Chapitre C Caractérisation d'une sélection de micro-organismes issus d'écosystèmes fromagers au regard de leur survie à un stress digestif *in vitro* et de leur effet immunomodulateur *in vitro*.

1. Présentation de l'article 1 accepté dans *International Journal of Food Microbiology*

Le double objectif de cette partie des travaux était de mettre au point (i) une méthode de digestion *in vitro* statique permettant le screening d'un nombre important de micro-organismes quant à leur capacité de survie à un stress digestif simulé et (ii) une méthode basée sur un technique immunologique *in vitro* afin d'obtenir un profilage de ces mêmes micro-organismes quant à leur caractère plutôt pro- ou anti-inflammatoire.

En effet, le grand nombre de modèle de digestion *in vitro* présents dans la littérature et leur design bien souvent lié à la nature de la matrice alimentaire à laquelle ils s'intéressent, a nécessité une analyse pointue de la littérature. La revue de Hur et al. (2010) a servi de base à cette analyse et a été complétée par un listing minutieux des conditions de digestion *in vitro* de manière à trouver un compromis entre des modèles expérimentaux parfois très différents. La difficulté à comparer des études réalisées dans des conditions parfois peu comparables, a fait émerger la nécessité d'établir un protocole qui fasse consensus parmi une communauté de scientifiques dont les objets d'étude sont parfois très variés. Pour cela, des consortia tels que le COST Infogest[3] ont été formés et celui-ci, en particulier, a abouti à un publication très récente dans le journal « Food and Function» (Minekus et al., 2014). L'équipe ADP, dont je dépends, a participé à l'élaboration de cet article. Ainsi la méthode de digestion *in vitro* que nous avons choisie d'adopter est en grande partie en adéquation avec les propositions d'Infogest. Elle se compose de deux compartiments : un estomac et un duodénum. Nous avons choisi d'exposer les micro-organismes à (i) un stress estomac, (ii) un stress duodénum et (iii) un stress estomac suivi d'un stress duodénum, afin de mettre en évidence lequel de ces deux stress (estomac ou duodénum) avait l'impact le plus important sur la viabilité des bactéries et levures testées. Les micro-organismes identifiés comme sensibles à l'un et/ou à l'autre de ces stress ont ensuite été inclus dans un gel présure, afin de mettre en évidence un éventuel effet protecteur de la présence d'une matrice laitière.

[3] https://www.cost-infogest.eu/ABOUT-Infogest

Les micro-organismes choisis étaient au nombre de trente-six (vingt-et-une bactéries, douze levures et trois champignons filamenteux). Parmi ces trente-six micro-organismes se trouvent des espèces dont la diversité potentielle de réponse a été évaluée en sélectionnant deux à trois souches par espèces. *Lactobacillus rhamnosus* GG, *Escherichia coli* Nissle 1917 et *Saccharomyces boulardii* utilisés comme probiotiques ont été choisis comme micro-organismes de référence.

La détermination du profil immunomodulateur des micro-organismes a fait appel à une technique éprouvée et reconnue, basée sur la réponse de cellules immunocompétentes – les Peripheral Blood Mononuclear Cells – isolées de différents donneurs humains (Foligné et al., 2007). Elle permet de classer les micro-organismes selon leur capacité à induire différents types de cytokines identifiées comme marqueurs d'une réponse plutôt pro- ou plutôt anti-inflammatoire.

Cet article a permis d'obtenir une caractérisation générale des micro-organismes choisis avant de procéder à (i) une étape de digestion toujours *in vitro* mais cette fois-ci dynamique, (ii) de procéder aux premières expérimentations *in vivo* en modèle murin et enfin (iii) de designer des écosystèmes aux profils immunomodulateurs *a priori* différents, basés sur les profils des souches individuelles.

2. Faits marquants

- ✓ Nous avons développé une méthode de digestion *in vitro* statique en deux étapes.
- ✓ Nous avons caractérisé le potentiel immunomodulateur *in vitro* de micro-organismes fromagers.
- ✓ Les bactéries à Gram-positif et à Gram-négatif ont différé par leurs réponses.
- ✓ La majorité des levures ont donné des profils immunomodulateur similaires.
- ✓ L'inclusion en gel présure a protégé du stress gastique.

In vitro characterization of the digestive stress response and immunomodulatory properties of microorganisms isolated from smear-ripened cheese

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Thirty-six microorganisms (twenty-one bacteria, twelve yeasts and three fungi) were isolated from surface-ripened cheeses and subjected to *in vitro* digestive stress. The approach mimicked gastric and/or duodenal digestion. *Lactobacillus rhamnosus* GG, *Escherichia coli* Nissle 1917 and *Saccharomyces boulardii* were used as reference strains. We studied the microorganisms grown separately in culture medium and then included (or not) in a rennet gel. The microorganisms' immunomodulatory abilities were also assessed by profiling cytokine induction in human peripheral blood mononuclear cells (PBMCs). The loss of viability was less than 1 log CFU/mL for yeasts under all conditions. In contrast, Gram-negative bacteria survived gastric and/or duodenal stress well but most of the Gram-positive bacteria were more sensitive (especially to gastric stress). Inclusion of sensitive Gram-positive bacteria in rennet gel dramatically improved gastric survival, when compared with a non-included cultured (with a 4 log CFU/mL change in survival). However, the rennet gel did not protect the bacteria against duodenal stress. The PBMC cytokine assays tests showed that the response to yeasts was usually anti-inflammatory, whereas the response to bacteria varied from one strain to another.

Highlights

- ✓ We performed a two-step, *in vitro* batch digestive stress experiment.
- ✓ We tested the immunomodulatory properties of microorganisms isolated from cheeses.
- ✓ Gram-positive and Gram-negative bacteria differed in their resistance to digestive challenge.
- ✓ All yeast species had very similar immunomodulatory profiles.
- ✓ Rennet gel inclusion protected bacteria against gastric challenge.

Keywords

Smear-ripened cheese microbiota, peripheral blood mononuclear cell, immunomodulation, *in vitro* digestive model, digestive stress.

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 live 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 microbiota depends on the type of cheese. In Cheddar and mozzarella, the microbiota 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 microbiota in soft, smear-ripened cheeses (such as Livarot and Munster) contains a broad, diverse range of bacteria and yeasts (Irlinger and Mounier, 2009). Thus, a fermented food product like cheese is an important, diverse source of microorganisms in the human diet. However, few studies have investigated the survival of the cheese microbiota 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 finding new probiotics or using cheese as a carrier for known probiotics (Saxelin *et* al. 2010). Indeed, cheese and (more generally) dairy matrices are often referred to as good vehicles for microorganisms, given their buffer properties and the physical barrier against digestive stress that they may provide (Lollo *et* al. 2012, Salaun *et* al., 2005, Sharp *et* al., 2008). 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). Likewise, cheese-ripening yeasts (such as *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Geotrichum candidum*) were able to survive *in vitro* challenges with acid and bile (Kumura *et* al. 2004, Lay *et* al. 2004, Psomas *et* al. 2001).

It is widely acknowledged that the intake of food-grade microorganisms influences the host's immune responses (both inside the gut and at distant sites). Indeed, many microbial-derived antigens, secreted compounds, surface molecules and cell-wall components (*e.g.* peptidoglycan, exopolysaccharides, teichoic acids, and mannans) have immunomodulatory properties (Lebeer *et* al., 2010). While it appears obvious that the type of immune-related response depends on intrinsic characteristics of each type of microbe (*e.g.* Gram-positive or Gram-negative bacteria, yeasts or fungi) and species, immune tuning also appears to be strain-specific - as has been demonstrated *in vitro* for probiotic LAB (Foligné *et* al., 2007, Nova *et* al., 2007), bifidobacteria (Hoarau *et* al., 2008, Riedel *et* al, 2006) and yeasts (Foligné *et* al., 2010, Maccaferri *et* al., 2012, Romanin *et* al., 2010). In contrast, only sporadic attempts have been made to characterize the immune patterns induced by a

very small number of bacterial or eukaryotic food strains isolated from cheese-ripening ecosystems (Rhaman *et* al., 2013).

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),. Whereas *in vivo* studies in animal models are quite expensive and intricate to perform, *in vitro* models offer greater reproducibility, few ethical issues and the ability to collect samples throughout the experiment. Most of the *in vitro* approaches have focused on aspects of food digestion, such as the bioavailability of nutrients (Salvia-Trujillo *et* al., 2013) and the release of food-borne toxins (Versantvoort *et* al., 2005). The lack of literature data on the fate of food microorganisms in general and ripened-cheese microbiota in particular prompted us to design a series of experiments on the strains' ability to survive simulated gastric and duodenal digestion.

We therefore isolated microorganisms from surface-ripened cheese (Mounier *et* al., 2008) and set up a two-step screening method consisting of (i) a batch-based *in vitro* gastric and/or duodenal challenge and ii) assays for cytokines released *in vitro* by human peripheral blood mononuclear cells (PBMCs). Lastly, some strains were included in a rennet gel, in order to assess the potential protective effect of a dairy food matrix.

2. Material and Methods

2.1 Microorganisms

The list of microorganisms used in the present study is available on Table 1. With the exception of *Hafnia alvei* GB01, all of the 36 microorganisms considered in our study (21 bacteria, 12 yeasts and three fungi), were isolated from dairy environments. Most were found on surface-ripened cheeses. Three commercially available probiotic strains– *i.e*. *Lactobacillus rhamnosus* GG ATCC53103 (Valio, Helsinki), *Saccharomyces boulardii (*Biocodex, Gentilly, France) and *E.coli* Nissle 1917 (Ardeypharm, Herdecke, Germany) were used for comparative purposes. Furthermore, five bacterial strains (*Bifidobacterium longum* Bb536, *Escherichia coli* TG1, *Lactobacillus acidophilus* NCFM, *Lactobacillus salivarius* Ls33 and *Lc. Lactis* MG1363) were used as references in the PBMC stimulation assay, as previously described (Foligne *et* al., 2007).

Table 1. List of the microbial strains and growth conditions used in the present study

Species	Strain	Origin	Media	Growth conditions, °C ; rpm
Lactobacillus delbrueckii subsp. bulgaricus	CNCM I-2809	Yogurt	MRS	37 °C - static
Lactococcus lactis	S3	Cheese	M17	30°C - static
Streptococcus thermophilus	CNCM I-2802	Yogurt	M17	42°C - static
Streptococcus thermophilus	LMD-9	Yogurt	M17	42°C - static
Streptococcus thermophilus	LMG-18311	Yogurt	M17	42°C - static
Arthrobacter arilaitensis	Re 117^T	Cheese(Reblochon)	BHI	25°C – 200 rpm
Arthrobacter arilaitensis	3M03	Cheese (Livarot)	BHI	25°C – 200 rpm
Arthrobacter arilaitensis	Ma107	Cheese (Maroilles)	BHI	25°C – 200 rpm
Brevibacterium aurantiacum	ATCC 9174	Cheese (Romadur)	BHI	25°C – 250 rpm
Brevibacterium aurantiacum	ATCC 9175	Cheese(Camembert)	BHI	25°C – 250 rpm
Brevibacterium aurantiacum	Ba 171	Cheese (Munster)	BHI	25°C – 250 rpm
Corynebacterium casei	2M01	Cheese (Livarot)	BHI	25°C – 200 rpm
Corynebacterium casei	DPC S298^T	Cheese (Gubbeen)	BHI	25°C – 200 rpm
Corynebacterium casei	1-3b	Cheese (Livarot)	BHI	25°C – 200 rpm
Escherichia coli	1E14	Cheese (Livarot)	BHI	25°C – 200 rpm
Hafnia alvei	GB01	Cheese	BHI	25°C – 200 rpm
Hafnia alvei	Type 2 n°920	Dairy products	BHI	25°C – 200 rpm
Hafnia alvei	B16	Cheese (Livarot)	BHI	25°C – 200 rpm
Staphylococcus equorum	Mu2	Cheese (Munster)	BHI	25°C – 200 rpm
Staphylococcus equorum	1265/GM16	Cheese (Camembert)	BHI	25°C – 200 rpm
Staphylococcus equorum	Mu206	Cheese (Munster)	BHI	25°C – 200 rpm
Debaryomyces hansenii	1L25	Cheese (Livarot)	PDB	25°C – 200 rpm
Debaryomyces hansenii	CLIB 623	Cheese	PDB	25°C – 200 rpm
Debaryomyces hansenii	CBS 767	Cheese	PDB	25°C – 200 rpm
Geotrichum candidum	ATCC 204307	Cheese (Pont l'évêque)	PDB	25°C – 200 rpm
Geotrichum candidum	UCMA 359	Cheese	PDB	25°C – 200 rpm
Geotrichum candidum	UCMA 103	Cheese	PDB	25°C – 200 rpm
Kluyveromyces lactis	CLIB 196	Cheese	PDB	25°C – 200 rpm
Kluyveromyces lactis	CLIB 531	Cheese	PDB	25°C – 200 rpm
Kluyveromyces lactis	CLIB 683	Cheese	PDB	25°C – 200 rpm
Yarrowia lipolytica	1E07	Cheese (Livarot)	PDB	25°C – 200 rpm
Yarrowia lipolytica	CLIB 632	Cheese	PDB	25°C – 200 rpm
Yarrowia lipolytica	CLIB 791	Cheese	PDB	25°C – 200 rpm
Penicillium camemberti	FM 13	Cheese	PDB	25°C – 200 rpm
Penicillium camemberti	FM 340	Cheese	PDB	25°C – 200 rpm
Penicillium camemberti	PcR	Commercial strain	PDB	25°C – 200 rpm
Saccharomyces boulardii	Ultralevure	Commercial strain	PDB	25°C – 200 rpm
Escherichia coli	Nissle 1917	Commercial strain	BHI	25°C – 200 rpm
Lactobacillus rhamnosus	LGG -ATCC53103	Commercial strain	MRS	37 °C – static

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). Prior to use in the experiments described below, all strains were grown until they reached the same growth phase (the late stationary phase, as defined in prior growth kinetics experiments; data not shown). 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. *Streptococcus thermophilus* was cultured in M17 medium supplemented up to 20 g/L of lactose in 100 mL Schott bottles at 42°C. Lactobacilli were grown in DeMan, Rogosa and Sharpe broth (MRS) in 100 mL bottles at 37°C. Yeasts were grown in 100 mL of PDB in 500 mL Erlenmeyer flasks at 25°C, with shaking at 200 rpm. Microorganisms were counted on the agar-based media used for liquid cultures, *i.e*. BHI agar for strains grown in BHI broth, for example. Eukaryotes were plated on yeast extract glucose chloramphenicol plates. The incubation temperatures were the same as in broth cultures. Prior to plating, cultures were diluted in Maximum Recovery Diluent (MRD, 9 g/L) (Difco).

With respect to the reference strains for immune cell stimulation, lactobacilli were grown with limited aeration at 37℃ in MRS broth, the *Bifidobacterium* strain was grown anaerobically in MRS supplemented with 0.05% L-cysteine-hydrochloride (Sigma), *L. lactis* MG1363 was grown at 30℃ in M17 broth supplemented with 0.5% glucose, and *E.coli* was grown at 37℃ in lysogeny broth (Difco).

Counting was performed on the corresponding agar-based media used for liquid culture, *i.e.* BHI agar for strains grown in BHI broth, for example. Anaerobic strains were grown in jars using Genbox anaer medium (Biomérieux, Marcy-l'Etoile, France). The same incubation temperatures were used as for broth cultures. Colony-forming units were counted using ComptatPétri software (Guillemin *et* al. 2014).

2.3 The cytokine release assay

Peripheral blood mononuclear cells were isolated from four healthy donors, as previously described (Foligné *et* al., 2007). Related to the above-mentionned study, interleukin 10 (IL-10), interleukin 12 (IL-12), interferon gamma (INFγ) and tumor necrosis factor alpha (TNFα) were chosen as biomarker of the *in vitro* immunomodulatory response. Briefly, after Ficoll gradient centrifugation (Pharmacia, Uppsala, Sweden), PBMCs were collected, washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Paisley, Scotland) and adjusted to $2x10^6$ cells/mL in RPMI 1640 supplemented with gentamicin (150 µg/mL), L-glutamine (2 mM), and 10% fetal calf serum (Gibco-BRL). The PBMCs ($2x10^6$ cells/mL) were seeded in 24-well tissue culture plates (Corning Inc, Corning, NY, USA). Next, ten microliters of thawed microbial suspensions of each of the reference strains and the microorganisms to be tested were added. Stationary-phase microbes were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS containing 20% glycerol. A portable photometer (Densimat, BioMérieux, Craponne, France) was used to adjust the cell density to McFarland 3 (Araujo *et* al., 2004). The microbial preparations were thus standardized in terms of cell biomass (corresponding to approximately $2×10^8$ CFU/mL, for *E. coli*) and stored at–80°C until subsequent use within three months. Survival rates of over 90% were observed for all frozen samples (data not shown). This resulted in a bacterium-to-PBMC ratio of approximately 10:1 for *E. coli*. Phosphate-buffered saline containing 20% glycerol was used as a negative (non-stimulated) control. On the basis of preliminary time-course experiments, 24 h of stimulation was determined to be the optimal time point for cytokine responses of bacterium-stimulated PBMCs. After 24 h of stimulation at 37°C in an atmosphere of air with 5% CO_2, culture supernatants were collected, centrifuged and stored at –20°C prior to cytokine analysis. Neither acidification of the medium nor bacterial proliferation was observed. Cytokine levels were measured with ELISAs (from BD Biosciences (San Jose, CA, USA) for IL-10, IFNγ and IL-12p70, and from R&D Systems (Minneapolis, MN, USA) for TNFα), according to the manufacturers' instructions. The results were first expressed as pg/L for each donor and then as a mean value across all donors, as a percentage of the value for a reference strain (*e.g. B. longum* Bb536 for IL-10 and *Lc. Lactis* MG1363 for IL-12, TNFα and IFNγ). This means of normalization (*i.e*. using strains as internal standards) has been shown to be accurate and reproducible for experiments with small number of donors and large numbers of strains (Foligné *et al.*, 2007).

2.4 *In vitro* gastric and duodenal batch challenges

The stress conditions and the stress medium's composition were adapted from the work of Lo Curto et al. (2011). 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 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). Simulated gastric and duodenal juices were made fresh daily. Gastric juice consisted in pepsin (0.025 g/L or 1.10^6U/mL), lipase from *R. oryzae* (0.2 g/L or 6.10^4U/L) and NaCl (2,75 g/L) suspended in a 0.020 M glycine-HCl buffer pH 3 at 37°C prior to experiments.

Duodenal juice was prepared by suspending pancreatin (9 g/L, bile (30 g/L) and NaCl (7 g/L) in 0.1 M phosphate buffer pH 6.5 at 37°C, prior to the experiments. One milliliter of a late-stationary phase culture was added to 9 mL of either gastric or duodenal juice in a 45 mL sterile screw-cap tube. The pH was checked again after inoculation and (if necessary) re-adjusted to either pH 3 or pH 6.5. The incubation times were respectively 1 h and 2 h for gastric and duodenal juices at 37°C, with moderate shaking (100 rpm). The serial stress condition (*i.e*. gastric stress followed by duodenal stress) was performed by adjusting the pH of a one-hour gastric juice incubation to 6.5 with 1M Na_2CO_3, and then adding bile and pancreatin under sterile conditions for the subsequent 2 h duodenal incubation. To assess cell survival, strains were counted on the corresponding agar-based media before and after each stress condition.

2.5 Rennet gel inclusion and the combined batch challenge

Rennet gel inclusion was carried out in two steps. Firstly, 0.2 mL of a 10 g/L $CaCl_2$ solution was added to 225 mL of standard, 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 of active chymosin, 145 international milk-clotting units/mL; 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.

Formation of the rennet gel was monitored by measuring the shear storage modulus (G'), the shear loss modulus (G") and the damping factor (tan δ, defined as the ratio of the shear loss to the storage modulus, G"/G', adapted from Morand *et* al. (2011)). We used a stress-controlled rheometer (MCR 301 (Anton Paar, Courtaboeuf, France), equipped with a coaxial cylinder geometry (CC27, inner cylinder: 27 mm)) in oscillatory mode (1 Hz) and with a strain of 1%. Before the experiment, we checked that the chosen association angular frequency/oscillatory shear value was located within the linear part of the gel response.

We considered the gelation process was complete when G' and G'' reached a plateau. It was obtained for a 40-minute gelation period for G' and G'' values of respectively (2.15 ± 0.39) Pa and (0.60 ± 0.09) Pa (n=3). One gram of rennet gel matrix with entrapped microorganisms was used in each new series of experiments, as described in the previous section.

2.6 Statistical analysis

Results are expressed as the mean ± standard deviation of three biologically independent experiments and were compared using a two-tailed Student's t-test. The statistical significance of a given test result is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

3.1 Immunomodulatory profiles of the selected microorganisms

We measured the ability of 35 cheese-ripening microorganisms to induce cytokine responses by PBMCs *in vitro*. Up to three strains per species were tested. As expected from previous research on the biodiversity of environmental and food-isolated microbes, we observed substantial inter-group variations in anti-inflammatory IL-10 production. Although lactobacilli, streptococci and *E. coli* were quite weak IL-10 inducers, some members of the *Arthrobacter* and *Staphylococcus* genera were strong IL-10 inducers (Fig. 1). It is noteworthy that a strain of *B. aurantiacum* (Ba 171) and the three *Staphylococci* induced significantly higher levels of IL-10 than the well-known probiotic strain *B. longum* Bb536 ($p<0.05$). The latter *B. longum* reference strain had much the same *in vitro* anti-inflammatory potential as *A. arilaitensis* RE 117^T and 3M01. For several species (including *C. casei*, *B. aurantiacum* and *H. alvei*), the cytokine release appeared to be strain-dependent, with low inducers and high inducers from the same species. In terms of eukaryotic strains, the three *P. camembertii* and *G. candidum* strains were all unable to induce substantial immune responses but other species of yeast induced moderate levels of cytokine production (and IL-10 production in particular). This contrasted with the results described above for bacteria, where cytokine production was substantially higher and often differed from one strain to another for a given species.

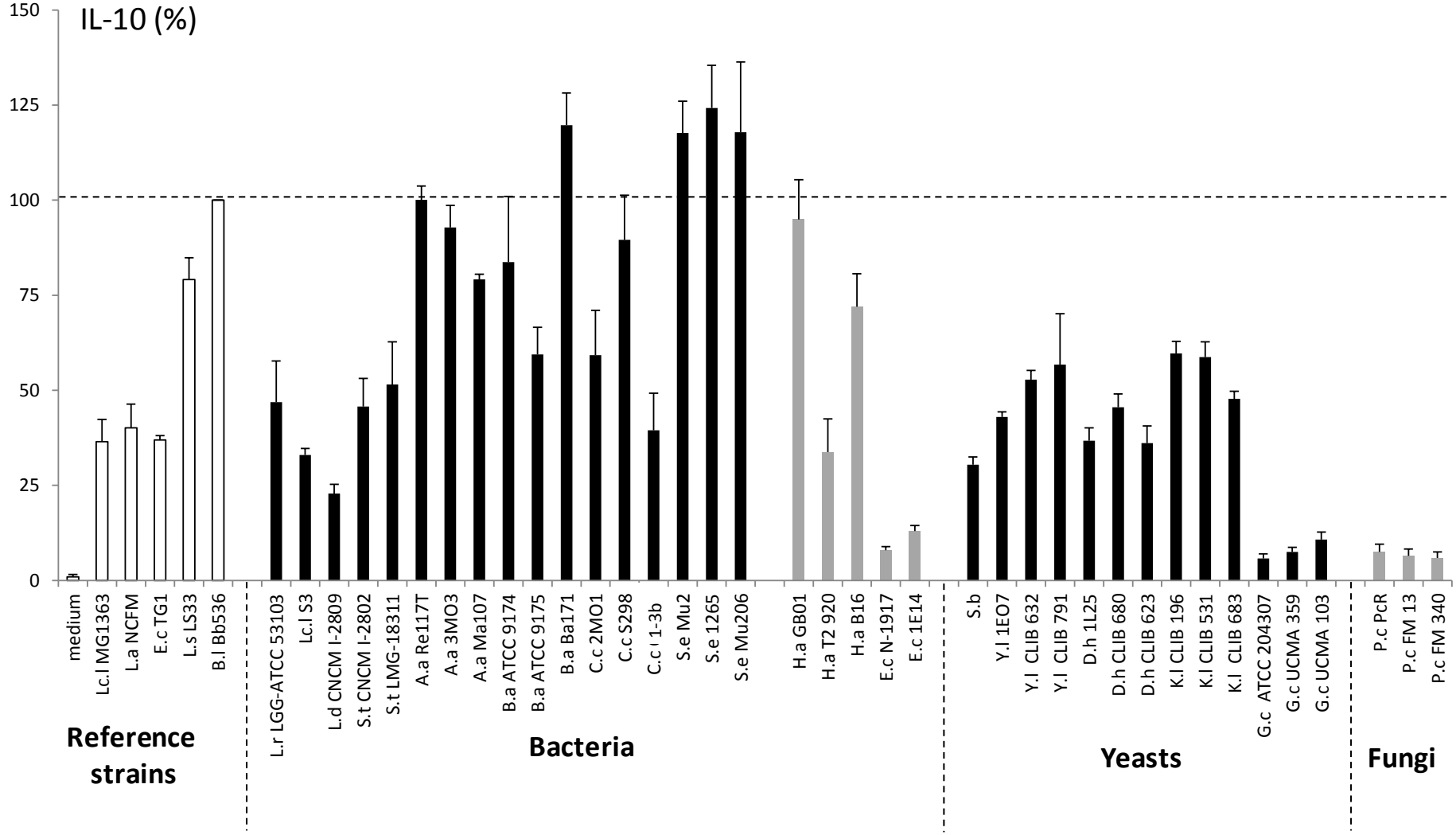

Figure 1. Production of IL-10 in supernatants of cultured human PBMCs (from 4 donors) stimulated (for 24h) with an equivalent biomass of various live strains of dairy bacteria, yeasts, fungi or lab-cultured probiotics. Data (mean ± SEM) are expressed as a percentage of a strongly IL-10-inducing reference strain.

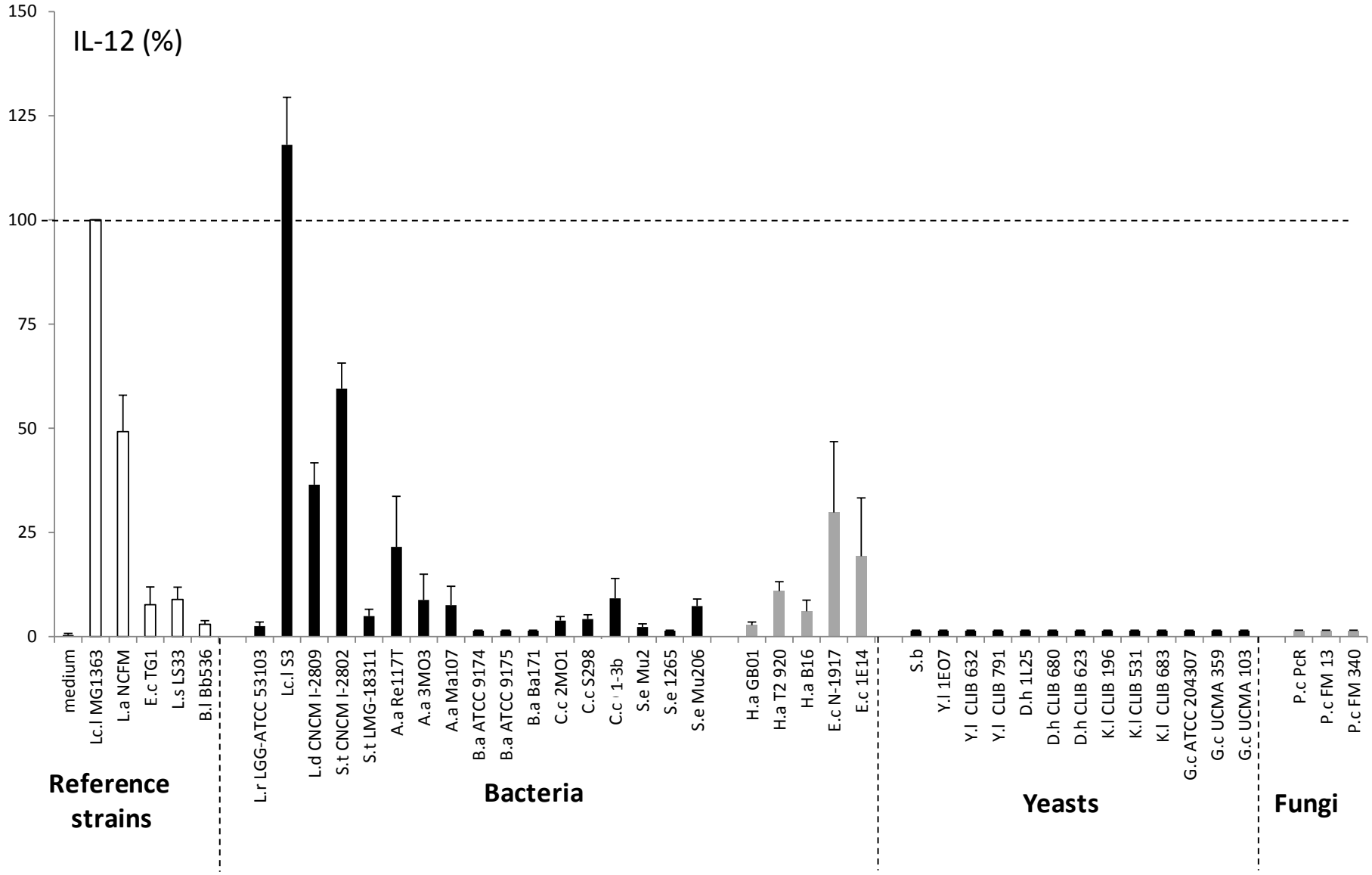

Figure 2. Production of IL-12 in supernatants of cultured human PBMCs (from 4 donors) stimulated (for 24h) with an equivalent biomass of various live strains of dairy bacteria, yeasts, fungi or lab-cultured probiotics. Data (mean ± SEM) are expressed as a percentage of a strongly IL-12-inducing reference strain.

When considering the induction of pro-inflammatory cytokines, most of the cheese-ripening bacteria induced low level release of pro-inflammatory mediators (with the exception of *Lc. lactis S3, Lb. delbrueckii ssp bulgaricus* CNCM I-2809 and *S. thermophilus* CNCM I-2802 for IL-12 release and *Staphylococcus equorum* Mu206 for IFNγ release). No pro-inflammatory cytokine release could be detected for any of the fungi and yeasts (Fig. 2 and 3). Accordingly, the levels of TNFα released by the PBMCs (which are less discriminant at the genus and strain levels) were fairly similar for Gram-negative and Gram-positive bacteria. The levels of TNFα released after incubation of PBMCs with eukaryotes were about half those measured after incubation with bacteria (Fig. 4). Overall, cheese-ripening bacteria and (to a lesser extent) yeasts and fungi show inter-species and inter-strain differences in their ability to drive immune cell responses *in vitro.* Although these individual microbial responses were mainly related to IL-10 release, some variations in the anti-inflammatory balance were due to the specific release of pro-inflammatory mediators.

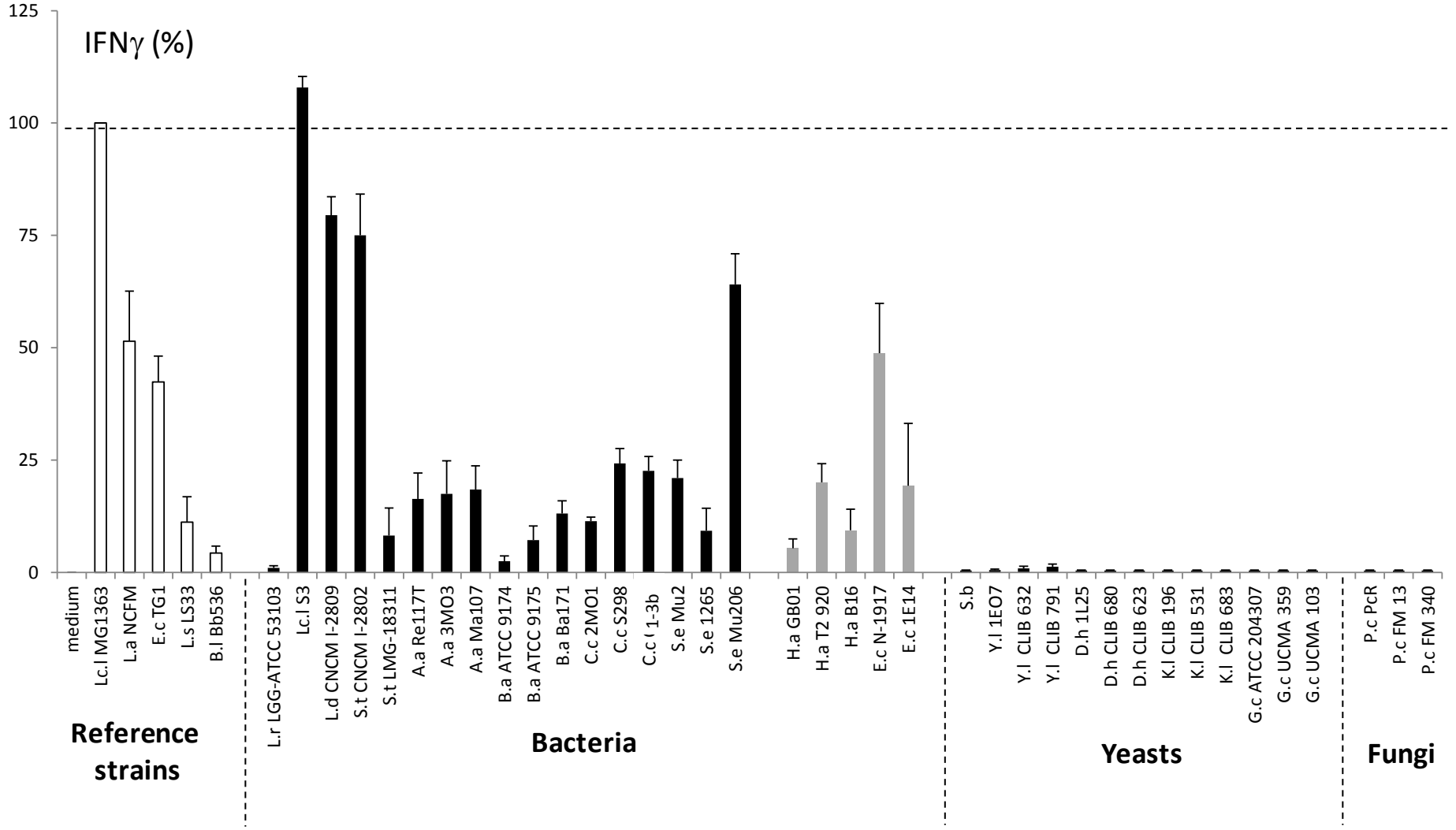

Figure 3. Production of IFNγ in supernatants of cultured human PBMCs (from 4 donors) stimulated (for 24h) with an equivalent biomass of various live strains of dairy bacteria, yeasts, fungi or lab-cultured probiotics. Data (mean ± SEM) are expressed as a percentage of the value observed for the strongest IFNγ–inducing reference strain (a *Lc. lactis*).

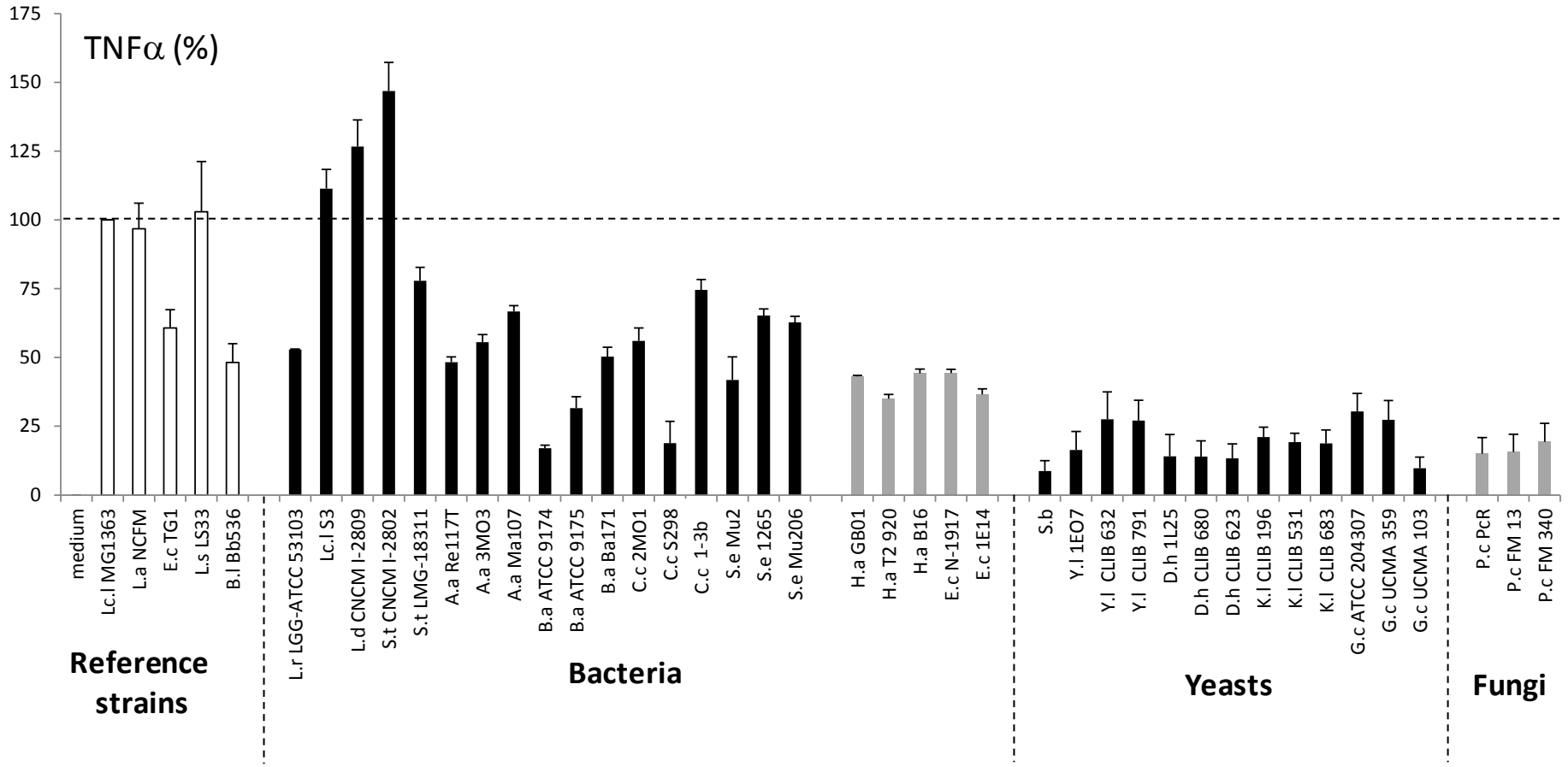

Figure 4. Production of TNFα in supernatants of cultured human PBMCs (from 4 donors) stimulated (for 24h) with an equivalent biomass of various live strains of dairy bacteria, yeasts, fungi or lab-cultured probiotics. Data (mean ± SEM) are expressed as a percentage of the value observed for the strongest TNFα-inducing reference strain (a *Lc. lactis*).

3.2 Survival of cheese-ripening microorganisms following *in vitro* gastric and duodenal batch challenges

We assessed the resistance of a number of surface-ripened cheese isolated microorganisms to incubation under gastric-like stress conditions (for 1 h with pepsin, at 37°C and pH 3) and duodenal-like stress conditions (for 2 h with bile and pancreatic enzymes, at 37°C and pH 6.5). Incubation under gastric-like conditions and then under duodenal-like conditions was also performed. The reference strains *S. boulardii* Biocodex and *E.coli* Nissle (Table 2) did not display any decrease in viability when submitted any of the stress conditions. *Lb. rhamnosus* GG survived a gastric challenge but did not withstand a duodenal challenge.

Neither *Y. lipolytica* nor *G. candidum* showed a significant decrease in viability when exposed to gastric and/or duodenal challenges ($p<0.05$) (Table 2). For a given species, there were no inter-strain differences in sensitivity to gastric or duodenal challenges. *Debaryomyces hansenii* strain CLIB 623 and 1L25 displayed a statistically significant drop in viability ($p<0.05$) after exposure to gastric-like stress and combined gastric- and duodenal-like stress, respectively. However, the difference in count between the reference and challenge conditions was only 0.25 log CFU/mL (for strain CLIB623) and 0.26 log CFU/mL (for strain 1L25). These values are below the generally accepted accuracy limit for plate counting (0.5 log CFU/mL). *Kluyveromyces lactis* showed a slightly higher sensitivity, with statistically significant differences ($p<0.01$ or $p<0.001$) between control and stress groups for all strains and all challenge conditions. The strain CLIB 196 displayed a loss of viability of 0.81 log CFU/mL, 0.86 log CFU/mL and 0.74 log CFU/mL under gastric, duodenal and combined challenge conditions, respectively. CLIB 531 showed similar behavior. The *K. lactis* strain CLIB 683 was the most sensitive to in vitro digestion, with a loss of viability of 1.66 log CFU/mL, 1.05 log CFU/mL and almost 2 log CFU/mL under gastric, duodenal and combined challenge conditions, respectively.

Table 2. *S*urvival of reference microorganisms and selected surface-ripened cheese yeasts and fungi in gastric, duodenal and combined *in vitro* challenges

Reference strain	Gastric challenge			Duodenal challenge			Combined challenge		
	Viable count (log CFU.mL^{-1})		**Loss of viability** (log CFU.mL^{-1})	Viable count (log CFU.mL^{-1})		**Loss of viability** (log CFU.mL^{-1})	Viable count (log CFU.mL^{-1})		**Loss of viability** (log CFU.mL^{-1})
	No digestion	1h challenge		No digestion	2h challenge		No digestion	3h challenge	
L. rhamnosus									
LGG - ATCC53103	7.18 ± 0.05	6.87 ± 0.12 *	**– 0.31**	7.04 ± 0.28	0	**T**	6.92 ± 0.19	0	**T**
S. boulardii									
Ultralevure	6.99 ± 0.12	7.09 ± 0.19	**+ 0.10**	6.86 ± 0.17	6.98 ± 0.11	**+ 0.12**	6.84 ± 0.14	7.12 ± 0.05 *	**+ 0.28**
E. coli									
Nissle 1917	9.31 ± 0.13	9.52 ± 0.05 *	**+ 0.21**	9.47 ± 0.11	9.35 ± 0.09 *	**–0.12**	9.21 ± 0.16	9.15 ± 0.08	**– 0.06**
Yeasts and fungi									
D. hansenii									
1L25	6.89 ± 0.21	6.92 ± 0.25	**+ 0.03**	6.85 ± 0.11	6.70 ± 0.16	**+ 0.05**	6.54 ± 0.11	6.80 ± 0.01 *	**+ 0.26**
CLIB 623	6.87 ± 0.07	7.11 ± 0.01 *	**+ 0.25**	7.06 ± 0.13	7.17 ± 0.22	**+ 0.11**	7.17 ± 0.06	7.15 ± 0.15	**+ 0.08**
CBS 767	6.86 ± 0.15	6.83 ± 0.15	**– 0.03**	7.01 ± 0.07	7.09 ± 0.17	**+ 0.08**	6.98 ± 0.05	7.05 ± 0.08	**+ 0.07**
G. candidum									
ATCC 204307	3.69 ± 0.26	3.73 ± 0.25	**+ 0.04**	3.68 ± 0.19	3.76 ± 0.23	**+ 0.08**	3.69 ± 0.25	3.56 ± 0.26	**– 0.13**
UCMA 359	3.76 ± 0.14	3.72 ± 0.13	**– 0.04**	3.75 ± 0.24	3.68 ± 0.15	**– 0.07**	3.73 ± 0.16	3.57 ± 0.17	**– 0.16**
UCMA 103	3.68 ± 0.23	3.70 ± 0.15	**+ 0.02**	3.67 ± 0.27	3.62 ± 0.22	**– 0.05**	3.68 ± 0.33	3.70 ± 0.22	**+ 0.02**
K. lactis									
CLIB196	8.27 ± 0.09	7.46 ± 0.07 ***	**– 0.81**	8.46 ± 0.20	7.60 ± 0.17 **	**– 0.86**	8.27 ± 0.11	7.53 ± 0.15 ***	**– 0.74**
CLIB 531	8.09 ± 0.01	7.21 ± 0.06 ***	**– 0.88**	8.14 ± 0.06	7.24 ± 0.14 ***	**– 0.90**	7.96 ± 0.09	7.40 ± 0.06 **	**– 0.56**
CLIB 683	8.53 ± 0.18	6.87 ± 0.20 ***	**– 1.66**	8.42 ± 0.25	7.37 ± 0.11 **	**– 1.05**	8.34 ± 0.12	6.37 ± 0.13 ***	**– 1.97**
P. camemberti									
PcR	4.93 ± 0.21	5.06 ± 0.16	**+ 0.13**	5.03 ± 0.26	3.71 ± 0.16 ***	**– 1.32**	4.93 ± 0.21	3.45 ± 0.07 ***	**– 1.48**
FM 13	3.98 ± 0.32	4.20 ± 0.22	**+ 0.22**	3.71 ± 0.19	2.55 ± 0.22 ***	**– 1.16**	3.98 ± 0.32	2.78 ± 0.11 ***	**– 1.20**
FM 340	4.57 ± 0.31	4.22 ± 0.12	**– 0.35**	4.22 ± 0.23	3.15 ± 0.19 ***	**– 1.07**	4.57 ± 0.26	3.21 ± 0.21 ***	**– 1.36**
Y. lipolytica									
1E07	7,20 ± 0.05	7,09 ± 0.03	**– 0.11**	7.13 ± 0.15	7.14 ± 0.11	**+ 0.01**	7.22 ± 0.16	7.20 ± 0.13	**– 0.02**
CLIB 632	6.90 ± 0.09	6.79 ± 0.02	**– 0.11**	6.91 ± 0.07	6.84 ± 0.05	**– 0.07**	6.86 ± 0.15	6.84 ± 0.12	**+ 0.02**
CLIB 791	6.69 ± 0.11	6.73 ± 0.11	**+ 0.04**	6.87 ± 0.16	6.89 ± 0.11	**+ 0.02**	6.85 ± 0.28	6.82 ± 0.35	**– 0.03**

With reference to the LAB*, Lc. lactis subsp*. *lactis* showed a loss of viability of 1 log CFU/mL following exposure to gastric stress and did not survive the duodenal challenge or a combined gastric-duodenal challenge. Along with *Lb*. *delbrueckii* subsp. *bulgaricus*, *S*. *thermophilus* did not survive any of the challenge conditions (Table 3). The various cheese-ripening bacteria (Table 3) showed three different responses to *in vitro* digestion. One subgroup (including *H. alvei*, *E. coli* and the Mu206 and 1265 strains of *S. equorum*) was strongly resistant to all three stress conditions. Indeed, strain *H. alvei* 920 Type 2 did not display a significant loss of viability following exposure to any of the stress conditions. Strain B16 was only slightly sensitive to duodenal challenge and the combined challenge, with a loss of viability of 0.13 log CFU/mL and 0.19 log CFU/mL, respectively. Strain GB01 was slightly sensitive to the gastric challenge but not the combined challenge. A second subgroup of bacteria (comprising the three strains of *B. aurantiacum*, *S. equorum* Mu2 and *A. arilaitensis*) was rather resistant to duodenal conditions. Indeed, *S. equorum* Mu2 and *B. aurantiacum* ATCC 9175 respectively lost only 0.32 log CFU/mL and 0.06 log CFU/mL of viability after duodenal challenge. Strains of *A. arilaitensis* were more sensitive to duodenal conditions, with a loss of viability of almost 3 log CFU/mL. Within this subgroup, only *B. aurantiacum* ATCC 9174 survived the gastric conditions (with a count of 2.77 ± 0.22 log CFU/mL after the 1-hour challenge). However, the loss of viability was dramatic and *B. aurantiacum* ATCC 9174 (like all of the strains in the subgroup) did not survive the combined challenge. These results emphasized the value of testing a microorganism's resistance to duodenal stress as well as to gastric stress. The third subgroup comprised *C. casei*, which survived neither gastric nor duodenal stress. It is important to bear in mind that the present study is the first to have investigated the respective abilities of *S. equorum, B. aurantiacum, C. casei* and *A. arilaitensis* to withstand digestive stress.

Table 3. *In vitro* survival of selected surface-ripened cheese bacteria in gastric, duodenal and combined *in vitro* challenges

Bacteria	Gastric challenge			Duodenal challenge			Combined challenge		
	Viable count (log CFU.mL^{-1})		Loss of viability (log CFU.mL^{-1})	Viable count (log CFU.mL^{-1})		Loss of viability (log CFU.mL^{-1})	Viable count (log CFU.mL^{-1})		Loss of Viability (log CFU.mL^{-1})
	No digestion	1h		No digestion	2h		No digestion	3h	
E. coli									
1E14	9.11 ± 0.06	9.23 ± 0.15	**+ 0.12**	9.19 ± 0.01	9.13 ± 0.09	**– 0.06**	9.32 ± 0.06	9.10 ± 0.08 *	**– 0.22**
H. alvei									
GB01	9.32 ± 0.07	8.91 ± 0.20 *	**– 0.41**	9.17 ± 0.04	8.93 ± 0.07	**– 0.24**	9.14 ± 0.08	8.89 ± 0.06	**– 0.25**
Type 2 n°920	8.73 ± 0.04	8.68 ± 0.11	**– 0.05**	8.66 ± 0.12	8.71 ± 0.05	**+ 0.05**	8.68 ± 0.11	8.66 ± 0.21	**– 0.02**
B16	8.55 ± 0.05	8.61 ± 0.07	**+ 0.06**	8.54 ± 0.04	8.41 ± 0.05 *	**– 0.13**	8.37 ± 0.22	8.56 ± 0.11 *	**+ 0.19**
S. equorum									
Mu2	8.54 ± 0.01	0	**T**	8.60 ± 0.01	8.28 ± 0.04 ***	**– 0.32**	8.62 ± 0.04	0	**T**
1265/GM16	8.31 ± 0.07	8.40 ± 0.15	**+ 0.09**	8.65 ± 0.15	8.53 ± 0.05	**– 0.12**	8.65 ± 0.12	8.79 ± 0.05	**+ 0.14**
Mu206	8.71 ± 0.20	8.88 ± 0.08 *	**+ 0.17**	8.21 ± 0.02	7.34 ± 0.06 ***	**– 0.87**	8.64 ± 0.15	7.98 ± 0.09 **	**– 0.75**
B. aurantiacum									
ATCC 9174	7.28 ± 0.23	2.77 ± 0.22 ***	**– 4.51**	7.59 ± 0.05	6.25 ± 0.03 ***	**– 1.34**	7.61 ± 0.14	0	**T**
ATCC 9175	8.73 ± 0.21	0	**T**	8.74 ± 0.14	8.68 ± 0.06	**– 0.06**	8.74 ± 0.13	0	**T**
Ba 171	8.46 ± 0.17	0	**T**	8.66 ± 0.02	7.48 ± 0.50 **	**– 1.18**	8.81 ± 0.12	0	**T**
A. arilaitensis									
Re 117^{T}	8.42 ± 0.13	0	**T**	8.46 ± 0.14	4.50 ± 0.21 ***	**– 2.96**	8.45 ± 0.14	0	**T**
3M03	8.48 ± 0.18	0	**T**	8.60 ± 0.14	4.00 ± 0.16 ***	**– 3.60**	8.58 ± 0.05	0	**T**
Ma107	8.53 ± 0.10	0	**T**	8.61 ± 0.12	4.09 ± 0.13 ***	**– 3.52**	8.60 ± 0.12	0	**T**
C. casei									
2M01	8.54 ± 0.16	0	**T**	8.73 ± 0.23	2.08 ± 0.11 ***	**– 5.65**	8.72 ± 0.12	0	**T**
DPC S298^{T}	8.50 ± 0.13	0	**T**	8.63 ± 0.14	0	**T**	8.51 ± 0.13	0	**T**
1-3b	8.51 ± 0.11	0	**T**	8.55 ± 0.12	0	**T**	8.65 ± 0.15	0	**T**
L. lactis									
S3	8.37 ± 0.11	7.37 ± 0.12 ***	**– 1**	8.04 ± 0.31	0	**T**	7.99 ± 0.22	0	**T**
S. thermophilus									
CNCM I-2802	8.06 ± 0.16	0	**T**	8.01 ± 0.23	0	**T**	7.97 ± 0.23	0	**T**
LMD-9	8.04 ± 0.22	0	**T**	8.07 ± 0.15	0	**T**	8.02 ± 0.31	0	**T**
LMG-18311	8.12 ± 0.12	0	**T**	8.16 ± 0.19	0	**T**	7.89 ± 0.21	0	**T**
L. delbrueckii subsp. bulgaricus									
CNCM I-2809	7.96 ± 0.69	0	**T**	7.92 ± 0.33	0	**T**	7.89 ± 0.32	0	**T**

3.3 Ability of matrix-included microorganisms to survive a combined gastric- and duodenal-like challenge

Five bacteria (*S. equorum* Mu2, *B. aurantiacum* ATCC 9174, *A. arilaitensis* Re117^T, *C. casei* 2M01 and *Lc. lactis* S3) were selected for rennet gel inclusion experiments. Although all were sensitive to the combined challenge, the sensitivity profiles differed (Table 4). *Staphylococcus equorum* Mu2 was extremely sensitive to gastric challenge but not at all sensitive to duodenal challenge. Likewise, *B. aurantiacum* and *A. arilaitensis* were highly sensitive to gastric challenge but fairly resistant to duodenal challenge. *C. casei* did not withstand any of the challenges. When included in rennet gel, the viability of *B. aurantiacum* ATCC 9174 and *C. casei* 2M01 fell by 1.09 and 0.52 log CFU/mL, respectively (*i.e*. much less than the dramatic falls of 4.51 and 5.68 log CFU/mL seen with pure cultures). *Staphylococcus equorum* Mu2, and *A. arilaitensis* Re117T (neither of which survived the gastric challenge in pure cultures) did not show any significant loss of viability (0.13 log CFU/mL and 0.08 log CFU/mL, respectively) when included in rennet gel. After the gastric challenge, the viability of *Lc. lactis* S3 fell by 1 log CFU/mL when tested as a pure culture and by 0.65 CFU/mL when tested as a rennet gel inclusion.

Hence, *S. equorum* Mu2, *B. aurantiacum* ATCC 9174 and *A. arilaitensis* survived a combined gastric and duodenal challenge when included in a rennet gel but not as pure cultures. In contrast, neither gel-included nor pure cultures of *Lc. lactis* S3 survived the combined challenge.

Table 4. *In vitro* survival of selected rennet-gel included microorganisms in gastric, duodenal and combined *in vitro* challenges

Rennet gel inclusion	Gastric challenge			Combined challenge		
	Viable count (log CFU.mL^{-1})		**Loss of Viability** (log CFU.mL^{-1})	Viable count (log CFU.mL^{-1})		**Loss of Viability** (log CFU.mL^{-1})
	No digestion	1h		No digestion	3h	
A. arilaitensis RE117^{T}						
Reference	8.42 ± 0.13	0	**T**	8.45 ± 0.14	0	**T**
Matrix inclusion	8.03 ± 0.03	7.92 ± 0.08	**– 0.08**	7.99 ± 0.03	4.91 ± 0.09 ***	**– 3.08**
B. aurantiacum ATCC 9174						
Reference	7.28 ± 0.23	2.77 ± 0.22 ***	**– 4.51**	7.61 ± 0.14	0	**T**
Matrix inclusion	7.37 ± 0.15	6.28 ± 0.44 *	**– 1.09**	7.37 ± 0.15	5.79 ± 0.13 ***	**– 1.58**
C. casei 2M01						
Reference	8.54 ± 0.17	1.86 ± 0.19 ***	**– 5.68**	8.72 ± 0.12	0	**T**
Matrix inclusion	8.47 ± 0.19	7.95 ± 0.16 **	**– 0.52**	8.47 ± 0.19	0	**T**
S. equorum Mu2						
Reference	8.54 ± 0.01	0	**T**	8.53 ± 0.04	0	**T**
Matrix inclusion	8.53 ± 0.06	7.66 ± 0.26	**+ 0.13**	8.53 ± 0.04	8.26 ± 0.28	**– 0.36**
L. rhamnosus LGG						
Reference	8.18 ± 0.07	7.36 ± 0.03 ***	**– 1.00**	8.22 ± 0.13	0	**T**
Matrix inclusion	8.15 ± 0.12	7.32 ± 0.10 ***	**– 0.83**	8.02 ± 0.19	0	**T**
L. lactis S3						
Reference	8.37 ± 0.17	7.37 ± 0.11 **	**– 1.00**	7.99 ± 0.05	0	**T**
Matrix inclusion	8.23 ± 0.22	7.58 ± 0.15 *	**– 0.65**	8.32 ± 0.19	0	**T**

4. Discussion

Scientific interest in food digestion and nutrient bioavailability has grown markedly over the last decade. Indeed, several journals dedicated to this new field of science have been launched and the number of published articles has increased dramatically (from 400 in 1995 to nearly 1400 in 2013). In this context, the fate of food microbial ecosystems is of a great interest in terms of both their provision of nutrients and their potential effects on gut immunity.

One of the objectives of the present work was to obtain basic information about whether or not microorganisms (n=35) isolated from several surface-ripened cheeses and dairy environments were able to withstand simulated digestive stress. Given the broad variety of *in vitro* batch digestion models described in the literature, we first had to determine which parameters of the gastric and duodenal digestive media (composition, temperature, pH, etc.) were likely to have an impact on the microorganisms' viability. We then maintained these parameters throughout our experiments. However, *in vitro* batch methods cannot replace *in vivo* experiments, especially since the former do not take account of dynamic aspects of the digestive process. Nevertheless, our *in vitro* digestive model served as a quick, convenient means of screening a large number of microorganisms and will enabled us to select a smaller number of strains for further experimentation based on both sensitivity to *in vitro* digestive stress and *in vitro* immunomodulating properties.

The reference strains *E. coli* Nissle and *S. boulardii* were resistant to our *in vitro* experiments, in contrast to *L. rhamnosus GG*. Indeed, the latter microorganism is reportedly quite sensitive to digestive stress when not encapsulated or included in a dairy food matrix (Burgain *et* al., 2013, de Vos *et* al., 2009). On the basis of the literature data, we expected *E. coli* and *S. boulardii* to be resistant (Czerucka *et* al., 2007, Krulwich *et* al., 2011). Yeasts tend to be strongly resistant to digestive challenge; our results for these microorganism are in substantial agreement with previous reports (Lay *et* al., 2004, Psomas *et* al., 2001) in which a number of *D. hansenii*, *Y. lipolytica* and *G. candidum* strains survived well when exposed to similar stress. In Psomas et al.'s study (2001), the strain of *K. lactis* 531 displayed a drop in viability of about 2 log CFU/mLs following gastric stress, whereas the strain 570 was not sensitive to gastric stress at all. In our present experiments, the three selected *K. lactis* strains lost respectively 0.81, 0.88 and 1.66 log CFU/mL - reflecting the fairly good resistance highlighted in the literature. 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). Consequently, our present results on *Lc. lactis* subsp. *lactis'* resistance to synthetic gastric juices are consistent with the species' physiology and the literature data. The other two LAB (*S*. *thermophilus* and *Lc*. *delbrueckii* subsp. *bulgaricus*) did not resistant any the stress conditions tested here. These findings are consistent with Lay *et* al.'s (2004) report that neither *S*. *thermophilus* nor *Lb*. *delbrueckii* subsp. *bulgaricus* survived after

Camembert cheese was consumed by rats hosting human microbiota. *Escherichia coli* is a human commensal and thus was expected to survive to exposure to simulated gastric juices. Given that *H. alvei* is closely related to *E.coli* (Janda *et* al., 2002) from genetic and phenotypic standpoints, its ability to withstand digestive stress is not surprising. However, our study is the first to have confirmed this profile.

As mentioned above, there is a dramatic lack of data on how Gram-positive microbiota from surface-ripened cheeses responds to digestive stress. However, studies of cheese ripening itself provide some clues. Indeed, *S. equorum* is part of the early smear-ripened cheese microbiota, when the cheese curd is still acidic (pH 5) (Bockelman *et* al., 2002). In contrast, *C. casei* and *B. aurantiacum* only start to grow when pH 7 is reached during ripening, and so one can legitimately expect these two species to be quite acid-sensitive. By analogy, we expected some of the selected *S. equorum* strains to be able to survive the gastric challenge, and expected *C. casei* and *B. aurantiacum* to be quite acid-sensitive. We indeed found that two of the three *S. equorum* strains survived the simulated gastric challenge fairly well, whereas *C. casei* and *B. aurantiacum* did not survive exposure to the acidic environment. Unexpectedly, gastric stress neither sensitized to nor protected against subsequent duodenal stress under our experimental conditions. In other words, gastric and duodenal stress did not have a cumulative effect.

It is generally assumed that the presence of a food matrix will protect microorganisms against digestive stress. The matrix's buffering properties, structure and composition are often presented as the main factors that enhance the survival of microorganisms subjected to digestive stress (Salaun *et* al., 2005, Sharp *et* al., 2008). In the present study, the simulated gastric survival of Gram-positive bacteria was dramatically improved by inclusion in a rennet gel (when compared with non-included cultures). Conversely, only strains that were already resistant to duodenal challenge in pure culture were able to withstand a combined gastric and duodenal challenge when entrapped in a rennet gel. These results suggest that the cheese curd used during our experiments provided protection against a gastric challenge but not a duodenal challenge. Our results corroborates the work by Heidebach et al. (2009), in which encapsulation in rennet gel improved the ability of two *Lactobacillus paracasei* strains and a *Bifidobacterium lactis* strain to survive a similar type of *in vitro* gastric stress. However, it should be noted that microorganisms did not actually grow inside the rennet gel but were included prior to the *in vitro* digestive challenge. Development as part of an ecosystem (i.e. interaction with other microorganisms and with cheese matrix constituents) - is very likely to change a microorganism's physiological properties. It is possible that in addition to the putative structural, protective effect of the rennet gel, changes in growth conditions might influence a microorganism's survival of a digestive challenge. Nevertheless, we consider that these initial data

are worthy of interest and now intend to characterize the cheese matrix's influence on the ability of surface-ripened microbiota to survive digestive stress.

Our research on cytokine release from PBMCs provided new information regarding on the microorganisms' potential immunomodulatory (pro- or anti-inflammatory) properties – most of which had not been explored previously. Our present results highlight the broad diversity of immune-stimulating activities among cheese-ripening organisms. Although these microbial strains are frequently found in dairy products (either as part of the native microbiota in cheese factories or after deliberate introduction into the dairy matrices by manufacturers), their health potential has not been well characterized. This is partly due to the lack of in-depth data on the strains' respective abilities to survive in the gastrointestinal tract - a prerequisite for prospective probiotics. The few available studies were limited to survival and adhesion properties and focused on yeast strains (Binetti *et* al., 2013; Kumura *et* al., 2004) or lactobacilli spp (Pitino *et* al., 2012; Solieri *et* al., 2014). However, one of the objectives of the present study was to address the potential impact of extensively consumed live microorganisms, rather than to screen cheese microorganisms organisms as new probiotics or to address the key role of cheese and dairy matrices as carriers for inoculated, well-characterized probiotics. Our results clearly show that bacteria and yeasts isolated from cheese (including *Arthrobacter*, *Brevibacterium*, *Staphylococcus*, *Corynebacteria* and *Hafnia* spp, together with *Yarrowia*, *Debaryomyces*, *Kluyveromyces* and *Geotrichum*) are not inert from an immunological perspective. Whereas strains exhibiting a higher propensity to induce the anti-inflammatory cytokine IL-10 may contribute to immunomodulation and the alleviation of inflammatory events (such as arthritis and colitis), other strains with a pro-inflammatory profile (triggering the release of IL-12, IFNγ and TNFα) may have immunostimulatory functions, such as anti-infective or adjuvant. Due to the huge dietary intake of these species through cheese consumption, we cannot afford to neglect the likely impact on the immune balance and, more generally, the consumer's overall health status. Although common traits may occur within particular groups, each individual strain from a given genus and species displayed its own cytokine release ratio in *in vitro* PBMC assays. This ratio may be used as a guide to the strain's ability to trigger a specific response *in vivo*. However, a strain's survival aptitudes and immuno-enhancing/immunomodulatory properties may differ according to whether it is growing in isolation or (in contrast) interacting with other microorganisms in a cheese ecosystem. Thus, the study of the resulting net immune influence of ripened-cheese ecosystems is crucial for understanding the currently unsuspected effects of surface-ripened cheese microbiota on the host.

In conclusion, we found that *in vitro* simulated gastric stress was more deadly to the microorganisms tested here than duodenal stress was, (ii) there was no cumulative effect of sequential gastric and duodenal exposure, and (iii) inclusion in a cheese-like matrix (a rennet gel) protected sensitive microorganisms against the *in vitro* gastric challenge. Our present findings