres that are variable among fermenters and whose constitution influences the microbial metabolism. In consequence, it is believed to simulate more closely the physiology of a human gut. It also enables microbiota differentiation between the compartments from a common fecal bacterial repertoire, and leads to similar microbial signatures between the distal compartment and the fecal inoculum.\textsuperscript{18} In the present study, we increased the retention time of media in each compartment in order to simulate an increase of transit time, from a normal intestinal transit time of 48 hours to a slow one of 96 hours. This was realized by adjusting media supply rates, with no other parameters changes in the entry of the in vitro system.

This retention time change induced modifications to the reconstructed gut microbiota such as a reduction of biomass, which is in line with in vivo modifications reported with a medication-induced reduction of transit time in humans.\textsuperscript{11} However, in vivo data on the effect of constipation and slow transit time in the general population and on their gut microbiota are sparse.\textsuperscript{1} Moreover, it is difficult to differentiate the effects due to transit time from other factors such as diet. The gut microbiota of elderly people is studied much more and more data are available.\textsuperscript{7-10,30} Aging is frequently associated with fecal microbiota modifications which can be explained by many different factors.\textsuperscript{7,31} Diet is a key factor\textsuperscript{11} and aging has been associated to a decrease sensitivity of taste and smell,\textsuperscript{32,33} so these changes could lead to the consumption of a restricted, nutritionally imbalanced diet and in fine the modification of the gut microbiota. Here, we hypothesized that an increase of transit time which is frequently associated with elderly people could be one of those factors.\textsuperscript{34} Indeed, in baboons, aging leads to an abnormal neuronal stimulation from different factors.\textsuperscript{5,6} Furthermore, increasing the retention time decreased the gut microbiota diversity in the transversal and distal compartment of the system comparably to the fecal microbiota of elderly people.\textsuperscript{8,9,35-37} Consequently, an important metabolic shift, mainly at a quantitative level was observed, the proximal colon simulating reactor being the most affected.

The important metabolic shift in the proximal part together with the increase of total SCFA when retention time increases suggests an increase of carbohydrate fermentation in this compartment as previously reported.\textsuperscript{15} This has several consequences: first, carbohydrate availability become lower in the next compartments, resulting in increased putrefaction by specialized groups, and, second, this induces a decrease in microbial diversity: this is also confirmed by an increase of concentrations of longer and branched fatty acids (isovalerate and isobutyrate typical of protein fermentation), and with the limited metabolic activity in the compartments simulating the transversal and distal colon.

\textit{Clostridium} cl. IX spp. increased in the 3 compartments of our system, principally in the first compartment: this family includes bacteria belonging to the \textit{Veillonella} genus which are however not capable to use carbohydrates as an energy substrate: they metabolize lactates into propionates and use amino acids as an energy source.\textsuperscript{38} Therefore their increased abundance in the first and second reactors likely increased the level of propionate. Furthermore, in the second and third fermentors the increase in branched fatty acids would suggest an increase of proteolytic bacterial activity that is frequently reported in elderly people.\textsuperscript{10,29} Also, our results support the decrease of \textit{Porphyromonadaceae} and of \textit{Rikenellaceae}\textsuperscript{8,31,40-42} and the increase of \textit{Enterobacteriaceae}\textsuperscript{8,31,40-42} reported in the gut microbiota of elderly people. Last, no changes in \textit{Bacteroidaceae} were observed in any reactors, agreeing with the recent finding that these bacterial species were associated with loose stool.\textsuperscript{3} Therefore, in vitro results obtained in this study provide strong elements that transit time seems a major driven force of the gut microbiota composition.

It also provides clues about the prevalence and abundance of methanogenic archaea, and among them, \textit{Methanobrevibacter smithii}, the most important one, is increased in elderly people.\textsuperscript{43,44} They are described as being correlated with an increase of transit time and inversely correlated to sulfate reducing bacteria.\textsuperscript{12} Here, the initial stool from a healthy adult was chosen without methanogens, which was confirmed by the absence of CH\textsubscript{4}, in the gas produced by any reactor. In a recent study, we showed that the inoculation and development of \textit{Mb. smithii} induced a specific increase of \textit{Clostridium} cl. IV and cl. XIV,\textsuperscript{46} agreeing with one of the 3 human enterotypes, the so-called \textit{Ruminococcus-Mb. smithii} one.\textsuperscript{1} Here also, without methanogens, and mainly in the second reactor, the increase of retention time induced an increase of \textit{Clostridium} cl. XIV,
possibly providing a favorable ecological nest for methanogens: 2 complementary non-exclusive mechanisms may be proposed: (1) in a similar way described for this methanogen and _Bacteroides the- taiotaomicron_, a mutualistic partnership may exist between these 2 groups, as _Mb. smithii_ in turn favors the communities that degrade cellulose. Moreover, (2) increasing the retention time led to the decrease of one major competitor group of methanogenic archaia, the sulfate-reducing bacteria (disappeared in the T96 reactor), where _Clostridium_ cl. XIV is increased the most. This has important physiological issues as it has been proposed that methane, one end-product of the gut metabolic processes, mediates slower transit time. If this is true, it would therefore sustain or even favor the slower transit time of elderly people. In consequence, we also tested the liquid media from reactors at various dilutions toward a direct action on the gut motility: the generated metabolites either retrieved from the elevated retention time or the normal retention time did not affect rats’ duodenum motility ex vivo on isolated organs (data not shown). Therefore, it seems that these metabolite changes do not in turn affect the transit time.

In vitro systems have several limitations with their reproduction of the colonic environment. The main limitation is that the interactions between the microbiota and the host are not reproduced: among them, the absorption of water and of nutrients leads in vivo to fecal consistency modifications, and together with peristalsis (also not simulated in ECSIM) has likely an effect on microbial development. However, our results show that such systems can replicate modifications seen in the gut microbiota of elderly people. The development of such models could help in understanding the impact of a factor on the gut microbiota and studying how some treatments could overcome deleterious bacterial developments. In vitro systems do not require the validation by ethic committees and could allow pre-clinical tests.

In conclusion, although in vitro systems present some limitations, they allowed the independent study of the effect of a single factor. The one used here revealed that transit time and simulated through retention time is a driven force of the gut microbiota and has very likely a physiological incidence in slow transit-time or constipated patients. The transit time explains, at least partly, modifications seen in the gut microbiota of elderly people, inducing the structural and metabolic changes of the gut microbiota with other capital elements such as nutrition and health.

**Supplementary Material**

Note: To access the supplementary table mentioned in this article, visit the online version of _Journal of Neurogastroenterology and Motility_ at http://www.jnnjournal.org/, and at https://doi.org/10.5056/jnm16042.

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