Chapitre I Impact de la croissance en écosystème sur la résistance au stress *in vitro* de micro-organismes fromagers et comparaison avec les conditions de croissance de laboratoire.

1. Présentation de l'article 2 accepté avec révisions dans *Food Microbiology*

L'objectif de cette partie des travaux était de comparer la résistance au stress digestif *in vitro* de micro-organismes d'intérêt fromager (i) cultivés séparément en milieu synthétique, (ii) cultivés en milieu synthétique puis mélangés, (iii) cultivés séparément en milieu synthétique puis mélangés et inclus dans un gel présure, (iv) cultivés en conditions d'affinage réelles. Nous avons ainsi eu accès à des micro-organismes rentrant en contact avec les mêmes stress digestifs mais différant soit par leur mode de croissance, soit par leur environnement lors de la mise en contact. Le protocole a aussi inclus une validation *in vivo* sur modèle murin concernant la condition (iv).

A l'heure actuelle, il existe une petite dizaine de dispositifs permettant de reproduire artificiellement le tube digestif en intégrant son aspect cinétique (pour revue, voir Guerra et al., 2012). La diversité de ces modèles comprend des designs relativement simples (e.g. mono-compartimental comme le Dynamic Gastric Model (Mercuri et al., 2008)mais comprend également des dispositifs très complexes et difficile à prendre en main (e.g. la combinaison des TIM-1 et TIM-2 et ses 8 compartiments couvrant le tractus digestif quasiment au complet avec notamment 4 de ses réacteurs ensemencés par un microbiote (Minekus et al., 1995 ; Minekus et al., 1999). Le choix de l'utilisation d'un dispositif ou d'un autre est lié aux objectifs de travail. Dans notre cas, le dialogue entre microbiote intestinal et les micro-organismes d'origine fromagère, l'incidence du péristaltisme, ou encore la déconstruction en bouche, n'ont pas été étudiés. Ainsi, nous avons fait le choix de développer notre propre digesteur. Le Digesteur Dynamique Gastro-Intestinal (DIDGI) est constitué de trois compartiments indépendants simulant l'estomac, le duodénum et la suite de l'intestin grêle (jéjunum-iléon). L'ensemble du système est relié à une armoire électronique assurant l'interface entre le dispositif et l'ordinateur. Le pilotage des différents paramètres ainsi que l'affichage des valeurs délivrées par les capteurs est réalisée par le logiciel STORM (Guillemin et al., 2010). Ce dispositif nous permet de contrôler à tout instant : la température, le pH, la composition des fluides digestifs ainsi que le transfert du bol d'un compartiment à l'autre. Le dispositif, entièrement stérilisable, permet de prélever des échantillons dans chacun des compartiments. L'annexe I détaille la composition du DIDGI ainsi que ses paramètres de fonctionnement lors des expérimentations réalisées.

La première partie des travaux nous a permis de constituer deux mélanges de micro-organismes aux potentiels immunomodulateurs opposés et permettant d'obtenir un fromage affiné de type pâte molle. Ces fromages affinés ont ensuite été introduits dans le digesteur selon les mêmes séquences de digestion que les autres conditions listées précédemment. En lien avec la troisième partie des travaux, la capacité de survie des micro-organismes à un stress digestif *in vivo* a été testée grâces à des souris BALB/c au microbiote standardisé.

2. Faits marquants

- ✓ Nous avons conçu et réalisé un digesteur *in vitro* gastro-intestinal dynamique composé de 3 étages.
- ✓ Le simulateur a été utilisé pour caractériser la résistance au stress digestif d'un microbiote fromager cultivé en conditions d'affinage réelles.
- ✓ Les résultats des expérimentations en modèles murins ont confirmé les observations faites *in vitro*.
- ✓ Le pouvoir tampon de la matrice fromagère s'est avéré avoir une importance dans le choix de futurs paramètres de digestion

Survival of cheese-ripening microorganisms in a dynamic simulator of the gastrointestinal tract

Nadège Adouard[ab], Laurent Magne[b], Thomas Cattenoz[b], Hervé Guillemin[b], Benoît.Foligné[c], Daniel Picque[b], Pascal Bonnarme*[b]

[a] AgroParisTech, Centre de Biotechnologies Agroindustrielles, AgroParisTech INRA, UMR 782 Genie & Microbiologie des Procedes Alimentaires, F-78850 Thiverval Grignon, France

[b] INRA, Centre de Biotechnologies Agroindustrielles, AgroParisTech INRA, UMR 782 Genie & Microbiologie des Procedes Alimentaires, F-78850 Thiverval Grignon, France

[c] Institut Pasteur de Lille, Lactic acid Bacteria & Mucosal Immunity, Center for Infection and Immunity of Lille UMR 8204, 1 rue du Pr Calmette, BP 245, F-59019 Lille, France

* Corresponding author Pascal BONNARME; pascal.bonnarme@grignon.inra.fr Phone: +33 130 815 388

Abstract

A mixture of nine microorganisms (six bacteria and three yeasts) from the microflora of surface-ripened cheeses were subjected to *in vitro* digestive stress in a three-compartment "dynamic gastrointestinal digester" (DIDGI). We studied the microorganisms (i) grown separately in culture medium only (ii) grown separately in culture medium and then mixed, (iii) grown separately in culture medium and then included in a rennet gel and (iv) grown together in smear-ripened cheese. The yeasts *Geotrichum candidum*, *Kluyveromyces lactis* and *Debaryomyces hansenii*, were strongly resistant to the whole DIDGI process (with a drop in viable cell counts of less than < 1 log CFU.mL^{-1}) and there were no significant differences between lab cultures and cheese-grown cultures. Ripening bacteria such as *Hafnia alvei* survived gastric stress less well when grown in cheese (with no viable cells after 90 min of exposure of the cheese matrix, compared with 6 CFU.mL^{-1} in lab cultures). The ability of *Corynebacterium casei* and *Staphylococcus equorum* to withstand digestive stress was similar for cheese and pure culture conditions. When grow in a cheese matrix, *Brevibacterium aurantiacum* and *Arthrobacter arilaitensis* were clearly more sensitive to the overall digestive process than when grown in pure cultures. *Lactococcus lactis* displayed poorer survival in gastric and duodenal compartments when it had been grown in cheese. *In vivo* experiments in BALB/c mice agreed with the DIDGI experiments and confirmed the latter's reliability.

Keywords

Smear-cheese microflora, cheese, *in vitro* model of digestion, murine *in vivo* model

1. Introduction

Cheese is one of the oldest ways of conserving milk: in Northern Europe, evidence of cheese-making activity has been found at sites dating from the sixth millennium BC (Salque et al, 2012).
At present, Europe produces around 9000 thousand tons of cheese per annum (Eurostat, 2013), and Europeans eat between 25 and 30 kg of cheese per capita per annum. Given that a gram of cheese contains 10^8 to 10^9 living microorganisms on average (Beresford et al. 2001), the annual intake of viable cells can be estimated at 10^{13} to 10^{14} per capita per annum. The complexity of microflora depends on the type of cheese. In Cheddar and mozzarella, the microflora is relatively simple and consists mainly of lactic acid bacteria (LAB) and a few species of yeast (Kindstedt et al. 2004; Lawrence et al. 2004). In contrast, the microflora in soft, smear-ripened cheeses such as Livarot or Munster contains a broad, diverse range of bacteria and yeasts (Bockelmann 2002, Irlinger and Mounier, 2009). Thus, a fermented food product like cheese is an important source of diverse microorganisms in the human diet. However, few studies have investigated the survival of the cheese microflora in the gastrointestinal tract. A review of the literature shows that most of the research in this field has focused on Lactobacilli, Bifidobacteria and Propionibacteria (Cousin et al. 2011, Saarela et al. 2000) with a view to find new probiotics or using cheese as a carrier for known probiotics (Gardiner et al. 1999, Saxelin et al. 2010).

It has been reported that pH is the major stress factor in the gastric compartment, whereas the presence of enzymes has a negligible effect on the microorganisms (Sumeri et al. 2012). The impact of the stomach's hydrochloric acid (HCl) on both Gram-positive and Gram-negative bacteria has been well characterized (Krulwich et al. 2011). In contrast, the impact of bile has been less documented (Begley et al. 2005) and has focused on food-borne pathogens (e.g. *Escherichia coli* and *Salmonella typhimurium* (Merrit et al. 2009)) or probiotic candidates (such as the Bifidobacteria and the Lactobacilli (Ruiz et al. 2013). One of the few studies related to cheese-ripening bacteria found that the genus *Corynebacterium* survived passage through the gastrointestinal tract in human microbiota-associated rats (Lay et al. 2004). Cheese-ripening yeasts (such as *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Geotrichum candidum*) were found to be able to survive *in vitro* challenges with acid and bile (Kumura et al. 2004, Lay et al. 2004, Psomas et al. 2001). A recent study of the human gut microbiome by David et al. 2014 showed that live bacteria and fungi from cheese are present and metabolically active in the distal colon. This novel approach to assessing the potential impact of food-related microorganisms and their ability to reach the distal colon needs to be consolidated.

Furthermore, Plé et al.'s work (2014, *in press*) on the potential immunomodulatory effect of the microorganisms selected in this study, sought to developed experimental cheeses with bacteria and yeasts that have opposite immune potentials and then studied their impacts in a mouse colitis.

As the interest in whether food microorganisms are able to withstand digestive stress grows, many batch-based models of *in vitro* digestion have been developed (for a review, see Hur et al. (2010)). Several "dynamic" models (intended to reproduce the time course of digestion) have also been designed (for reviews, see Guerra et al. 2012, Ménard et al. 2013). In fact, *in vivo* studies in animal models are quite expensive and intricate to perform. In contrast, *in vitro* models offer greater reproducibility, few ethical issues and the ability to collect samples throughout the experiments. Dynamic models take account of the time course of digestion, the pH profiles at each stage, the transfer between compartments and the progressive addition of digestive juices and other components (e.g. bile, enzymes and electrolytes). After reviewing the literature, we found only two recent studies (Pitino et al. 2012, Sumeri et al. 2012) dealing with the survival of cheese-grown microorganisms namely lactic acid bacteria (LAB) - in pasta filata and semi-hard cheeses, respectively -in a dynamic *in vitro* system. Use of a smear-ripened cheese model (Mounier et al. 2009) would thereby provide novel, relevant information on the little-studied field of cheese microorganisms and dynamic *in vitro* models of digestion. Building on the results of our previous batch-based screening experiments [Adouard et al. 2014 submitted], we adapted the "dynamic gastrointestinal digester" ("DIDGI") designed and developed by the French National Institute for Agricultural Research (*Institut National de la Recherche Agronomique*) (Ménard et al. 2013). This computer-controlled digester focuses on the upper parts of the digestive tract, i.e. the stomach, duodenum and small intestine. It reproduces the pH profile and the time course of emptying in each compartment, the sequential addition of digestive juices and the stirring of the compartments' contents.

We adapted a model of a smear-ripened cheese ecosystem (Mounier et al. (2008)) containing five bacteria and three yeasts from the surface microflora plus a lactic acid bacteria used as a starter. We compared the microbiota's resistance to dynamic *in vitro* digestive stress under three different conditions, in which the microorganisms were (i) grown separately in culture medium only (ii) grown separately in culture medium and then mixed, (iii) grown separately in culture medium and then included in a rennet gel and (iv) grown together in smear-ripened cheese. A series of experiments using BALB/c mice were also performed, in order to obtain data on the selected strains' ability withstand full digestive transit in an *in vivo* model.

2. Material and Methods

2.1 Microorganisms

The mixture of six bacterial strains and three yeast strains studied here (Table 1) was chosen on the basis of the smear-ripened cheese microbiota described by Plé et al. 2014 (*in press*).

Table 6: List of the microbial strains and growth conditions used in the present study

Species	Strain	Origin	Medium	Growth conditions
Lactococcus lactis	S3	Cheese	M17	30°C - static
Brevibacterium aurantiacum	ATCC 9174	Cheese (Romadur)	BHI	25°C – 250 rpm
Corynebacterium casei	1-3b	Cheese (Livarot)	BHI	25°C – 200 rpm
Arthrobacter arilaitensis	Re 117^T	Cheese(Reblochon)	BHI	25°C – 200 rpm
Hafnia alvei	920	Cheese	BHI	25°C – 200 rpm
Staphylococcus equorum	Mu206	Cheese (Munster)	BHI	25°C – 200 rpm
Debaryomyces hansenii	CLIB 623	Cheese	PDB	25°C – 200 rpm
Kluyveromyces lactis	CLIB 683	Cheese	PDB	25°C – 200 rpm
Geotrichum candidum	ATCC 204307	Cheese (Pont-l'évêque)	PDB	25°C – 200 rpm

2.2 Growth and plate count media

All growth media were purchased from Biokar Diagnosis (Beauvais, France), with the exception of potato dextrose broth (PDB: Difco, Pessac, France). With the exception of LAB, all bacteria were cultured in 100 mL of brain heart infusion (BHI) broth in 500 mL Erlenmeyer flasks at 25°C, with shaking at 200 rpm. The LAB *Lactococcus (Lc.) lactis subsp. lactis* was cultured in M17 medium in 100 mL Schott bottles at 30°C. Yeasts were grown in 100 mL of PDB in 500 mL Erlenmeyer flasks at 25°C, with shaking at 200 rpm. All strains were grown until they had reached the late stationary phase, as determined in previous time course experiments (data not shown).

Bacteria were counted on the same agar based media used for liquid cultures, i.e. BHI agar for strains grown in BHI broth. Eukaryotes were plated on yeast extract glucose chloramphenicol (YEGC) plates. The incubation temperatures were the same as in broth cultures.

The bacteria and yeast have specific and distinct colonies' shapes and morphologies when grown in their respective agar medium (e.g. M17 for L.lactis, BHI for the ripening bacteria and YEGC for yeasts). Therefore, we were able to differentially count all of them using the three above-mentioned agar media, eventhough several species were present in the same Pétri dish.
Prior to plating, cultures were diluted in Maximum Recovery Diluent (MRD, containing NaCl and peptones, 9 $g.L^{-1}$) (Difco, Pessac, France).

2.3 Rennet gel inclusion of microorganisms

The rennet gel matrix used for microorganism inclusion was produced in a two-step procedure. First, 0.2 mL of a 10 $g.L^{-1}$ $CaCl_2$ solution was added to 225 mL of commercial, pasteurized semi-skimmed milk (Lactel, Laval, France) and preheated for 30 min at 32°C. Secondly, 25 mL of a mixture of all the microorganisms and 0.1 mL of rennet (Naturen 450, containing 555 $mg.L^{-1}$ of active chymosin, 145 $IMCU.mL^{-1}$; Chr. Hansen, Arpajon, France) were added to the matrix, which was gently stirred for a few seconds before being left to gel unstirred at 32°C for 40 min.

2.4 Pilot-scale cheese-making

Pilot-scale, aseptic cheese production (namely coagulation, cutting, draining, and molding of the curd) was performed in a 14 L pilot tank, according to the procedures used for Livarot cheese. Pasteurized milk (12 liters) was standardized at 29 $g.L^{-1}$ fat by mixing semi-skimmed milk and full-fat milk (Alsace Lait, Hœrdt, France). Ten mL of a filter-sterilized 100 $g.L^{-1}$ $CaCl_2$ solution was added to the milk and the mixture was pre-heated at 34°C prior to addition of 10^6 $CFU.mL^{-1}$ *Lc. lactis*. Once the milk's pH had reached 6.3, a mixture of the ripening bacteria and yeasts was added (yielding final concentrations of 10^6 and 10^4 $CFU.mL^{-1}$, respectively, for each kingdom). Next, filter-sterilized rennet (Naturen, Chr. Hansen, Arpajon, France) containing 520 $mg.L^{-1}$ of active chymosin was added (0.3 $mL.L^{-1}$ of milk). After 20 min of coagulation at 34°C and 30 min of hardening, the curds were cut and then stirred for 5 min at 10 $stirs.min^{-1}$. After the curd had stood for 15 min, 6.5 L of whey were removed prior to molding. The cheeses were shaped into two circular, polyurethane molds (200 x 150 x 40 mm) and weighed approximately 900 g each. The molds were then stored at 20°C and inverted four times (30 min, 2.5 h, 6 h, and 22 h after the molding step). Twenty-four hours after molding, the cheese curds were removed from the mold. After standing for two hours, the cheeses were cut into circular pieces (diameter: 50 mm; thickness: 15 mm; weight: approximately 20 grams).

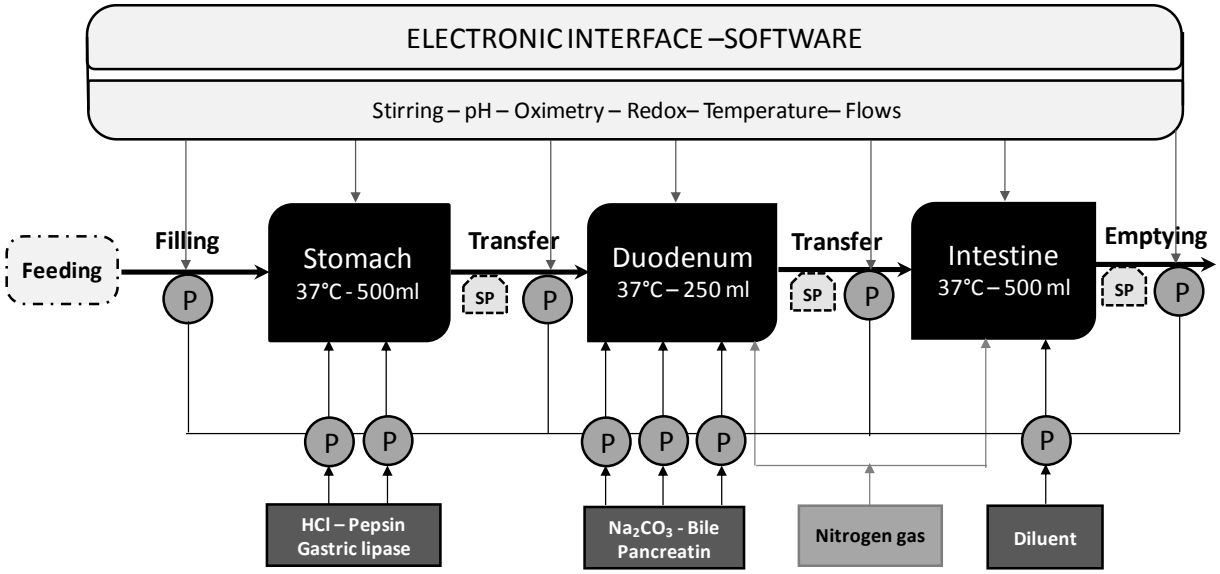
Hence, 32 small cheeses were obtained and placed in a sterile environment at 14°C and 97% relative humidity for a 28-day ripening period. All nine bacteria and yeasts were able to grow in the cheese. The concentration of each species in the final, ripened cheeses is given in Table S1.

2.5 *The in vitro* digestive challenge

2.5.1 The dynamic *in vitro* simulator of gastrointestinal digestion

The original DIDGI digester (Ménard et al. (2013)) comprised two compartments (a stomach and a duodenum/small intestine). We decided to add a third compartment (Figure 1) by separating the duodenum from the small intestine, since these compartments are known to have different volumes, fluid compositions and transit times *in vivo*. Each compartment was surrounded by a glass jacket filled with temperature-controlled water, enabling us to mimic the human's physiological body temperature (37°C). Each glass vessel was equipped with temperature, pH and redox sensors (Electrode InPro 4801i/SG/120, reference 52003581, Mettler Toledo, Viroflay, France) and computer-controlled peristaltic pumps (Verder, Eragny-Sur-Oise, France) for controlling the various inflows (HCl, Na_2CO_3, bile and enzymes) and transferring the meal from one compartment to the next. A Teflon membrane (pore size: 2 mm) was placed between the gastric and the duodenal compartment, in order to mimic the sieving effect of the pylorus (Kong & Singh (2008)). A sampling device was placed at the outlet of each compartment. Anaerobic conditions were simulated by flushing out the air with nitrogen gas. In each experiment, 50 mL of MRD was added to 100 g of broth culture, rennet gel or cheese, in order to mimic the effect of dilution by the saliva. Therefore, 150 mL of simulated food bolus were introduced into the gastric compartment. In the first phase of our study, the simulated food consisted of a broth culture of each of the selected microorganisms alone. We then worked with a mixture of all nine selected microorganisms (*i.e.* cultured separately in broth and mixed immediately before the experiment). Thirdly, tests were carried out using the rennet gel-included mixture of the microorganisms that had been identified in a smear-ripened cheese mix (Plé et al. 2014, in press). Lastly, smear-ripened ripened cheese was used in the final set of experiments.

Figure 1: Block diagram of the Digesteur Dynamique Gastro-Intestinal (DIDGI).



P: peristaltic pump; **SP**: sampling pump.

2.5.2 Computer software and mathematical modeling

The DIDGI's parameters were based on a review of the literature on digestion in humans and pigs. Secretion rates and volumes and the nature and quantity of enzymes in the DIDGI's various compartments were based on previous *in vivo* studies (Blanquet et al., 2004, Bouzerzour et al., 2012 and Minekus, 1995).The pH curve in the stomach was obtained by combining data from *in vivo* studies in humans and in piglets (Calbet et al. 2004 Chiang et al., 2008, Moughan et al., 1991). For the experiments with culture broth alone, the pH in the gastric compartment was defined as −0.063 x t + 6.5 until it reached pH 2. The latter value was then maintained until the end of the experiment. Since the pH of the model cheeses was slightly higher than that of broth, the pH was controlled in two steps: pH = - 0.11 x t + 7.5 from t= 0 min to t= 10 min, and then pH = −0.063 x t + 6.5 until it reached pH 2. Again, the latter value was then maintained until the end of the experiment. The pH of the duodenum compartment was kept constant at 6.5. The variables $t_{1/2}$ and β reflect the transit time of the bolus in the stomach and the duodenum and were adapted from Minekus (1995): we used $t_{1/2}$ = 70 min ; β = 2 for gastric transfer and $t_{1/2}$ = 120 min ; β = 1.8 for duodenal transfer. Table 2 summarizes the parameters used during a DIDGI run.

Table 7 : Composition and flow rates of the various inputs used in the DIDGI experiments

Compartment	Input	Constituents	Origin	Concentration/quantity of the input	Flow rate ($mL.min^{-1}$)	Time lapse (min)
Stomach	Culture/Matrix/Cheese + maximum recovery diluent as "saliva"	Microorganisms + Peptone/NaCl	-	100 g + 50 g (9.5 $g.L^{-1}$)	15	[0;10]
	HCl	-	Chemical	1 $mol.L^{-1}$	pH regulation	-
	Gastric enzymes	Pepsin	Porcine	0.5 $g.L^{-1}$ (~2000 $U.mL^{-1}$)	0.4	[10;90]
		Gastric lipase	Recombinant	6.5 $g.L^{-1}$ (~200 $U.mL^{-1}$)		
Duodenum	Na_2CO_3	-	Chemical	1 $mol.L^{-1}$	pH regulation	-
	Bile 40	Bile	Porcine	40 $g.L^{-1}$	0.5	[0;80]
					0.15	[80;100]
	Bile 20	Bile	Porcine	20 $g.L^{-1}$	0.2	[80;220]
	Pancreatin	Pancreatin	Porcine	9 $g.L^{-1}$	0.25	[0;50]
					0.1	[50;220]
Intestine	Diluent	Peptone NaCl	Chemical	9.5 $g.L^{-1}$	0.8	[20;100]
					0.5	[100;250]

Data on the change over time in the various compartments' volumes are given in the supplemental data section (Figure S1). Data collected in the above-mentioned *in vivo* studies (such as the quantity of ingested food, the meal duration, the pH curves for the stomach and small intestine, the secretion rates into the different compartments and the gastric and small intestine emptying) were fed into the *STomach Regulation and Monitoring* software package (Guillemin et al. 2010). A power-exponential model for gastric and intestinal delivery was used (Elashoff et al. 1982) to control the chyme's transit time in each compartment:

$$f = 1 - 2^{-(\frac{t}{t_{1/2}})^{\beta}}$$

Where f is the fraction of chyme remaining, t is the time, t½ is the half-emptying time and β is a coefficient that describes the shape of the curve. The pH values were computer-controlled by injecting either 1 M HCl to decrease the pH in the stomach or 1 M Na_2CO_3 to raise the pH in the small intestine.

All digestive juice components were purchased from Sigma (Saint-Quentin-Fallavier, France) and diluted in MRD. Pepsin (P6887, EC 3.4.23.1, activity: 3300 $U.mg^{-1}$ of protein, calculated using hemoglobin as a substrate), pancreatin (P1750), and bile (B8631) were of porcine origin. The “gastric lipase” was a recombinant enzyme produced in the fungus *Rhizopus oryzae* (80612, EC 3.1.1.3, activity: ≥30 $U.mg^{-1}$). After rehydration, the enzymes were kept on ice throughout the experiment, in order to avoid autolysis. Digestion experiments were performed in duplicate and samples from each compartment were collected throughout the experiment.

2.6 The *in vivo* digestive challenge

2.6.1 Animal care and use and ethical aspects

Ten BALB/c female mice (aged 7 weeks on arrival) were obtained from Charles River (Saint-Germain-sur-l'Arbresle, France). The animals were housed in a controlled environment (with a temperature of 22°C, a 12 h/12 h light/dark cycle and *ad libitum* access to food and water). All animal experiments were performed according to the guidelines of the Institut Pasteur de Lille Animal Care and Use Committee and complied with the Amsterdam Protocol on Animal Protection and Welfare and the Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes (updated in the Council of Europe's Appendix A). The animal work was also compliant with French legislation (the French Act 87–848, dated 19-10-1987) and the European Communities Amendment of Cruelty to Animals Act 1976. The study's objectives and procedures were approved by the local Ethics and Welfare Committee for Experiments on Animals in France (Nord-Pas-de-Calais region; approval number: 19/2009R and 21/2009R).

2.6.2 Assessment of the survival of ripening microflora

Ten mice were given 1 g of the cheese via the intragastric route. This procedure was performed daily (in the late afternoon) for five consecutive days. On each of the first three days, feces were collected eight hours after feeding. Feces were also collected after a seven-day washing period. The fecal samples were dissolved in MRD and plated on BHI agar or PDA medium for counting bacteria and yeasts, respectively. The results were expressed as the mean ± standard deviation for the group of 10 animals for each of the three consecutive days of feces collection.

3. Results

3.1 *In vitro* experiments: pure culture

The ability of the nine cheese-ripening microorganisms (six bacteria and three yeasts) to withstand *in vitro* digestive stress was tested in the DIDGI under three different conditions. We would like to emphasize the fact that the main characteristic of the DIDGI is its "dynamic" approach of the digestive process. Indeed, the bolus introduced in the gastric compartment starts its journey to the duodenum while the artificial stomach still contains sample, as it occus during *in vivo* digestion. It goes alike with the transfer between the duodenual and the intestinal compartment.
Figure 2 displays the viability curves for the six selected bacteria during transit through the DIDGI's gastric, duodenal and small intestine compartments when administered as pure cultures. In order to compensate for the effect of the added components, the data on viability was corrected by a dilution factor according to the residence time in each compartment. Data on the change over time in the yeasts' viability are given in the supplemental data section (Figure S2).

It is to note that no morphological change in the shape of the colonies was observed throughout the experiments. It enabled us to keep the same numeration method disregarding the stage of the *in vitro* digestion process.

3.1.1 *The gastric phase*

Both *H. alvei* and *Lc. lactis* displayed strong resistance to gastric stress (Figure. 1A). *H. alvei* did not become less viable until the pH reached 2.5 (after 60 min). The count of 9 log CFU.mL^{-1} then fell to 6 log CFU.mL^{-1} at 90 min and remained relatively constant until the gastric compartment had emptied completely at 110 min. *Lc lactis* also remained viable for 60 min, although the decrease was sharper than for *H. alvei*; viability had fallen to 4 log CFU.mL^{-1} at the end of the gastric phase. *B. aurantiacum*, *S. equorum* and *C. casei* were much more sensitive to gastric stress and displayed similar changes over time in viability. The viability started to decrease after 40 min of exposure to gastric stress (pH 4), and then fell rapidly to zero after 70 min for *C. casei* and 80 min for the other two species. Lastly, the viability of *A. arilaitensis* started to fall from the beginning of the gastric phase onwards, with a decrease of 1 log CFU.mL^{-1} every ten minutes over the first 40 min and a value of zero after 50 min.
All the yeasts were strongly resistant to gastric exposure, since *G. candidum* and *D. hansenii* did not show any changes in viability. The *K. lactis* count fell by just 1 logCFU.mL^{-1} over the duration of the experiment (Figure S2).

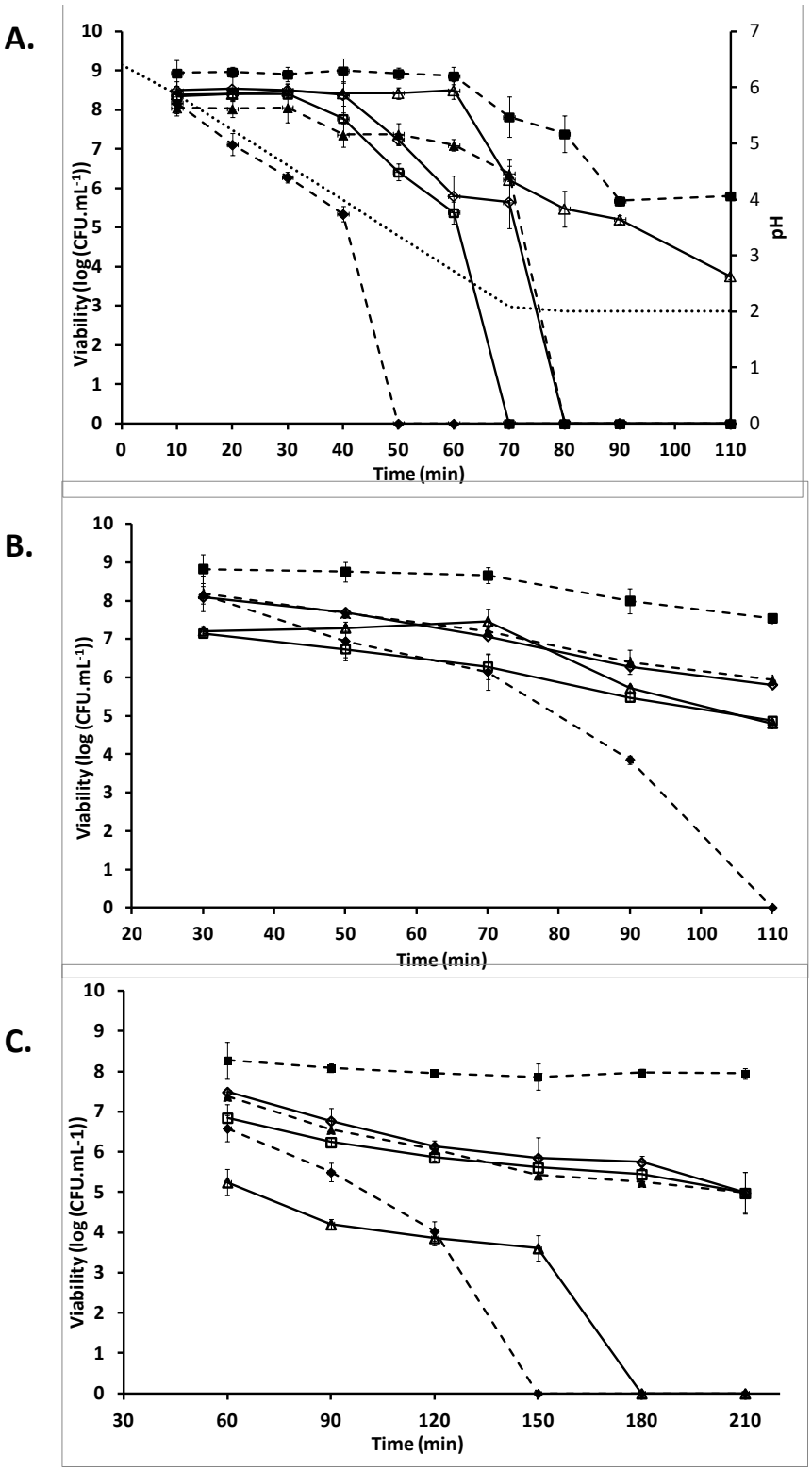


Figure 2 : Effect of in vitro dynamic, simulated digestion on the viability of the selected smear-ripened cheese micro-organisms. (A) the gastric compartment; (B) the duodenal compartment; (C) the small intestine compartment. Inoculation levels in the gastric compartment are given between brackets. *Brevibacterium aurantiacum* (◇, solid line, 8.5 log CFU/mL); *Corynebacterium casei* (□, solid line, 8.3 log CFU/mL); *Lactococcus lactis* (△, solid line, 8.4 log CFU/mL); *Arthrobacter arilaitensis* (◆, dotted line, 8.2 log CFU/mL); *Hafnia alvei* (■, dashed line, 8.9 log CFU/mL); *Staphylococcus equorum* (▲, dashed line, 8.1 log CFU/mL).
The pH in the gastric compartment is plotted as a dotted line against the right-hand axis.

3.1.2 The duodenal phase

H. alvei also showed strong resistance to the duodenal conditions (Figure. 2B). Its viability level did not change over the first 40 min and then fell from 8.7 log CFU.mL^{-1}to 7.5 log CFU.mL^{-1} by the end of the experiment (110 min). The initial viability level of *Lc. lactis* (7.2 log CFU.mL^{-1}) remained constant over 70 min of exposure and fell to a value of 4.8 log CFU.mL^{-1} by 110 min. *B. aurantiacum* and *S. equorum* displayed similar viability curves; the counts fell steadily from 8.2 log CFU.mL^{-1} at the beginning of the duodenal phase to 6.0 log CFU.mL^{-1} by the end. *C. casei* displayed a similar pattern, except that the initial value of 7.0 log CFU.mL^{-1} had fallen to 5 log CFU.mL^{-1} by the end of the duodenal phase. The initial viability level of *A. arilaitensis*' was 8 log CFU.mL^{-1}. This value fell by almost 2 log CFU.mL^{-1} over the first 40 min of duodenal exposure and was zero at the end of the experiment. As seen in the gastric compartment, the yeasts viability was not affected by the duodenal phase of the experiment. The counts of *G. candidum, K. lactis* and *D. hansenii* remained constant (at 3.8, 6.5 and 6.0 log CFU.mL^{-1}, respectively) throughout the duodenal phase.

3.1.3 The small intestine phase

We observed three different bacterial viability profiles in the small intestine phase. The viability of *H. alvei* remained constant, at 8.5 log CFU.mL^{-1}. *S. equorum*, *B. aurantiacum* and *C. casei* displayed a viability value of 7.0 log CFU.mL^{-1} at the beginning of the small intestine phase and fell to a value of 5 CFU.mL^{-1} by the end. The viability of *A. arilaitensis* and *Lc. lactis* had fallen to zero by the end of the small intestine phase. However, *Lc. lactis* survived for at least 150 min, whereas *A. arilaitensis*' viability fell to zero after 120 minutes. As seen in the gastric and duodenal compartments, the yeasts' viability remained constant throughout the small intestine phase, with values of 3.5, 6.5 and 6.2 log CFU.mL^{-1} for *G. candidum*, *K. lactis* and *D. hansenii*, respectively.

3.1.4 Overall DIDGI process

Overall, the yeasts appeared to be strongly resistant to the stress encountered in the DIDGI's three compartments. Indeed, between the start of the gastric phase and the end of the small intestine phase, none of the yeasts lost more than 1.5 log CFU.mL^{-1} in viability. The counts were fairly high at the end of the experiment as a whole (3.5, 6.5 and 6.2 log CFU.mL^{-1} viable cells for *G. candidum*, *K. lactis* and *D. hansenii*, respectively). When considering the bacteria, *H. alvei* was extremely resistant to the overall process and only lost 1 log CFU.mL^{-1} in viability between the first sample in stomach and the last sample in the small intestine. *S. equorum, C. casei* and

B. aurantiacum were fairly resistant, since live cells were still present at the end of the experiment. In contrast, *Lc. lactis* and *A. arilaitensis* did not withstand the *in vitro* digestive process.

3.2 *In vitro* experiments: a liquid mixture of microorganisms and a rennet gel inclusion

Importantly, we found that mixing the nine bacteria and yeasts immediately before the beginning of the DIDGI experiment or including this mixture in a rennet gel did not change the species' viability patterns in any of the compartments, when compared with the pure individual cultures described above (data not shown) every other things being equals (i.e. DIDGI parameters, sampling time, plating and count method).

3.3 *In vitro* experiments with cheese

We next sought to assess the influence of growth in a simplified but realistic smear-ripened cheese environment on the microorganisms' abilities to withstand digestive stress, relative to a mixture of the 9 microorganisms in broth.

Figure 3 compares the viability of *H. alvei* in pure cultures and cheese-grown conditions for the DIDGI's three compartments. The starting concentration was higher for the pure-culture condition than for the cheese condition. For the cheese condition (Figure 3A), the viability in the gastric compartment remained constant for 50 min and then started to dropped to zero over 70 minutes. As mentioned above, the viability of the pure-culture condition was still above 6 log CFU.mL^{-1} at the same time point; *H. alvei* was less resistant to gastric conditions when grown in cheese than in a pure culture. In the duodenal compartment (Figure 3B), the pure culture and cheese conditions displayed the same changes over time in viability. Likewise, the viability in both conditions remained stable in the small intestine compartment (Figure 3C). The slightly lower viability for the cheese condition (less than 1 log CFU.mL^{-1}) was not statistically significant (as judged by the standard deviations). In the case of *S. equorum* (Figure 4), the change over time in viability was the same for the pure culture and cheese conditions in all three compartments. Hence, growth in cheese conditions did not appear to change *S. equorum*'s ability to resist *in vitro* digestive stress in the DIDGI.

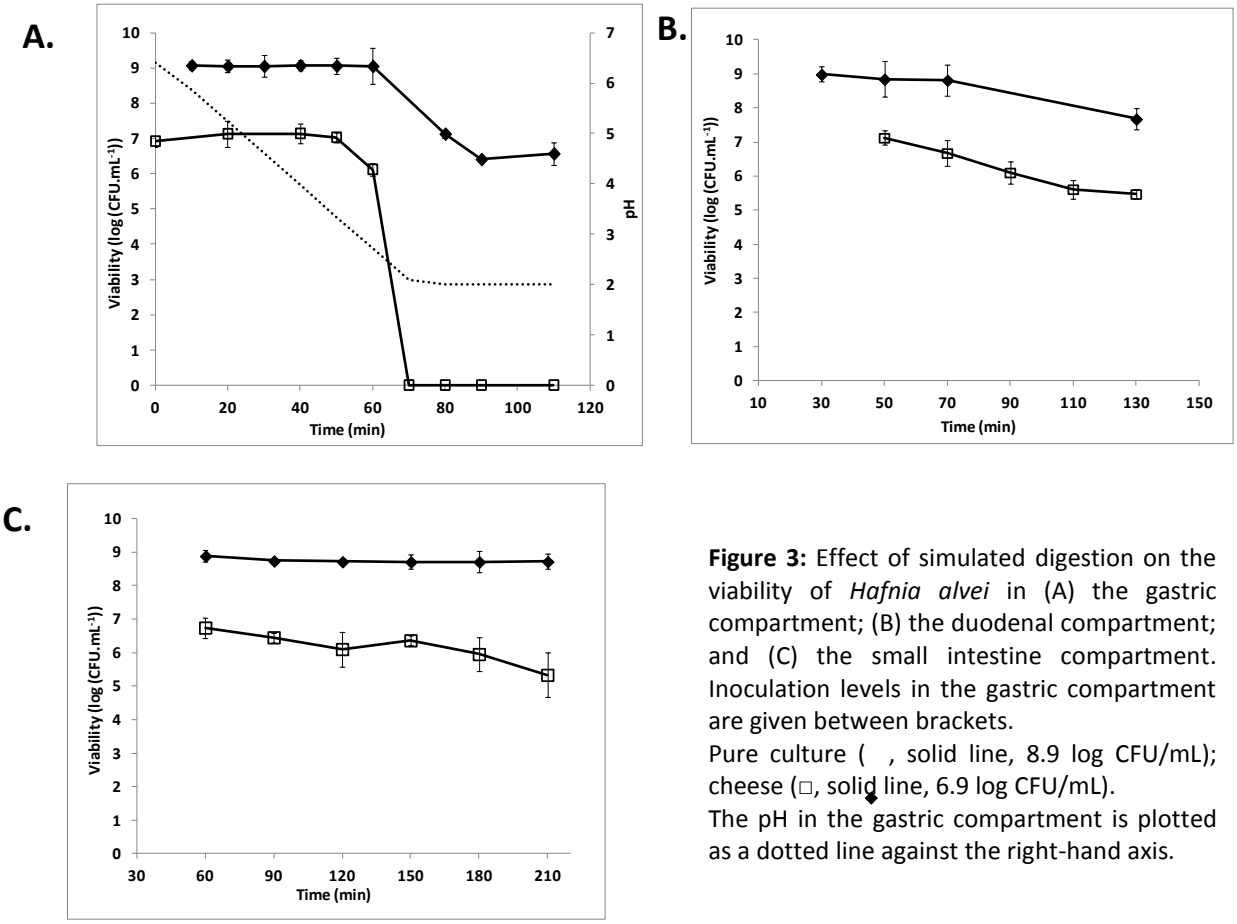


Figure 3: Effect of simulated digestion on the viability of *Hafnia alvei* in (A) the gastric compartment; (B) the duodenal compartment; and (C) the small intestine compartment. Inoculation levels in the gastric compartment are given between brackets.
Pure culture (, solid line, 8.9 log CFU/mL); cheese (□, solid line, 6.9 log CFU/mL).
The pH in the gastric compartment is plotted as a dotted line against the right-hand axis.

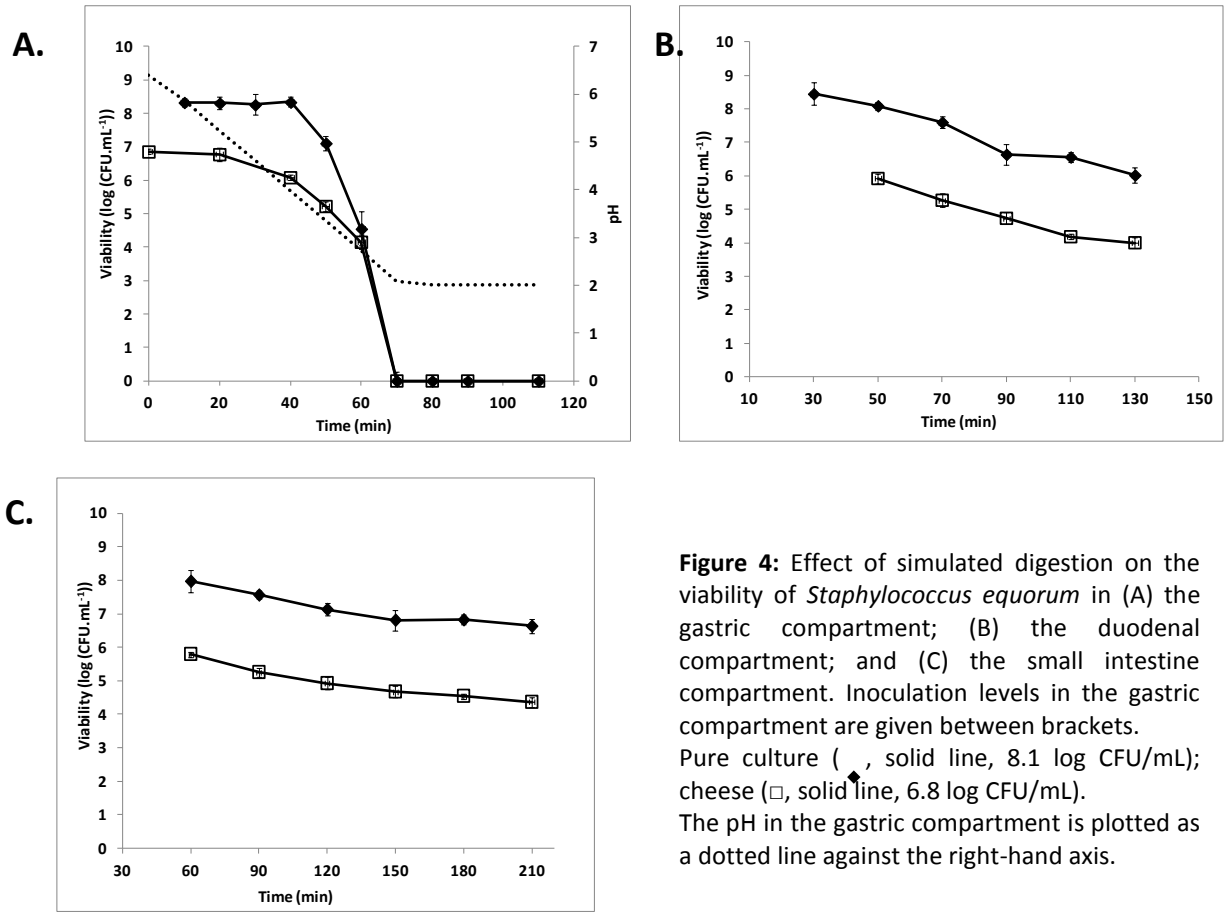


Figure 4: Effect of simulated digestion on the viability of *Staphylococcus equorum* in (A) the gastric compartment; (B) the duodenal compartment; and (C) the small intestine compartment. Inoculation levels in the gastric compartment are given between brackets.
Pure culture (, solid line, 8.1 log CFU/mL); cheese (□, solid line, 6.8 log CFU/mL).
The pH in the gastric compartment is plotted as a dotted line against the right-hand axis.

The change over time in *G. candidum*'s viability in the three compartments differed slightly when comparing the pure culture condition with the cheese condition (Figure 5). From 60 min to the end of gastric exposure, the viability level in the cheese condition dropped from 6.5 log CFU.mL^{-1} to 4.5 log CFU.mL^{-1}, whereas that of the pure culture conditions was unchanged (Figure 5A). In the duodenal phase (Figure 5B) and the small intestine phase (Figure 5C), *G. candidum*'s viability in the pure culture condition was stable. In contrast, the viability *G. candidum* in the cheese condition fell from 5.8 log CFU.mL^{-1} to 3 log CFU.mL^{-1} at the end of the duodenal phase and from 5.8 log CFU.mL^{-1} to 4 log CFU.mL^{-1} at the end of the small intestine phase. In light of these findings, *G. candidum* appears to be more sensitive to digestive stress when it is grown in cheese than in a pure culture.

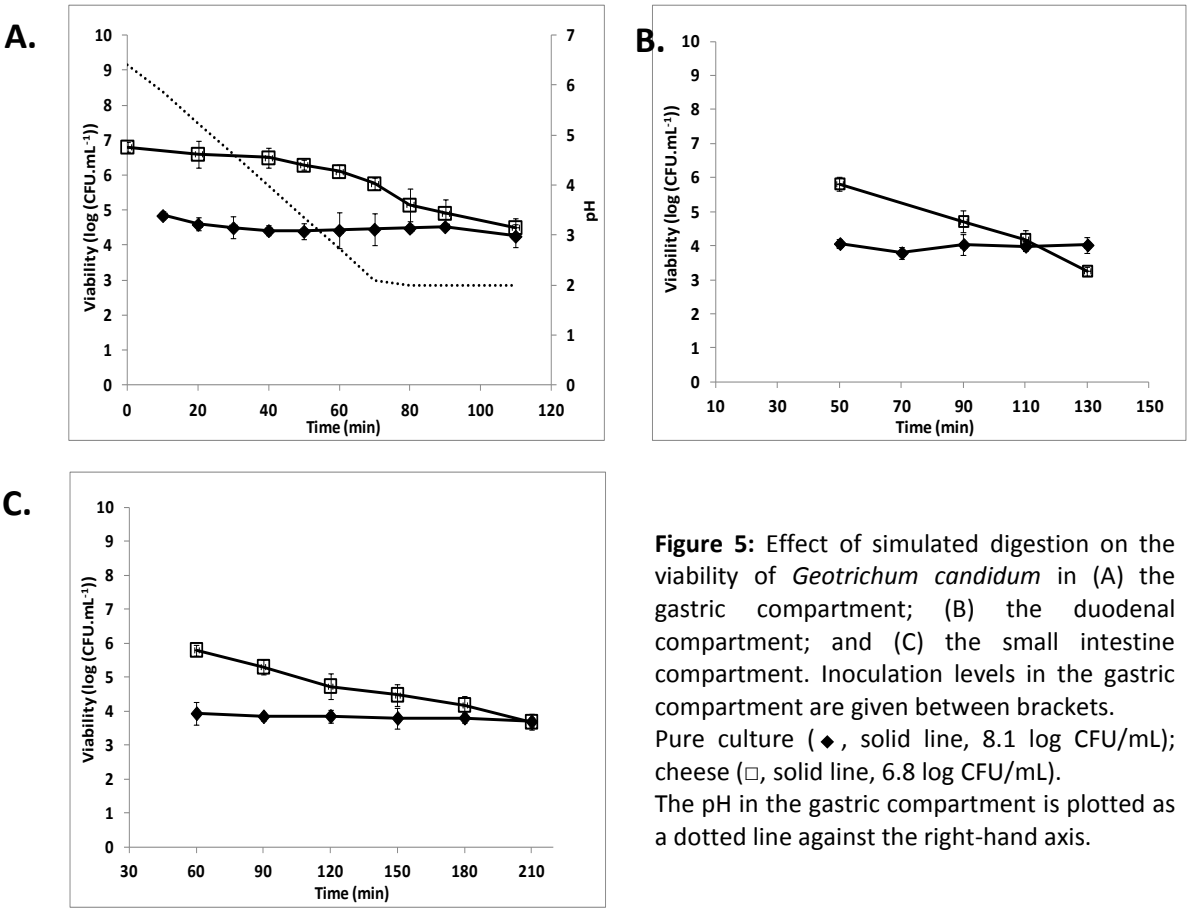


Figure 5: Effect of simulated digestion on the viability of *Geotrichum candidum* in (A) the gastric compartment; (B) the duodenal compartment; and (C) the small intestine compartment. Inoculation levels in the gastric compartment are given between brackets.
Pure culture (◆, solid line, 8.1 log CFU/mL); cheese (□, solid line, 6.8 log CFU/mL).
The pH in the gastric compartment is plotted as a dotted line against the right-hand axis.

The viability of *C. casei* (Figure S3) for pure-culture and cheese conditions displayed the same patterns in the DIDGI's three compartments. The gastric-phase viability of *B. aurantiacum* (Figure S4) in cheese fell from 6 log CFU.mL^{-1} to less than 3 log CFU.mL^{-1} (i.e. below the detection limit of the plating method) within 50 min, whereas the value in a pure culture took 80 min to drop from 8.5 log CFU.mL^{-1} to less than 3 CFU.mL^{-1}. Although the viability of a pure culture fell during the duodenal and small intestine phases, the final concentration of live cells was still significant at the end of the experiment 6.5 log CFU.mL^{-1} at 210 min in the small intestine compartment.

In the cheese condition, *B. aurantiacum*'s viability had fallen to zero long before the end of the experiment. *Lactococcus lactis*' viability (Figure S5) in gastric conditions fell to zero within 60 min for cheese but remained above 3.5 log CFU.mL^{-1} in the pure culture condition. The duodenal phase led to complete loss of viability for *Lc. lactis* in cheese, whereas a pure culture still displayed a value of 5 log CFU.mL^{-1} at the end of the phase. Lastly, both cheese and pure cultures contained viable *Lc. lactis* cells at the end of the small intestine phase 3.5 log CFU.mL^{-1} and 5 log CFU.mL^{-1}, respectively.
In summary, the culture conditions of *K. lactis* and *D. hansenii* did not significantly affect the change over time in viability when the yeasts were exposed to digestive stress in the DIDGI. In contrast, *G. candidum* appeared to be slightly more sensitized when grown in cheese. This was also the case for bacteria such as *H. alvei* (albeit in the gastric phase only). *Corynebacterium casei's* and *S. equorum's* respective abilities to withstand digestive stress were not modified by growth in cheese, relative to growth in broth. *Brevibacterium aurantiacum* was clearly more sensitive to the overall digestion experiment when grown in cheese than when grown in pure culture. This was also the case for *A. arilaitensis*. *Lactobacterium lactis* was more sensitive to the conditions in the gastric and duodenal compartments when grown in cheese.

3.4 Acid curves

Since acid is likely to be a significant stress factor, we monitored the addition of HCl during each experiment. The DIDGI was set up so that the pH curve was the same for experiments with pure cultures, the rennet-gel inclusion and the cheese matrix. Figure 6 clearly shows that the quantity of HCl varied markedly from one condition to another; the final volumes were 3.5 mL, 11 mL and 50 mL for the pure culture, the rennet gel and cheese, respectively.

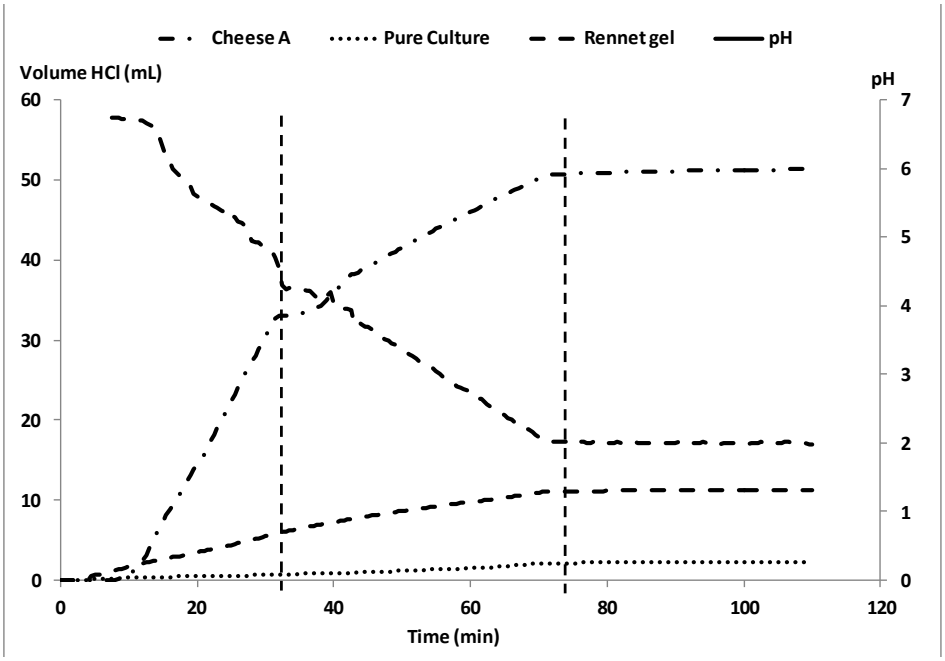


Figure 6 : The change over time in the volume (mL) of 1 M HCl added during the gastric phase of a DIDGI experiment, for each of the three matrixes.
pH (solid line); pure culture (dotted line); rennet gel (dashed-line); cheese (dashed-dotted line)

3.5 *In vivo* experiments with microorganisms grown in pilot-scale cheeses

These experiments were designed to assess the *in vivo* resistance of surface-ripened cheese microorganisms grown in pilot-scale cheeses. In general, it appeared that the microorganisms grown in cheese were able to survive passage through the murine digestive tract (Table 3). With the exception of *Lc. lactis* and *A. arilaitensis*, live bacteria were found in mice feces at counts ranging from almost 4 log CFU.g^{-1} feces (for *B. aurantiacum*) to 7.5 log CFU.g^{-1} feces (for *C. casei*). The yeasts also withstood passage through the murine digestive tract: live *D. hansenii*, *G. candidum* and *K. lactis* were all found in the feces at counts close to 4 log CFU.g^{-1} feces.

Table 8 : Survival of cheese-ripening microorganisms after ingestion by BALB/c mice (n=10)

Microorganisms	**Diluted cheese (feeding)**	
	Viable cells (log CFU.g^{-1})	**Feces (8 h)** (log CFU.g^{-1})
Bacteria		
A. arilaitensis Re117^{T}	5.69 ± 0.09	ND
B. aurantiacum ATCC9174	6.51 ± 0.15	4.29 ± 0.39
C. casei 1-3b	8.52 ± 0.12	7.52 ± 0.25
H. alvei 920	6.61 ± 0.19	5.21 ± 0.29
Lc. Lactis S3	7.04 ± 0.09	ND
S. equorum Mu206	6.32 ± 0.12	5.13 ± 0.32
Yeasts		
D. hansenii CLIB 623	5.93 ± 0.08	4.16 ± 0.56
G. candidum ATCC 204307	6.32 ± 0.30	3.73 ± 0.35
K. lactis CLIB 683	6.42 ± 0.18	4.65 ± 0.20

ND : Not detected

4. Discussion

Scientific interest in food digestion and nutrient bioavailability has grown rapidly in the last ten years. For example, several new journals dedicated to this new field of research have been launched, and the number of published articles increased dramatically from 400 in 1995 to nearly 1400 in 2013. In this context, the fate of food microbial ecosystems in humans is of a great interest in terms of nutrient availability and potential effects on gut immunity. One of the main objectives of the present work was to evaluate the survival of cheese-ripening microorganisms in conditions that would approximate digestive stress, by using a recently developed *in vitro* digester. Given the broad variety of models described in the literature (Guerra *et* al. 2012), we had to determine which digestive compartments and parameters (e.g. pH, transit time, and digestive juice components) were likely to have an impact on the microorganisms' viability. We therefore designed a three-stage system (modelling the stomach, duodenum and small intestine) and decided to use the same operating parameters for all matrices (i.e. a pure culture, a rennet gel or cheese) in order to compare the respective datasets. Our *in vitro* digestive model was a convenient way of comparing the viability of microorganisms exposed to reproducible stress conditions while changing the input conditions. Overall, a variety of cheese-isolated microorganisms were found to be alive at the end of the DIDGI experiment. It is noteworthy that the gastric compartment produced a greater drop in the microorganisms'viability than the downstream duodenal and small intestine compartments did. This observation suggests that HCl is indeed the most intense stress encountered by microorganisms during the digestive process. Yeasts were strongly resistant to the *in vitro* digestive process as whole, confirming previous studies (Lay *et* al., 2004, Psomas *et* al., 2001) in which a selection of *D. hansenii*, *K.lactis* and *G. candidum* strains survived well when exposed to digestive stress. When considering bacteria, the genetic and phenotypic similarities between *E. coli* and *H. alvei* (Janda *et* al., 2002) prompted us to assume that *H. alvei* would resist digestive stress, as reported in both *in vivo* and *in vitro* experiments. Furthermore, the greater sensitivity to gastric stress observed when *H.alvei* had been grown in cheese may be explained by the poorer availability of some amino acids. Indeed, growth in an ecosystem generates inter-strain competition for nutrients such as glutamic acid. The latter's key role in resistance to acid stress in (among others) Gram-negative bacteria has been widely documented (Krulwich *et* al. 2011). Further research will seek to confirm the presence of this mechanism of action in *H. alvei*. Kim *et* al. (1999) reported that *Lc. lactis* subsp. *lactis* strains were able to survive a pH as low as 2.5 in M17 medium acidified with HCl. The resistance of *Lc. lactis* subsp. *lactis* to the gastric phase of the present study is consistent with the species' physiology and the literature results (i.e. maintenance of viability above pH 2.5).

However, the viability of *Lc. lactis* fell quickly below this pH threshold, highlighting the great effect of a small pH variation when a strain is close to its limit.

As mentioned above, the lack of published studies on the response to digestive stress of the surface-ripened cheese Gram-positive microflora makes it difficult to compare our present results with the literature. However, some information on the response to acid stress can be gleaned from studies of cheese ripening. The work by Bockelman et al. (2002) showed that *S. equorum* is part of the early smear-ripened cheese microflora that is established when the cheese curd is still acidic (pH 5). In contrast, *C. casei* and *B. aurantiacum* start to develop when a pH of 6 is reached. It was therefore not surprising that *S. equorum* was better able to resist a gastric-type challenge than *C. casei* and *B. aurantiacum*, both of which failed to survive this phase.

In contrast to the static model used in our previous studies (Adouard et al. 2014, accepted), the DIDGI's continuous fermentation enabled a certain proportion of otherwise acid-sensitive microorganisms to survive the gastric compartment and pass into the downstream compartments. In dynamic experiments, the stress is applied gradually and so part of the bolus is transferred before the stress becomes stringent. Hence, a proportion of the gastric-sensitive microorganisms are able to stay alive until the end of the experiment if they can resist conditions in downstream compartments. Clearly, this kind of approach models the real digestion process more closely. Indeed, almost all of the microorganisms selected in the present work were able to withstand the full digestive process in the murine gastrointestinal tract. These findings corroborate our initial hypothesis, i.e. that a dynamic *in vitro* model is more accurate than a batch model (even though the former still does fully reproduce what happens *in vivo*).

We originally assumed that the presence of a barrier-like, buffering rennet gel (Upreti et al. 2006) and (especially) cheese-grown microorganisms would enable acid-sensitive strains to better survive the digestive stress. However, our experimental results did not reveal an obvious protective effect. The time course of acidification was the same for all input matrices, which therefore forced the system to add more HCl for matrices with a greater buffering capacity. The hypothesis that a given amount of HCl would be secreted regardless of the nature/amount of the matrix is therefore quite improbable. On the contrary, one can reasonably expect acid secretion in the stomach to be stimulated when the bolus has a higher buffering capacity (as occurred in the DIDGI). This increase raises the question of whether our *in vitro* results faithfully mimic *in vivo* digestion. We therefore calculated the mean acid flow rate and the maximum acid concentration in the gastric compartment in the DIDGI in the presence and absence of the cheese matrix and them with *in vivo* available data (Table 4). We found that the DIDGI's observed acid concentrations and flow rates during cheese experiments exceeded *in vivo* values.

Firstly, the cheese matrix's buffering capacity of the matrix led to an increase in the amount of secreted HCl *in vitro*. Secondly, acid secretion *in vivo* may be influenced by other physiological parameters such as gastric emptying, which is known to be strongly influenced by the nature of the bolus; the thicker the bolus, the slower the gastric emptying (Low et al. 1990).

Table 9: Flow rates and acid concentrations in the stomach *in vivo* and in the DIDGI's gastric compartment.

	Physiological data	**Experimental data**		
	Smith and Morton, (2010)	Pure culture	Rennet gel	Cheese
HCl flow rate during acidification ($mmol.h^{-1}$)	25	2.1	10	44.6
Maximum HCl concentration ($mmol.L^{-1}$)	150	13	51	225

The presence of fat globules and proteins also changes the time course of emptying (Turgeon et al. 2011). Our findings can be compared with those of an interesting study by Gardiner et al. (1998), in which microorganisms included in cheddar cheese and submitted to *in vitro* digestive stress were better protected than those included in yogurt. The researchers' main explanation was based on the cheese's stronger buffering capacity (as a result of a higher peptide concentration than yogurt). However, the study's *in vivo* results failed to evidence a significant difference between cheese and yogurt matrices in terms of their ability to protect microorganisms throughout the digestive process. These various findings prompt us to think that the quantity of HCl in the DIDGI should be fixed and the change over time in the pH should be monitored (rather than the other way around). In fact, the physiological situation is likely a balance between these two means of regulating the stomach's acidity. Indeed, acidification is driven by the stomach's HCl sensors and depends on the nature of the bolus (Smith and Morton 2010). In future research with the DIDGI, we intend to study variations in other parameters (such as the inter-compartment transfer rate) and their relationships with the tested matrix's physical and chemical properties.

Taken as a whole, the present results enabled us to (i) reliably show that cheese microorganisms survive digestive stress, (ii) gain an initial picture of the impact of growth in a real cheese matrix on the microorganisms' survival ability, and (iii) validate the DIDGI as a more reliable tool than basic batch models. The growing interest in food microorganisms and their fate after ingestion prompts the question of how they might affect human health, since it is clear that a considerable proportion can survive passage through the gastrointestinal tract. These questions are being addressed in a gripping and challenging field of investigation at the intersection between microbiology, immunology and food science.

Acknowledgements

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Supplementary data

Table S10: Microbial concentrations in model cheeses after a 28-day ripening period

Species	Microbial concentration (log CFU.g-1)
Bacteria	
A. arilaitensis Re117^T	6.17 ± 0.18
B. aurantiacum **ATCC9174**	6.55 ± 0.12
C. casei **CC13b**	9.00 ± 0.50
H. alvei **920**	6.90 ± 0.25
Lc. Lactis ***S3***	7.52 ± 0.12
S. equorum **Mu206**	6.80 ± 0.17
Yeasts	
D. hansenii **CLIB 623**	6.41 ± 0.25
G. candidum **ATCC 204307**	7.81 ± 0.23
K. lactis **CLIB 683**	6.90 ± 0.18

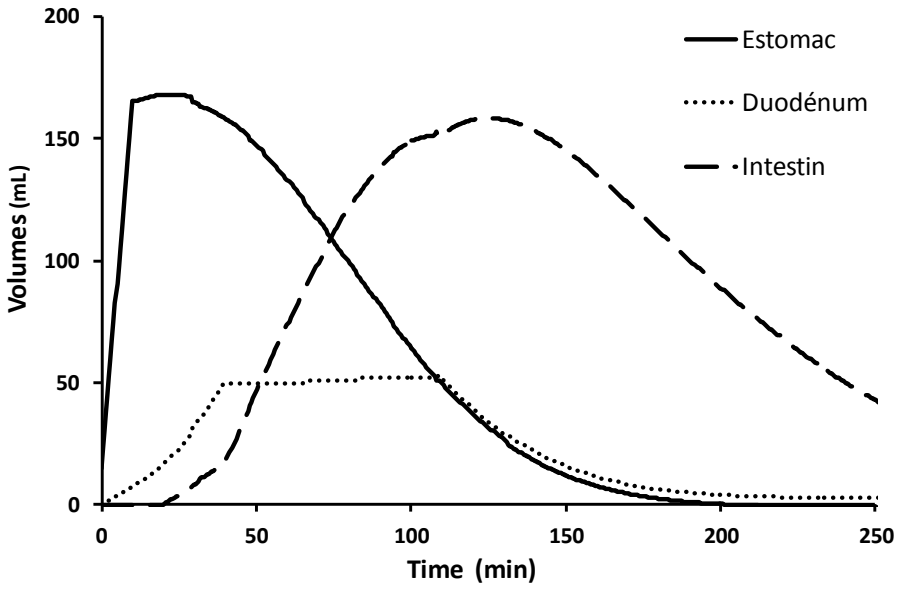

Figure S1: Filling/emptying curves for the DIDGI compartments during a 250-min experiment

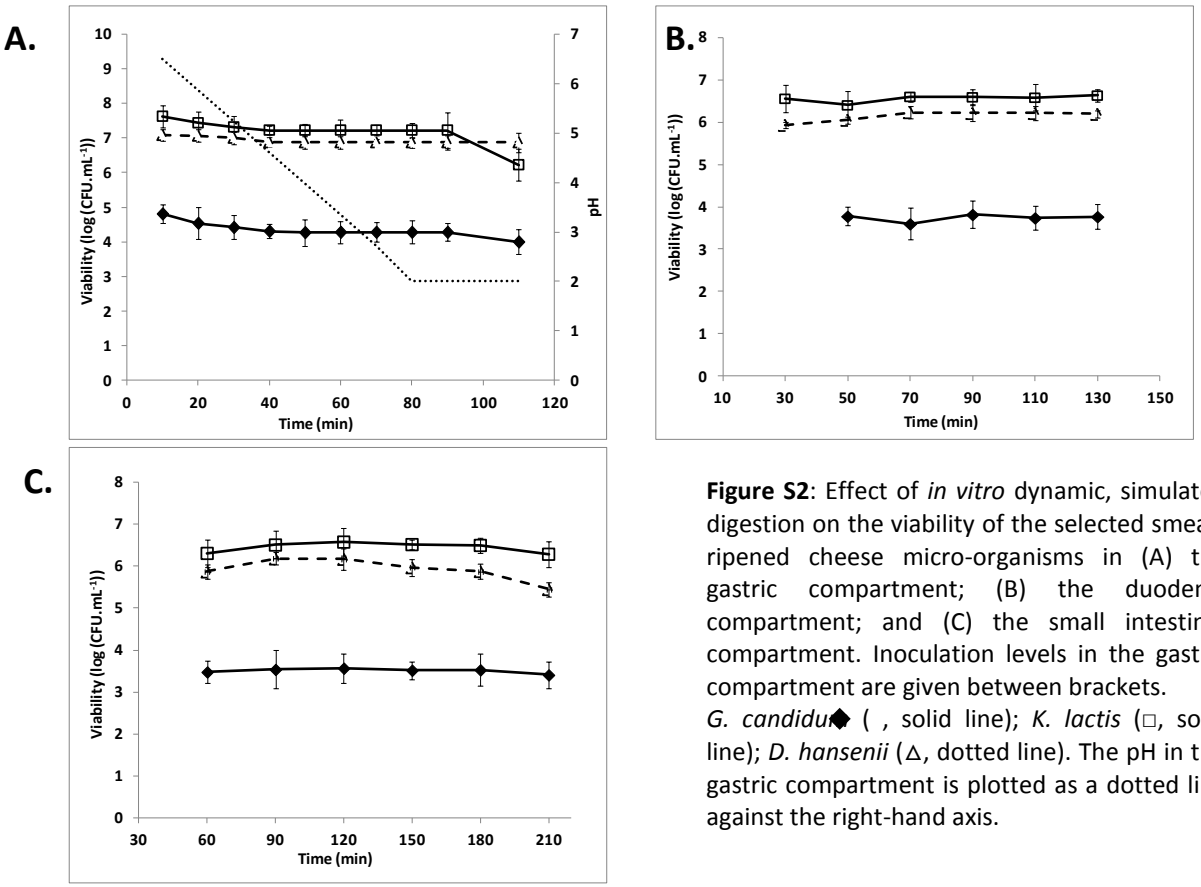

Figure S2: Effect of *in vitro* dynamic, simulated digestion on the viability of the selected smear-ripened cheese micro-organisms in (A) the gastric compartment; (B) the duodenal compartment; and (C) the small intestine. compartment. Inoculation levels in the gastric compartment are given between brackets.
G. candidum ◆ (, solid line); *K. lactis* (□, solid line); *D. hansenii* (∆, dotted line). The pH in the gastric compartment is plotted as a dotted line against the right-hand axis.