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# The ECSIM Concept (*Environmental Control System for Intestinal Microbiota*) and Its Derivative Versions to Help Better Understand Human Gut Biology

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## 1. Introduction

Each meal reminds us of our energy needs. As heterotrophs, we have a whole set of organs for food digestion and conversion into substances usable by our body. The breakdown of food (mainly proteins, carbohydrates and lipids) takes place in the gastrointestinal tract (GIT) which assimilates these elements and transfers them into the bloodstream. In a simplified manner, these digestion and absorption functions are provided by the following organs: the mouth, oesophagus, stomach, the small intestine (including the duodenum, jejunum and ileum), the large intestine or colon (formed from the right, transverse and left colon), ending with the rectum and anus. Various functions are required to facilitate the hydrolysis of food. They include a combination of physical factors (temperature, pH, grinding and friction, ...), chemical factors (acid secretions, bile salts, ...) and enzymes (salivary, gastric or pancreatic enzymes,...). Colonic physiology also benefits in parallel from a real contribution by micro-organisms, specifically bacteria.

Several factors contribute to our interest in the human digestive tract. For example, in Europe colorectal cancer is the second cause of cancer in women and the third in men (Boyle & Ferlay, 2005). Some pathologies can be chronic, infectious or even mortal. The digestive tract is also a simple and practical way of giving chemotherapy treatment, whether the pathology is of digestive origin or not.

Proposing more or less advanced simulation systems of the GIT, therefore, can overcome certain ethical, technical and/or financial difficulties in research. Furthermore, it is interesting to model the way the digestive tract works, in order to test various elements independently and/or concomitantly: such as drugs, food/nutrients, microbial agents, even physiological and physical elements.

Animal models work particularly well if they have a similar anatomical/physiological GIT. Rodent models, however, are used more in the laboratory for practical reasons. In order to enhance the resemblance to humans, "humanized" animal GITs have been developed from germ-free, newborn animals turned into gnotobiotic animals after being seeded by a human

microbiota (see for example (Samuel & Gordon, 2006). In a more controlled manner, *in vitro* models are also alternatives which may answer biological questions, in an easier, more complete and less partial manner. In all cases, however, *in vitro-in vivo* correlations have to be established.

Our laboratory is particularly involved in the development, elaboration and use of *in vitro* devices able to reproducibly simulate the behaviour of elements in the digestive tract, with special emphasis on the human digestive tract. This involves the study of food, medicines, prebiotics, probiotics and infectious agents in the digestive tract. In this article we will particularly address the workings of the colon and its metabolic and physiological roles which still remain largely unknown. We will also present a fermentation system called ECSIM (Environmental Control System for Intestinal Microbiota), which is a modular system consisting of three reactors from GPC (Global Process Concept, [www.gpcbio.com](http://www.gpcbio.com)). It can be used in various configurations to mimic the different functions of a human colon depending on whether a simulation of a portion of the colon (for example, P-ECSIM for a simulation of the proximal part) or the colon in its totality and continuity is needed (for example, 3S-ECSIM for 3-stages-ECSIM, for the proximal, then transverse, then distal part). Figure 1 shows the general principle of the equipment.

#### Computer-controlled Bioreactors

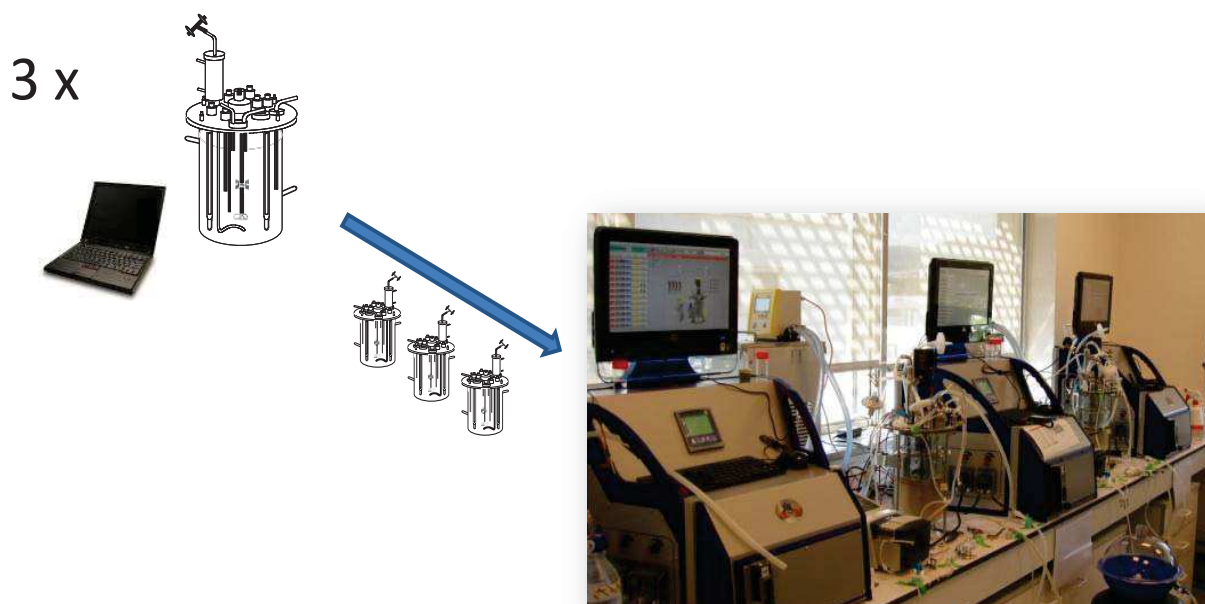


Fig. 1. A general view of the ECSIM platform permitting the simulation of the human gut.

Three computer-controlled bioreactors from Global Process Concept ([www.gpcbio.com](http://www.gpcbio.com)) are individually or collectively used as one modular system.

## 2. GIT, colon and gut microbiota

Simulating some or most of the functions of the digestive tract requires an accurate knowledge of the components of the GIT, at both the descriptive and functional levels. This is particularly true for the colon, which is different anatomically and functionally compared

to the other parts of the GIT. Water and electrolytes are reabsorbed at this level of the tract, leading gradually to a solidification of faecal matter all along the colon. It also involves an intensive microbial fermentation process: matter which was either only partially digested or not at all digested in the upper parts of the GIT (rapid transfer from the upper part preventing total digestion, absence of necessary enzymes, limiting physicochemical conditions, ...) or finally because these elements do not come from alimentation but from the digestive tract itself (digestive secretions like the epithelial mucins, dead bacterial and human cells,...).

There are 3 distinct parts in the colon: the right (or ascending) side consists of the caecum and proximal colon; the central transverse colon; and the left side (or descending colon) consisting of the distal colon, the sigmoid colon and the rectum.

An important and specific microbial population inhabits each of these levels, whose density is incommensurate with the previous stages (Table 1), reaching values of  $10^{12}$  bacteria per gram of colonic contents (Savage, 1977).

		Length (cm)	pH	Usual transit time (H)	Microbiota abundance per gram	
<b>Stomach</b>		12	1 - 2.5	2 - 6	0-10 <sup>4</sup>	
<b>Small intestine</b>	Duodenum	25	6-6.5		10 <sup>4</sup> - 10 <sup>5</sup>	
	Jejunum	160	6.5	3 - 5	10 <sup>5</sup> - 10 <sup>7</sup>	
	Ileon	215	7.5		10 <sup>7</sup> - 10 <sup>8</sup>	
<b>Large intestine</b>	caecum	6			10 <sup>10</sup> - 10 <sup>11</sup>	<b>Matter entering the colon</b>  <b>(g per day)</b>  <b>Polysaccharides</b> 10-60g <b>Proteins</b> 6-18g <b>Lipids</b> 5-8g
	Ascending colon	10 - 15	5.5-6.0		10 <sup>10</sup> - 10 <sup>11</sup>	
	Transverse colon	50	6.0-6.5	50 - 70	10 <sup>10</sup> - 10 <sup>11</sup>	
	Descending colon	25	6.5-7.0		10 <sup>10</sup> - 10 <sup>11</sup>	
	Sigmoid colon	40 - 80			10 <sup>10</sup> - 10 <sup>11</sup>	
	Rectum	18		1	10 <sup>10</sup> - 10 <sup>12</sup>	

Table 1. Some physical, physiological and microbial characteristics of the human GIT.

An estimation of the quantity of matter entering the large intestine is given in the column on the right (adapted from A. Mihajlovski and G. Macfarlane & J. Cummings (G.T. Macfarlane & Cummings, 1991; A. Mihajlovski, 2010)).

The term “microbiota” is used to define this set of micro-organisms living together within our bodies. The genomes of these “indigenous microbes” are known as the “microbiome”, the microbial counterpart of our genome. B. Zhu and his colleagues (Zhu et al., 2010) state that this was suggested in 2001 by the Nobel Laureate Joshua Lederberg and by Hooper & Gordon (2001). “Microbiome” can be substituted by the more general term “metagenome” when concerned with environmental genomic studies (“metagenomics” (Handelsman, 2004)). The authors of a recent article did not hesitate to use the terms “human gut microbiome” as “the second genome of human body” in their title (Zhu et al., 2010) and this

enthusiasm also accompanies the results of recently obtained gut metagenomics (Qin et al., 2010).

In the early 2000s, once the human genome had been decoded, some scientists called for a collective effort to study global metagenomics to decode all of the bacterial communities inhabiting the human body, from the external communities of the skin, to internal ones, such as oral, intestinal and vaginal flora. The creation of a second “human genome project” (Relman & Falkow, 2001), studying the whole human flora, would complete our knowledge of the human genome. In 2005, the French “National Institute of Agronomic Research” (INRA), initiated an international discussion on the opportunity of structuring an intestinal microbiota research effort. This led to the creation of the project MetaHIT, “Metagenomics of the Human Intestinal Tract”, supported by the Seventh Framework Program of the European Union ([www.metahit.eu](http://www.metahit.eu)). In parallel, in 2007 the American National Institute of Health created the Human Microbiome Project (HMP, <http://nihroadmap.nih.gov/hmp/>). Its purpose was to address not only the intestinal tract, but also the skin, mouth and vagina (Peterson et al., 2009). For the intestine, the approach was to directly sequence 1,000 bacterial genomes usually found in this habitat, and in parallel determine the metagenome of 250 human guts. The results are expected in 2014.

### **2.1 Diversity and repartition of human gut microbiota**

If we take a particular look at the microbes in the colon, a great diversity is observed in the part where the microbial density is highest. An overview of the various microbes found shows that planktonic forms predominate in the lumen, and biofilms are principally encountered on the epithelium. In addition, material in transit, in the process of being metabolized, is also colonized by micro-organisms, which move with the matter. Because of the gradual disappearance of oxygen along the digestive tract, and a very negative redox potential (ORP), the main bacterial populations are facultative or strict anaerobic species.

Before the creation and development of molecular microbial ecology tools, studies were based almost entirely on the use of selective or non selective culture media. Such studies were completed by biochemical, metabolic and morphological (microscopic examination and Gram stain) information. There were large discrepancies, however, between the data produced by culture and microbial counts performed by Fluorescent In Situ Hybridization (FISH). In fact, studies with DAPI staining (- 4', 6'-diamidino-2-phenylindole) revealed only 15% of cultured bacteria were found by counting (Langendijk et al., 1995). Since then, numerous molecular methods have been developed to overcome the limitations of microbial culture and assess the number of bacterial species in the digestive tract. The first methods used were based on the development and sequencing of the clone library of bacterial 16S DNA, using techniques that permitted to consider max 50% (even 10%) being cultivated species (Ley et al., 2006; Zoetendal et al., 2004).

It is particularly difficult to precisely define both the concept of species in bacteria and the justification of the rules used for these definitions. Molecular methods are used to define groups of micro-organisms based on their identity for sequences, principally those of 16S RNA considered in part or almost in its entirety. This defines operational taxonomic units (OTUs) on the basis of variations of 1, 2, 3 or 5% among these sequences (or 99%, 98%, 97% or 95% of their identity). It is generally accepted that 98% of the identity used to define phylotypes may be related to the concept of species. On this basis, 500 to more than

1,000 different species have been detected in the gut microbiota of 3 different individuals (Eckburg et al., 2005). M. Rajilić-Stojanović and her colleagues compiled all data available up to 2007 in a meta-analysis and identified 1,200 different phylotypes (Rajilic-Stojanovic et al. 2007). Recent technological advances in sequencing technology, such as the 454 technology of Roche Diagnostics Applied Science or Solexa/Illumina, have facilitated inventorying an even larger number of colonic bacteria (Claesson & O'Toole, 2010; Murphy et al., 2010; Tasse et al., 2010). D.N. Frank and N.R. Pace used metagenomics data to deduce the presence of 15,000 to 36,000 different bacterial species, with variations due to the molecular criteria chosen (Frank & Pace, 2008). It has recently been shown, however, that part of the bacteria are universally shared between individuals (Tap et al., 2009). The work defined a "universal core", more or less large, depending on the degree of sharing among humans. It is important to note that, although the number of non-cultivable species seems very large in these studies, metagenomics enables a "gene catalogue from the human gut microbiome" to be established (Qin et al., 2010). It is theoretically possible, therefore, to extrapolate the activity or the metabolic potential of microbiota.

Unfortunately, a very important part of colonic microbiota remains uncultivable as isolated species. This can be explained by a particularly high sensitivity to O<sub>2</sub>, even at low levels of exposure (amount, duration), a high dependence on certain elements not present in the media being used, and a strong dependence on other microbial species providing such elements. It is interesting, therefore, to develop cropping systems which maintain consortia in vitro, and allow the study of biological interactions and ecological and functional relationships between the various members of the colonic ecosystem. Baoli Zhu et al. (2010) indicate that recent results in in vitro systems can detect 50 to 70% of sequences belonging to "uncultivated bacteria". This new data makes current vocabulary inappropriate and requires a rapid evolution and/or a redefinition of the terms being used. For example, one can question the legitimacy of the finality of such terms as "non-cultivable".

The role of the bacterial community should not obscure the importance of other microorganisms. Two other domains of life which must not be forgotten can be found in this ecosystem: eukaryotic cells (e.g. *Blastocystis* spp.) and yeasts (e.g. *Candida* spp.) (Scanlan & Marchesi, 2008), and Archaea, which includes methanogens able to synthesize methane from CO<sub>2</sub> and H<sub>2</sub> from the metabolism of other microbiota microorganisms (Mihajlovski, Doré, Levenez, Alric, & Brugère, 2010; Strocchi, Furne, Ellis, & Levitt, 1991). Some Archaea may also possess other features, closely related, or not, with non-usual methanogens, i.e. potential Thermoplasmatales (Mihajlovski, Alric, & Brugere, 2008; Mihajlovski et al., 2010) or halophilic archaea (Oxley et al., 2010). Furthermore, the presence of a considerable diversity of viral genotypes is also important, as about 1,200 have been described to date (Breitbart et al., 2003). This could have a significant functional and metabolic implication, not per se, but through the role they play in regulating bacterial populations.

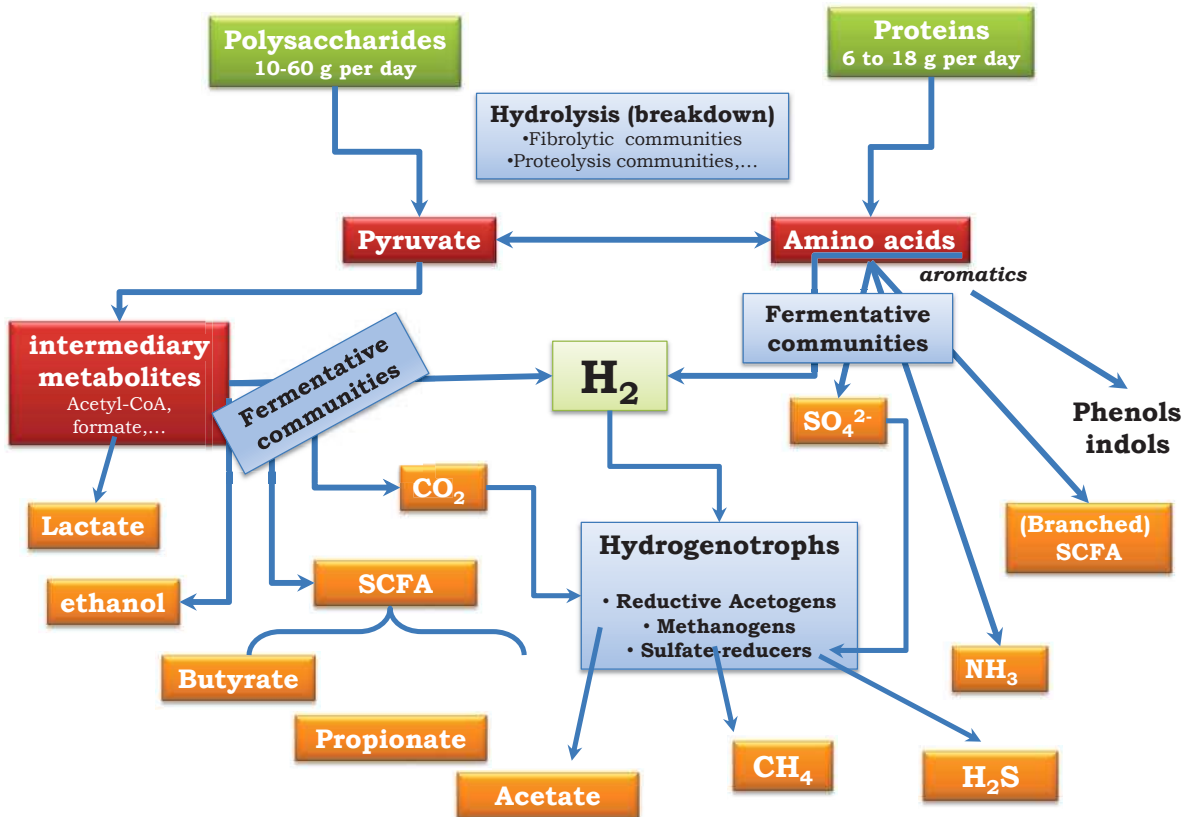
## 2.2 Properties, functions and evolution of gut microbiota

It is important to remember that the contribution of intestinal microbiota is a major, though not essential, factor in the life of the host (germ-free mice are viable but present various alterations). The microbiota acts as a physical barrier to exclude pathogens by coating the mucosa and by inducing antimicrobial peptide production by Paneth cells (Hooper et al., 2003). It also contributes to the maturation, morphology and maintenance of the digestive

tract (Hooper et al., 2002), reduces inflammatory response and the number of Peyer's patches, and promotes angiogenesis (Stappenbeck et al., 2002). Furthermore, it is responsible for the production of available vitamins, especially Vit. B9, Vit. B12 and Vit. K (Hill, 1997) and is a source of energy, *per se* and through its metabolism. For example, germ-free mice implanted with a microbiota show a 60% increase in body-fat (Backhed et al., 2004), mainly due to the resulting and excess of energy-release from the fermentation of undigested food residue. Many other research works are being undertaken to understand the relationships linking the microbiota to the regulation of fat storage, or more broadly, how gut microbiota may influence metabolism and body composition (Vrieze et al., 2010).

Microbiota metabolic properties have different functional groups which can be identified according to their metabolic capabilities (including the use of substrate), creating dependencies and mutualistic relationships between organisms. The colon is, therefore, the centre of an intense fermentation activity. The origin of fermented substrates may be due to the non-use, partial-use or total-use of food/nutrients in the upper compartments (right part of Table 1). This may be caused by the transit time being too rapid and not allowing full treatment in the upper parts. Most often though, it is due to the absence of necessary enzyme activities and to unfit physicochemical conditions (especially pH). Other substrates, such as endogenous substrates, may come from the host itself: for example secreted mucopolysaccharides lubricating the digestive tract (mucins), digestive enzymes, cellular debris and even microbial debris.

Less than 100 g of exogenous substrates reaching the colon and available for microbial conversion (Cummings & Macfarlane, 1997) can produce 10% of our energy needs through their metabolism by the colonic microbiota (G.T. Macfarlane & Cummings, 1991). As mentioned in Table 1, these substrates are mostly complex polysaccharides and proteins with some lipids and nucleic acids. Out of the 100 g the complex polysaccharides alone account for 10 to 60 g. These are the substrates for which metabolic pathways are best known. The many different microorganisms and enzymes which provide the first steps of microbial conversion (fibrolytic flora) are still being characterized. The contribution of multiple microbial groups is necessary at this stage (Figure 2). The hydrolysis of polysaccharides by fibrolytic bacteria leads to simple compounds which are, in turn, used by each member of the fermentative microbiota to produce pyruvate. Depending on which microorganism is present, different reactions will then lead to the formation of lactic acid and short chain fatty acids, with over 90% being three major compounds: propionate, butyrate and acetate (the last being in amounts two to three times greater than the other two). Although synthesis takes place mainly in the ascending part of the colon (main area of polysaccharides fermentation), the concentrations observed in humans are nevertheless fairly constant (around 120 mM) in the other parts due to a decreasing absorption along the large bowel (G. T. Macfarlane, Gibson, & Cummings, 1992). Another consequence of this fermentation is the production of gases, particularly CO<sub>2</sub> and H<sub>2</sub>. The presence of the latter limits feed-back on the effectiveness of this fermentation. It is necessary, therefore, to eliminate the presence of H<sub>2</sub>, which is realised by hydrogenotrophs. This dedicated microbiota is variable among individuals and can eliminate the H<sub>2</sub> in 3 different (sometimes combined) anaerobic reduction processes: sulfate-reduction (synthesis of H<sub>2</sub>S from sulfate as acceptor of electrons) realised by sulfate-reducing prokaryotes (SRP), reductive acetogenesis (reduction by a combination of CO<sub>2</sub> and a methyl) realised by acetogens, and hydrogenotrophic methanogenesis (CO<sub>2</sub> reduction to methane) realised by methanogenic archaea.



adapted from A. Bernalier-Donadille (2010).

Fig. 2. A schematic overview of the microbial fermentative metabolism in the human colon

The properties and functions of the intestinal microbiota are varied, complex and probably partly unknown. An *in-vitro* system, even with its limitations, could provide a simplified way of addressing various biological questions and problems. There is, therefore, a need to develop *in-vitro* models allowing a controlled approach to physical, chemical and microbiological parameters. As it appears to be extremely difficult to grow a significant part of the microbiota, artificially replicating a colon may provide a solution for growing microbiota as a consortium, and make it easier to determine the impact of environmental factors (*i.e.* all that is external to the microbiota) on its composition and metabolism. This requires a good knowledge of such environmental conditions in order to replicate them *in vitro*, and to have already measured various biological parameters *in vivo* in order to validate the *in vitro* results. Table 1 shows some of the results. The parameters vary with the specificity of nutrition, age and some diseases. The developed model, therefore, should ideally consist of a dynamic, multi-set system, easily adaptable to any simulation requirement, physiological or pathological situation.

### 3. The ECSIM paradigm

#### 3.1 Simulating gut fermentations: a rapid overview of existing systems

Many *in vitro* systems have been developed to answer biological problems relating to the gastrointestinal tract. For example, there are systems to simulate chewing and to



reproduce the size of masticated food in the presence of artificial saliva (Woda et al., 2010). Some tools focus on the digestive kinetics of food and drugs, others are limited to the study of *in-vitro* dissolution [see the various equipments of the pharmacopoeia, USP 1, 2, 3 and 4 (Pharmacopoeia, 2011)] or conversely, to replicate kinetic absorption by simple or more complex systems of intestinal cells. In general these systems replicate the different compartments of the GIT anatomically and physico-chemically, with or without a gradual/discontinuous evolution of the conditions encountered along the digestive tract. For example, a simple thermostatic beaker may be sufficient to replicate a basic stomach; a highly acidic pH, controlled/modulated in a liquid medium with ions, bile salts, gastric enzymes, may be sufficient to control the release kinetics of encapsulated active ingredients. The study of colonic fermentation is based on models of varying complexity. George & Sandra Macfarlane (G. T. Macfarlane & Macfarlane, 2007) describe the motivations for using such systems and the essential features found in these models. Generally, these tools range from simple batch cultures to continuous culture systems. The choice of the model is based on the advantages/disadvantages of each to respond to specific biological problems. Various factors must be taken into account and comparisons made relative to the degree of reliability of what actually happens *in vivo* : for example, the ease of experimentation, the amount of experiments (acquisition time and speed), the number of environmental factors which need to be controlled (few/many), the price. This chapter includes, in our opinion, the three systems most likely to replicate the complexity of the colon in a controlled manner.

The SHIME [Simulator of the Human Intestinal Microbial Ecosystem (Molly et al., 1993)], recently available in two identical parallel models (TWIN-SHIME (Van den Abbeele et al., 2010)), is a composite system consisting of five double-jacketed vessels of various volumes, each replicating a particular part of the gastrointestinal microbial ecosystem. It simulates the stomach and the small intestine with two vessels of 0.3 L, and the large intestine with three vessels reproducing three compartments at constant volume and with pH control.

A second system was developed by the TNO in Holland, with several types and two functions. The TIM-1 (TNO Intestinal Model 1) is an *in-vitro* model of the upper part, with compartments replicating the stomach and the three parts of the small intestine (duodenum, jejunum and ileum) (Minekus et al., 1999). This is mainly achieved by a simulation of the saliva flow, gastric and pancreatic juices, and the peristaltic mixing of the chyme. Physical and chemical factors such as the transit time, temperature and pH control, and removal of digested compounds using hollow fibre membranes, are also used. This model seems useful for studying what happens to macro/micro-nutrients and the survival/stability of probiotics in the upper part of the GIT. A second TIM (TIM-2 for TNO Intestinal Model 2) simulates the colon, by controlling pH, anaerobiosis, and the gradual intake of media, as it would be with the upper part metabolism of food (Rajilic-Stojanovic et al., 2010; van der Werf & Venema, 2000).

The final model was also developed in Europe, by the laboratories of George Macfarlane and Glenn Gibson in the United Kingdom (Macfarlane & Gibson, 1998). It is based on a compartmentalized system for simulating the different parts of the human colon (proximal, transverse and distal). An anaerobic continuous culture system is distributed among the different compartments and simulates the continuity of the colon. Our three-stage system (3S-ECSIM) is strongly inspired by this equipment, and is based on the same principles, except for maintaining anaerobic conditions.

These systems, however, do not integrate the epithelial, neuroendocrine and immune host components, but can include means for simulating bacterial biofilms such as those observed on the colonic epithelium or the food in transit (Macfarlane & Macfarlane, 2007).

Core Medium		Trace elements solution 1 mL per L of core medium		Vitamin solution 1 mL per L of core medium	
Elements	g.L <sup>-1</sup>	Elements	mg.L <sup>-1</sup>	Elements	mg.L <sup>-1</sup>
Mucin	4.0	MnSO <sub>4</sub> . 2H <sub>2</sub> O	500	Menadione	1
Starch	5.0	FeSO <sub>4</sub> . 7H <sub>2</sub> O	100	d-biotin	2
Pectin	2.0	CoSO <sub>4</sub>	100	Pantothenate	10
Guar gum	1.0	ZnSO <sub>4</sub>	100	Nicotinamide	5
Xylan	2.0	CuSO <sub>4</sub> . 5H <sub>2</sub> O	10	Vitamin B <sub>12</sub>	0.5
Arabinogalactan	2.0	AlK(SO <sub>4</sub> )	10	Thiamin	4
Inulin	1.0	H <sub>3</sub> BO <sub>3</sub>	10		
Cystein	0.8	Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	10	<i>para</i> -aminobenzoic	5
Casein	3.0	NiCl <sub>2</sub> . 6H <sub>2</sub> O	100	acid	
Peptone	5.0	Na <sub>2</sub> SeO <sub>3</sub>	10		
Tryptone	5.0				
Yeast extract	4.5				
Bile salts	0.4				
Tween 80	1.0				
Fe <sub>2</sub> SO <sub>4</sub> . 7H <sub>2</sub> O	0.005				
NaCl	4.5				
KCl	4.5				
KH <sub>2</sub> PO <sub>4</sub>	0.5				
MgSO <sub>4</sub> . 7H <sub>2</sub> O	1.25				
CaCl <sub>2</sub> . 6H <sub>2</sub> O	0.15				
NaHCO <sub>3</sub>	1.5				
Hemin	0.05				
<i>pH adjusted to 6.0</i>					

Table 2. Composition of the artificial gut medium

This medium is derived from those previously described (Macfarlane et al., 1998; Molly et al., 1993) and is a mix of three solutions: a trace elements solution, a vitamin solution (1 mL of each for 1 L of artificial gut medium) and the core-medium.

### 3.2 General description of the ECSIM systems

To complete these *in-vitro* systems, we have developed our own infrastructure to simulate the human colon, with the goal of having a modular system for maintaining a human microbiota in similar environmental conditions to those encountered physiologically and pathologically in the human gut. This system was developed with the technical support of Global Process Concept (France, see [www.gpcbio.com](http://www.gpcbio.com) for more information). It consists of three bioreactors which can operate independently, but also may be associated (see the next chapter 3.3). It is possible to supply each bioreactor with an identical nutritive medium from a unique tank or to supply each with a different medium. The usual medium faithfully replicates the contents of the terminal ileum from an individual with a Western diet, as

described by several groups (Macfarlane et al., 1998; Molly et al., 1993). This basal medium is shown in Table 2. If required, it can be modified to simulate other systems or to simulate specific cases, such as the uptake of prebiotics, the excessive presence of protein residues due to poor hydrolysis/absorption in the anterior parts, or an excess of bile acids, ...

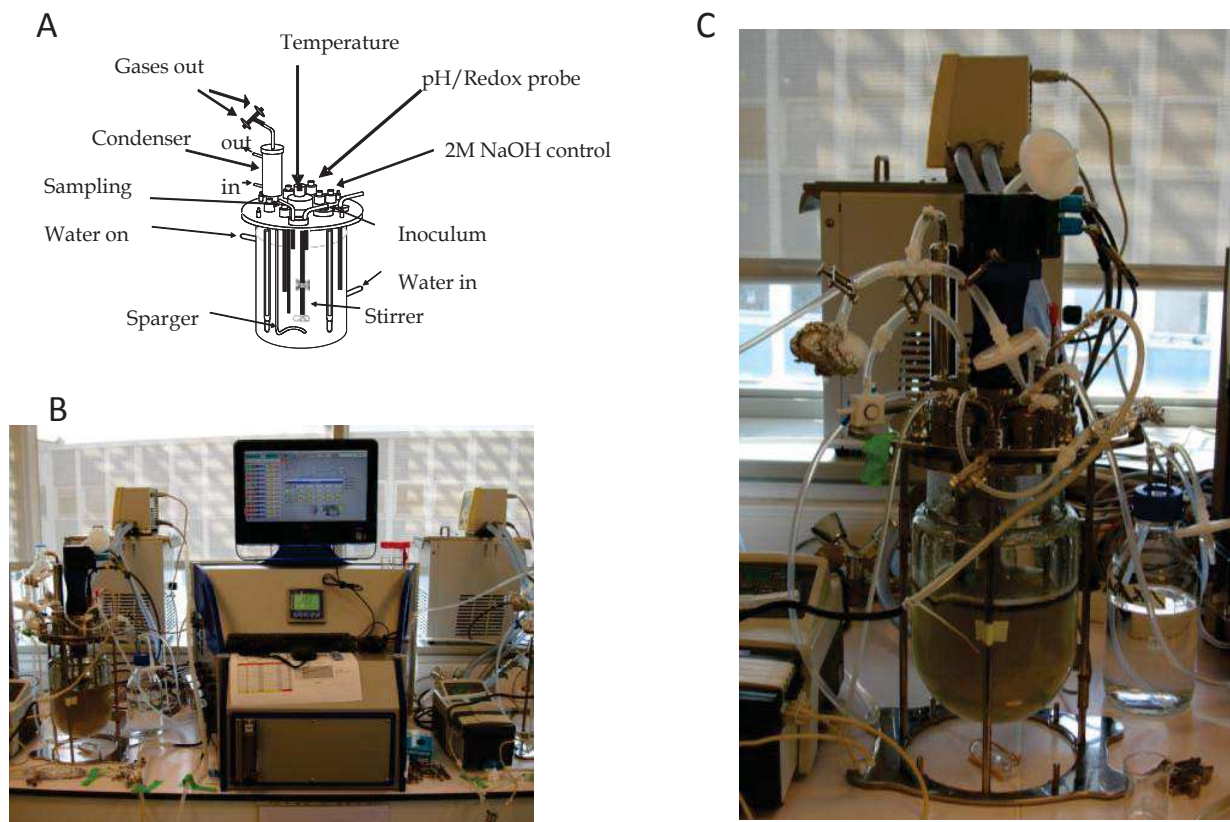


Fig. 3. Description of one module of the ECSIM, a GPC™ bioreactor.

A- Schematic representation of the tank and connections. B- General view of the complete module, command system and the heating/cooling water system (behind). C- Detailed view of the bioreactor.

Each modular system is based on a bioreactor that can operate autonomously if necessary (Figure 3). Each bioreactor is composed of a tank and an upper plate. The 2-L tank is made from borosilicated glass, mounted on a removable stainless steel frame. Its minimum useful volume is 0.5 L, however, it is used in all our different ECSIM variants with a volume of 1L. The tank has a jacket permitting the circulation of water from its own heating module (tank with electric heater and circulating pump) to maintain the temperature of the culture. The top plate (stainless steel) has various ports and accessories allowing the use of an agitation motor, an aeration at the tank bottom through a removable sparger, and a gas outlet equipped with a stainless steel condenser (for a max flow of 15 L/min) with an expansion chamber and internal coil for circulating cold water. The stirring inside the tank is made in a pendular motion using a Rushton turbine and a marine propeller (each with a diameter of 60 mm) with adjustable height on the stirrer shaft. The system incorporates a temperature sensor, pH electrode, redox electrode, a liquid level or foam (modular) sensor, and an injection input for pH correction (which can be used for the substrate). The plate has a sterile

sampling device for sterile medium inoculation, addition and sampling. Each bioreactor has its own control terminal connected to a PC via an Ethernet cable. Dedicated software is used to control the stirring speed (variable from 40 to 1000 rpm), temperature, pH, ORP and the level regulations and measurements. In parallel, the terminal controls four peristaltic pumps (to ensure the addition of nutrient solutions, neutralizing solutions and tests) and valves to automatically control the gas flow. The dedicated software (using Windows™) allows a hierarchical control of experiments (4 levels), ranging from single view measurement tables in real time without the possibility of intervention through access, to full functionality (detailed configuration and complete opportunity to exit the software and turned off the set).

### **3.3 Coupling/associating ECSIMs to generate other *in vitro* systems**

The following describes the use of ECSIM as a chemostat, although sometimes it may be (and is) used for batch cultures. One of the advantages of the ECSIM system is its modularity which allows a variety of different applications using the same components. Our approach consists of three initial modules which can produce three different scenarios depending on their settings (Figure 4). The first case considers these three systems as being totally independent, with each bioreactor having its own experiment controls (left, Figure 4).

This approach provides rapid responses to various issues. If the effect of a component (biotic or abiotic) on the particular microbiota of different individuals has to be addressed, each bioreactor is inoculated with a particular microbiota, and as experimental conditions are identical, the incubation medium is shared between the different systems. If the experiment concerns the dose-effect or formulation of a compound, each bioreactor is started with strictly identical conditions (culture medium, inoculum, pH,...), with each bioreactor pump providing a controlled release of different amounts of the compound from the same stock solution. Other alternatives can be implemented using the same principle. In these cases, the proper control of each bioreactor is provided by a comparison test carried out before, between and after any modification is made. Controls are sampled after obtaining stability, which is considered acceptable after at least 5-times the retention time of the medium in the bioreactor. The experimental measurements are then retrieved either kinetically, during the addition (progressive or not) of the compound, or after re-obtaining a new state of equilibrium, or both. The results can be complementary and depend on the desired information, the disruption of the system itself (specific metabolites of this phase, responsible mechanisms, ...) or the final long-term effect of waiting for the new induced equilibrium.

It is possible that the extreme sensitivity of these systems limits the comparisons which can be made between the different bioreactors and experiments. The apparatus, however, is designed to severely limit any technical disruption when working on the bioreactor (especially sampling). Another possible problem is confirming that the observed effect in a test does not result from a deviation of the system over time, independently from the test being performed. The realisation of a witness test and two bioreactor tests (see Figure 4, centre diagram) overcomes this problem by performing a co-inoculation of the three systems at T<sub>0</sub>, and by comparing these different samples for each bioreactor at different times (during the supposed equilibrium phase in the control, during the disturbance conducted in parallel on two bioreactors, during the equilibrium phase resulting from the disturbance, ...).

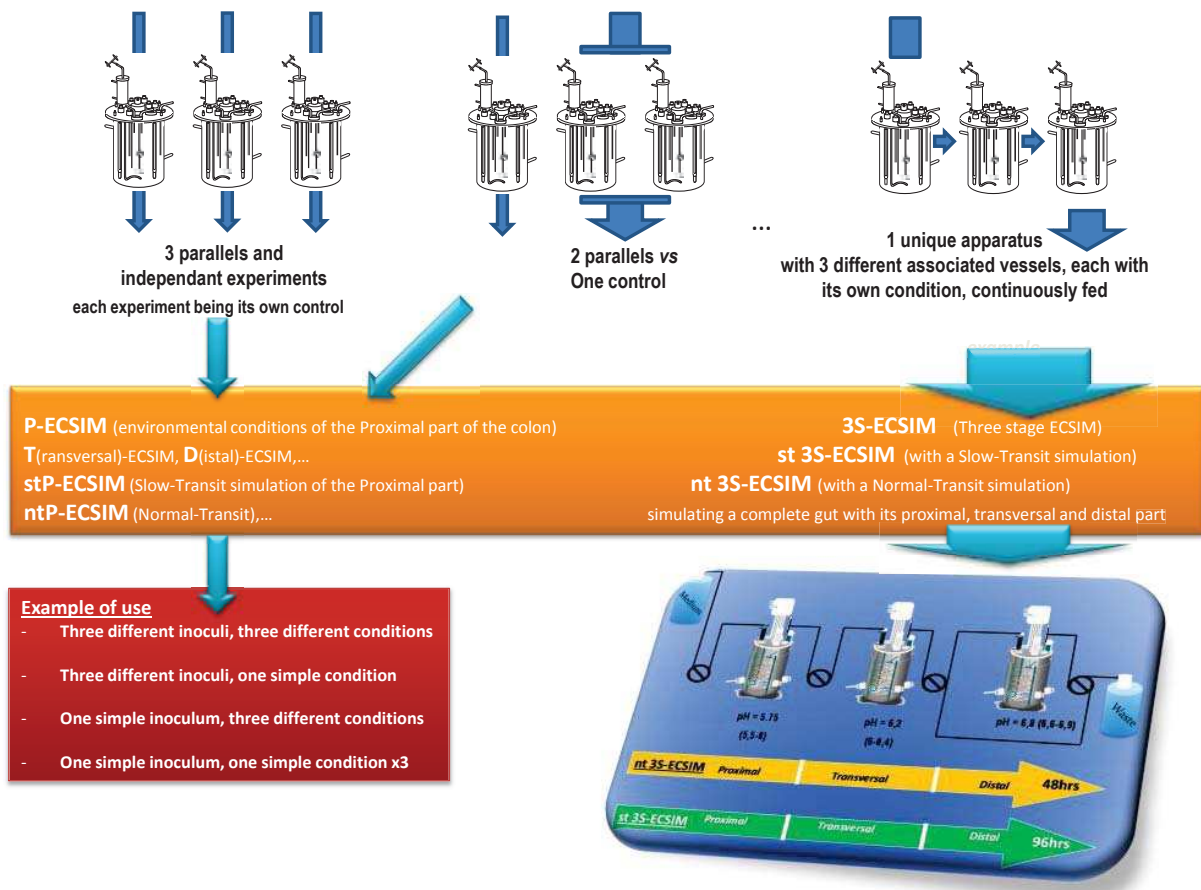


Fig. 4. The ECSIM paradigm: the modular components allow various organizations and various applications with identical elements from Global Process Concept.

Finally, the modularity of the system can be broken down as a grouping of bioreactors, ensuring continuity in the transition from a medium (and microbiota) from a medium stock, to a first bioreactor, then to a second, up to a third. The advantage of this system is the ability to modify the experimental conditions in each of the bioreactors. In all cases the experimental periods are long, extending up to several days in the last case presented. The duration time of a system disruption, therefore, is usually limited to a few seconds for a single injection, or a few hours for an incremental change. The need to obtain a steady state requiring incubation times equal at least to 5 residence times, however, may lead to long experiment times. For example, if we consider that a basic transit-time in the colon is 48 hours (nt, normal transit), and the length of the proximal colon being about 26% of the entire colon (vs 37% for each other of transverse and distal parts), we obtain around 13 hours of transit, only in the proximal part (Table 3). Therefore, a delay of 65 hours (nearly 3 days) is needed to reach a stabilised state. Furthermore, the transit time should be increased to nearly 18 hours in a transverse or a distal compartment, leading to a delay of 90 hours (almost four days) to obtain a steady state. Finally, the delay for two steady states separated by a disturbance (obtaining the first steady state/disturbance/result at stabilization) is, therefore, about a week for an experiment conducted in ntP-ECSIM (normal transit Proximal-ECSIM) and is two weeks for an experiment conducted in stP-ECSIM (slow transit Proximal-ECSIM, simulating a slow transit time of 96 hours for the colon).

When placed in a continuous 3-stage system (3S-ECSIM) to replicate the entire colon in these three functional parts, the completion time of the experiment increases accordingly, each state is expected to be stabilized individually and progressively. For example to simulate a 48-hours transit time, 10 days are needed to get a steady state in the last compartment (the distal part), and, as previously mentioned, the system was already stable in the first bioreactor (simulated proximal part) after 3 days. Therefore, if the effect of a disturbance is studied on the balance of the microbiota, the experiment will take approximately 20 days.

Another consequence would be the minimum doubling time that any microorganism must have to be kept in the reactor. If the time is greater than this minimal value, the microorganism will not be kept in the bioreactor but will be lost through the dilution effect. Table 3 indicates these values for of our system for various cases.

The working volume of each reactor is maintained at 1 L in order to lower the disturbance of the system by the sample intake itself. This means, however, that about 2 L of medium is needed to feed one chemostat bioreactor per day for a normal transit time simulation: either a P-ECSIM alone or a 3S-ECSIM composed of three vessels. This volume is naturally reduced twice while increasing the simulated transit time to a 96-hour simulation (Table 3).

Residence Time (h)	Dilution Rate <sup>1</sup> (h <sup>-1</sup> )	Feed rate <sup>2</sup> (vol=1L) (mL.min <sup>-1</sup> )	Medium (L per days)	Minimum doubling time <sup>3</sup> (h)
<b>A-Simulation of a 48-h transit time (nt)</b>				
12.48	0.0801	1.3355	1.9231	8.65
17.76	0.0563	0.9384	1.3514	12.31
17.76	0.0563	0.9384	1.3514	12.31
<b>B-Simulation of a 96-h transit time (st)</b>				
24.96	0.0401	0.6677	0.9615	17.30
35.52	0.0282	0.4692	0.6757	24.62
35.52	0.0282	0.4692	0.6757	24.62

<sup>1</sup>the dilution rate is calculated as the inverse of the residence time.

<sup>2</sup>in this case, the feed rate corresponds directly to the dilution rate, due to the 1-L volume.

<sup>3</sup>the minimum doubling time corresponds to the minimum period in which a microorganism should divide, in order to not be washed by dilution effect: obtained by dividing ln2 by the dilution rate.

Table 3. Characteristics of the dilution rate, feed rate, volume of media needed and the minimal doubling time for simulating the indicated residence time. A working volume of 1L in each vessel, in order to simulate a normal transit time (A, 48h) or a slow transit time (B, 96h).

### 3.4 Coupling/associating analytical systems

The ECSIM system is actually a very powerful sample generator because of the different controls it can perform. It can test the metabolic activity of microbiota in realistic terms, influenced by typical environmental constraints which may be encountered in humans, whether physiological or pathological or concerning functional, nutritional or other situations. It provides *in vitro* testing of the impact of a living, or an abiotic element, on the constitution and metabolism of a microbiota. It is a valuable tool, but has little or no interest if it is not coupled with an analytical infrastructure, particularly concerning the microbial, biochemical and molecular fields. Figure 5 shows what we feel to be the most important analyses.

Firstly, the analytical platform has to establish a quick inventory of the main microbiological and biochemical components. Classic microbiology, despite its limitations, especially in this

particular anaerobic ecosystem, remains of great help in identifying and enumerating total anaerobes, facultative anaerobes, and functional groups and/or some genus (bifidobacteria, lactobacilli, *Bacteroides* spp,...).

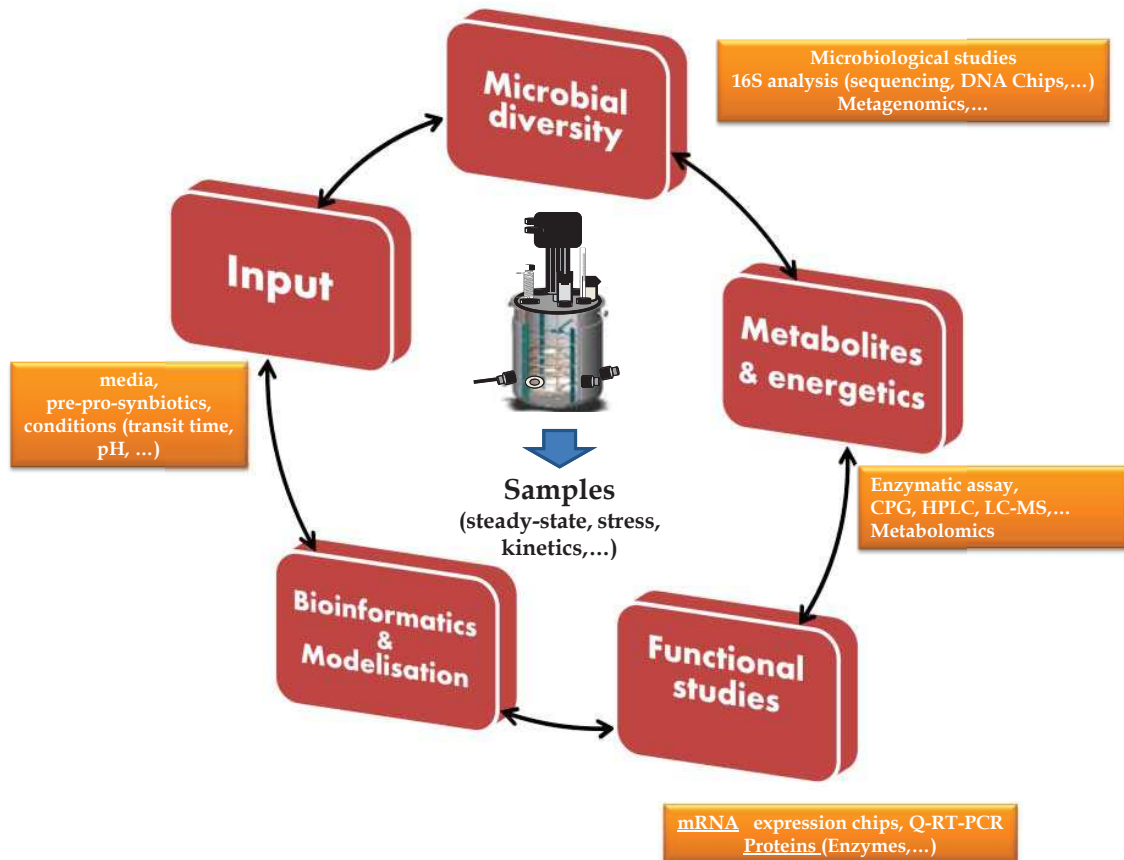


Fig. 5. Example of some analyses and their use to address questions about the gut biology from experiments developed from the ECSIM.

Anaerobic microbiology is technically easier when an anaerobic chamber is used. From a biochemical point of view, special importance is given to nutritional compounds (sugars, lipid and total proteins assays) and also to the major metabolites, such as those issuing from the fermentation process: gases ( $H_2$ ,  $CO_2$ ,  $H_2S$ , ...), lactic acid and short chain fatty acids. Gases can be detected and quantified using several methodologies, including Gas Chromatography (GC), and the other elements mainly by High Precision Liquid Chromatography (HPLC). If these elements are essentials, however, other more global approaches are now possible which provide a wealth of consistent information about microbial metabolites and microbes. At the metabolic level, therefore, a metabolomic approach will determine real metabolic identity cards before and after the testing, associated clearly with identified microbial populations.

Working with the platform "exploration du metabolisme" from the Clermont-Theix INRA centre (the French National Institute for Agronomic Research), we can already identify metabolic fingerprints and compare experiences between two situations (Feria-Gervasio et al., 2010). Using the same principle, global (comprehensive) approaches for determining microbial diversity are also in progress. For reasons of cost and speed, it uses an approach giving a more informational fingerprint. We usually perform the RISA techniques

(ribosomal intergenic spacer analysis, (Cardinale et al., 2004)) in order to obtain comparable fingerprint samples: Figure 6 shows an example. When necessary, rather than using pyrosequencing analysis of 16S rDNA banks, we use a phylogenetic DNA microarray called HuITMiCHIP. This biochip, which is presently being validated, was developed in collaboration with the laboratory of Pr Pierre Peyret (GIIM, UMR UBP-CNRS 6023, Clermont-Ferrand). It has about 5,000 specific oligonucleotides probes designed for detecting 67 bacterial families present in the human GIT. Depending on the method used, it provides a thorough determination of diversity associated with a semi-quantitative evaluation of populations (William Tottey, personal communication). The most interesting data can then be verified by quantitative PCR tools. In the near future we hope to be able to associate metabolic and microbial diversity data to expression analysis concerning quantitative changes of bacterial mRNAs expression, when studying an induced metabolic disturbance (Figure 5).

As a general example, Figure 6 shows an experiment conducted with P-ECSIM studying the impact of transit time on the nature and metabolism of the microbiota. The experiment was initiated from a faecal slurry at 20% (w/v) cultured at 37° C in several successive anaerobic batch with the colonic medium (Table 2), and then stored at -20°C as aliquots at 20% glycerol (v/v). One of these aliquots was unfrozen on ice and used to inoculate a preculture of 5 mL of artificial gut medium, grown at 37°C for 10 hours. It was then transferred for 15 hours into a 1L Erlenmeyer flask containing 95 mL of artificial gut medium. This was then transferred into 900 mL of complete artificial gut medium in two stirred 2-L tank bioreactors, previously N<sub>2</sub>-flushed, for a 24-hour batch culture. A continuous cultivation was subsequently launched in parallel in the two bioreactors. The initial dilution rate was of  $D=0.08\text{ h}^{-1}$ , followed by  $D=0.04\text{ h}^{-1}$  (Figure 6-A). A dilution rate of  $0.08\text{ h}^{-1}$  simulates a transit time in the proximal colon equivalent to 12.5 hours, *i.e.* a colonic transit time of about 48 hours, which is considered normal (ntP-ECSIM, normal transit time Proximal-ECSIM) (see Table 3). Conversely, a dilution rate of  $0.04\text{ h}^{-1}$  simulates a slow colonic transit time in proximal condition, approximately 25 hours (stP-ECSIM, slow transit time Proximal ECSIM), equivalent to a global gut transit of 96 hours. Controlling the pump allows for deceleration and acceleration ( $\alpha=0.0025\text{ h}^{-1}$ ), providing a gradual change from one state to another in a few hours. The C-BIO (Global Process Concept inc., France) acquisition and control software was used to adjust the stirring rate at 400 rpm, maintain the temperature at 37° C, and the pH at 5.75 by automatic addition of NaOH. The ORP was monitored every 5 minutes using an Argenthal reference probe from Mettler Toledo (3253i/SG/225 Inpro® probe). Samples were taken over each steady state, mimicking either a normal transit (nt, states 1 and 3) or a slow transit (st, state 2). As presented in Figure 6-B and C, the microbial metabolism and its diversity were affected by this modification of retention time (Feria-Gervasio et al., 2010). The part B highlights the variation of the diversity of the microbiota using the RISA (Ribosomal Intergenic Spacer Amplification). This technique (Cardinale et al. 2004) provides a rapid comparison of conserved and modified patterns. In Figure 6-C, a PCA (Principal Components Analysis) is shown, deduced from the metabolomic data retrieved from the LC-MS analysis. It indicates that the samples may all be differentiated by their origins, either the raw artificial gut medium, the ntP or the stP- ECSIM (E Pujos, JF Martin and JL Sebedio, personal communication). These results also indicate that there are fewer differences among samples from the same steady-state, highlighting the real stability of the so-called steady-state. Moreover, they also indicate that results are very similar between reactors used in the same conditions.



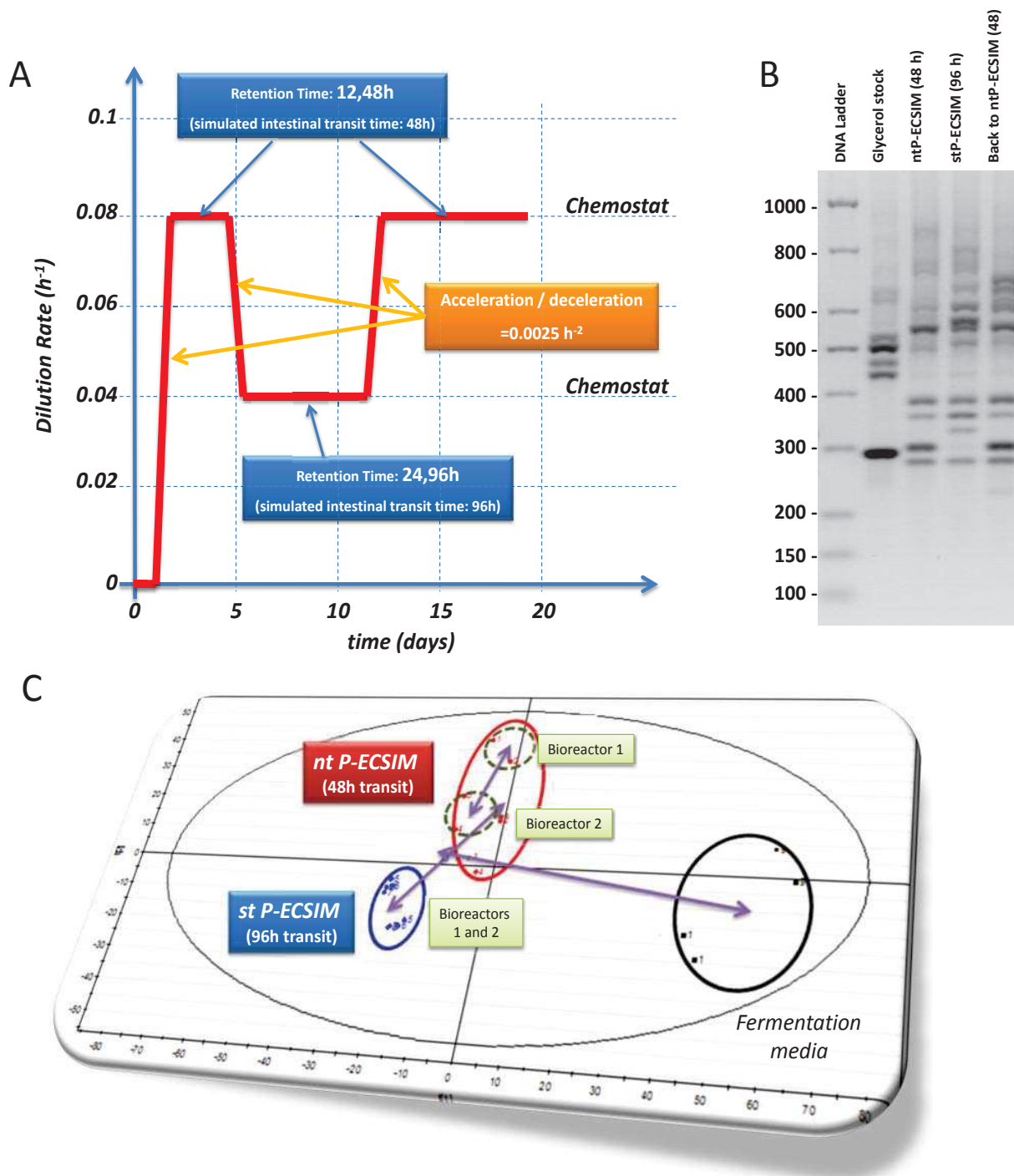


Fig. 6. An example of the use of the ECSIM system: an experiment in P-ECSIM addressing the question of effect of the transit time on the microbial diversity and its metabolism. A- Design of the experiment, with the modulation of the dilution rate in order to simulate a normal (nt P-ECSIM, 48h) or a slow (st P-ECSIM, 96h) transit in the proximal part of the colon. B- Fingerprints obtained from the RISA experiments, showing the impact of the retention time (i.e. the transit time) on the proximal gut microbiota studied in P-ECSIM. C- PCA analysis of the metabolites detected by LC-MS (courtesy of E Pujos, JF Martin and JL Sebedio), highlighting the different metabolic status when changing the residence time, and the similar metabolic fingerprint obtained with two different bioreactors in the same conditions.

#### 4. Conclusion

Studying the role of human microbiota in the digestive tract remains difficult because of various technical and ethical problems, however, in-vitro systems may overcome some of them. We have shown that the ECSIM system proposes a modular and adaptable solution when focusing only on the metabolic behaviour of the microbiota per se, without addressing its cellular and metabolic interaction with the host. The system can be seeded by a faecal microbiota, even to simulate a proximal part, or the whole 3 parts, making it easier technically and ethically throughout the experimentation.

The ECSIM system also helps to mitigate the causes of fluctuations among experiments, due to strict control of parameters. It, therefore, provides an effective means of helping us better understand the role of the human intestinal microbiota, and the role and fate of endogenous and exogenous factors (physico-chemical, nutrients, prebiotics, probiotics, synbiotics, ...). The future development of in-vitro systems, including human intestinal cells (epithelial, immune cells), would be of very great interest, but for the time being remains too technically difficult to undertake. This kind of apparatus, therefore, may also provide a means for safely producing a human microbiota to be reintroduced into a human GIT. This process could be of great interest for several pathologies (for example, *Clostridium difficile* diarrhoea), using a faecal bacteriotherapy (Bakken, 2009; Borody et al., 2004) provided by a specially designed in vitro production system.

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