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

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ABSTRACT

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 assay tests showed that the response to yeasts was usually anti-inflammatory, whereas the response to bacteria varied from one strain to another.

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 9 million t 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., 1993). 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-

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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 (Rahman 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 the 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., 2009) 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 in 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 *Escherichia coli* Nissle 1917 (Ardeypharm, Herdecke, Germany) were used for comparative purposes. Furthermore, five bacterial strains (*Bifidobacterium longum* Bb536, *E. coli* TG1, *Lactobacillus acidophilus* NCFM, *Lactobacillus salivarius* Ls33 and *Lc. Lactis* MG1363) were used as references in the PBMC stimulation assay, as previously described (Foligné et al., 2007).

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;

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	3 M03	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	2 M01	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	1 L25	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