Etude de l'effet immunomodulateur de deux fromages aux profils opposés dans deux modèles de colites murins

1. Présentation de l'article publié dans *Journal of Functional Foods*

L'objectif de cette partie du projet était d'évaluer l'impact de la consommation de deux fromages contenant des micro-organismes aux profils immunomodulateurs opposés, sur des modèles murins (i) sains, (ii) avec une colite induite au TNBS et (iii) avec une colite induite au DSS.

Bien qu'une quantité importante de micro-organismes soit ingérée lors de la consommation de fromage, l'influence de ces bactéries et levures d'affinage sur le consommateur n'a été que très peu étudiée. La majeure partie des travaux sur le sujet considère le fromage comme un bon candidat pour véhiculer des micro-organismes dont les caractéristiques probiotiques ont, par ailleurs, déjà été établies (Gardiner et al., 1999 ; Sharp et al., 2008 ; Lollo et al. 2012). Le fromage en tant que tel - et plus particulièrement les micro-organismes technologiques responsables de son affinage - n'a fait l'objet que d'un nombre très réduit de travaux. Ceux-ci concernent des technologies dont l'affinage n'implique presque qu'exclusivement des bactéries lactiques (Seong et al., 2012 ; Ricciardia et al. 2014) ; les derniers travaux en date font état d'une détection de ces micro-organismes vivants ainsi que d'une activité métabolique, dans la partie terminale du côlon(David et al., 2014,.

Au-delà de l'impact de la consommation de fromage sur des individus sains, ses conséquences ne sont que peu établies sur l'homéostasie intestinale de patients atteints de pathologie inflammatoire de l'intestin (ex. Inflammatory Bowel Disease (IBD) ou maladie de Crohn) et ne se concentrent pas sur les micro-organismes (Hosoya et al., 2012 ; Labonte et al., 2013).

Lors de nos précédents travaux, nous avons montré qu'une part non négligeable des micro-organismes d'affinage sélectionnés pouvait survivre à la digestion (articles 1 & 2) et que ces micro-organismes ne sont pas neutres d'un point de vue immunomodulateur. Un mélange de micro-organismes ne donnant pas toujours une réponse globale identique à la réponse des souches prises séparément (Chapman et al., 2011), les travaux développés dans cette partie du projet doivent permettre d'obtenir des pistes concernant les flores d'affinage fromagères lorsqu'elles se développent en conditions « réelles ».

Ici, nous avons utilisé la même technologie fromagère que lors des expérimentations réalisées pour l'article 2, en choisissant deux mélanges contenant les mêmes espèces de micro-organismes d'affinage (*Lactococcus lactis, Brevibacterium aurantiacum, Arthrobacter arilaitensis, Hafnia alvei, Staphylococcus equorum, Corynebacterium casei, Debaryomyces hansenii, Kluyveromyces lactis et Geotrichum candidum)*, dont certaines souches différaient par leur potentiel immunomodulateur – établi lors des expérimentations sur PBMCs de l'article 1.

Ceci a conduit à deux fromages dont les mélanges de souches avant affinage donnaient des réponses respectivement plutôt pro-inflammatoire (A) ou plutôt anti-inflammatoire (B). Les souris BALB/c ont été nourries avec des croquettes standards et gavées avec (i) un contrôle (PBS), (ii) un lait emprésuré, (iii) le fromage (A) et (iv) le fromage (B). Le gavage a permis de contrôler que la prise soit identique pour chacune des souris. La cohorte de 100 individus a été divisée en groupe « sain-contrôle » (n = 4 x 5), un groupe «colite induite TNBS » (n = 4 x 10) et un groupe « colite induite DSS » (n= 4 x 10). Ces modèles sont reconnus pour représenter une partie des symptômes retrouvés dans des pathologies du type IBD.

Afin de suivre la réponse immunomodulatrice provoquée par chaque matrice, le poids a été suivi quotidiennement pendant toute la durée de l'expérimentation. Les paramètres suivants ont été mesurés suite au sacrifice :

- Côlon : longueur et divers scores histologiques ; qPCR sur la portion distale (gènes marqueurs de l'inflammation) ; infiltration des macrophages.
- Rate : poids et aspect
- Sang : dosage de l'IL-6 et de la Serum Amyloid Protein (SAA)

L'article dans sa mise en page journal est disponible en Annexe III.

2. Faits marquants

- ✓ Des souches de micro-organismes isolées de fromages ont induit des réponses immunomodulatrices *in vitro* différentes.
- ✓ Des consortia de micro-organismes sélectionnés selon le critère précédent peuvent induire des réponses immunomodulatrice *in vitro* opposées.
- ✓ Certains cocktails de micro-organismes peuvent exacerber les colites induites chez des modèles murins.
- ✓ Le contrôle du design de fromage peut en partie prédire la nature de la réponse qu'ils engendreront *in vivo.*
- ✓ Le profil immunomodulateur du fromage peut s'avérer clé dans l'évaluation des risques de sa consommation chez des patients atteints d'IBD.

Designing specific cheese-ripening ecosystems to shape the immune effects of dairy products?

Coline Plé[1,*], Nadège Adouard[2,*], Jérôme Breton[1], Joëlle Dewulf[1], Bruno Pot[1], Pascal Bonnarme[2] and Benoit Foligné[1]

* shared first authorship

[1] Institut Pasteur de Lille, Lactic Acid Bacteria & Mucosal Immunity, Center for Infection and Immunity of Lille, U1019, UMR 8204, Université de Lille, Rue du Pr Calmette, BP 245, F-59019 Lille, France

[2] INRA-AgroParisTech UMR 782 Microbiology and Food Process Engineering (GMPA), Avenue Lucien Brétignères F-78850 Thiverval-Grignon, France.

Correspondance: Benoit Foligné, Institut Pasteur de Lille, 1, Rue du Pr Calmette, BP 245, F-59019 Lille, France. Tel: +33 320 871 191; Fax: +33 320 871 192; Email: benoit.foligne@ibl.cnrs.fr

Running title: Cheeses and colitis in mice

Funding

The work was funded by the Centre National Interprofessionnel de l'Economie Laitière (CNIEL) through by the Scientific Committee of Syndifrais/Cniel call for proposals, and is part of the EcoDige project (2012-2015 grant).

Abstract

Although large numbers of viable microorganisms are ingested through ripened-cheese consumption, little is known about the microbial ecosystems' influence on the host's immune responses. We designed experimental smear-ripened cheeses with bacteria and yeasts that have opposite immune effects (mixtures of selected *Lactococcus lactis*, *Brevibacterium aurantiacum, Arthrobacter arilaitensis, Hafnia alvei, Staphylococcus equorum, Corynebacterium casei, Debaryomyces hansenii, Kluyveromyces lactis* and *Geotrichum candidum*) and evaluated their impact in the acute dextran sulphate sodium (DSS) and trinitrobenzene sulphonic acid (TNBS) colitis mouse models. BALB/c mice were fed with a control diet, a milk matrix or with lab-designed, 28-day-ripened prototype soft cheeses A and B (CheA and CheB) from cow milk that respectively hosted consortia of immuno-enhancing and immuno-modulatory microbial strains. Inflammatory markers and transcriptional signatures were evaluated in healthy mice and DSS- and TNBS-treated colitic mice. In the DSS colitis model, there were no differences between CheA and CheB in terms of the inflammatory read-outs. In contrast, CheA (but not CheB) exacerbated weight loss, systemic inflammation and colon lesions in the TNBS model. In the mouse, nutritional intervention with selected strains in cheeses may influence immunomodulation. Our results suggest that designer cheeses may provide opportunities for diet management.

Abbreviations: CD, Crohn's disease; **DSS**, dextran sulphate sodium; **IBD**, inflammatory bowel disease; **MPO**, myeloperoxidase; **PBMC**, peripheral blood mononuclear cell; **SAA**, serum amyloid A; **TNBS**, trinitrobenzene sulphonic acid; **TNF-α**, tumor necrosis factor alpha; **UC**, ulcerative colitis.

Keywords: Smear-cheese microflora, PBMC, immunomodulation, colitis, mouse model

1. Introduction

The role of dietary habits as an environmental risk factor (Ng et al., 2013; Gentschew, & Ferguson, 2012) or a factor able to prolong remission in inflammatory bowel disease (IBD) (Rajendran, & Kumar, 2010) has not received a great deal of research attention. Diet may influence gut inflammation through several biologically plausible mechanisms, including antigenic responses to food and alteration of the host microbiota (Clarke, & Mullin, 2008; Viladomiu, Hontecillas, Yuan, Lu, & Bassaganya-Riera, 2013). However, the association between diet and IBD has not been clearly demonstrated - mostly because the etiology of both Crohn's disease (CD) and ulcerative colitis (UC) are not fully understood. The impacts of genetic factors, microbiome diversity and environmental factors have been well documented. Some risk factors and modulators, however, may vary in their influence; for example, smoking cessation improves CD but worsens UC (Cosnes, 2004; Birrenbach, & Bocker, 2004). Identification of the food types or components responsible for beneficial effects in IBD is tricky, since a range of doses and frequencies of macronutrients and micronutrients (including trace elements and pollutants) have to be considered. Although discriminating between causes and effects is challenging, there is a clear association between the incidence of IBD and loss of diversity within gut microbiota. This association fits with the hygiene hypothesis (Bach, 2002; Khosravi et al., 2014), in which the use of antibiotics and the increased consumption of refined food in our diet depletes the amount and activity of raw and fermented foods (Petrof, Claud, Gloor, & Allen-Vercoe, 2013). Thus, both nutritional food supplements and/or diets containing "healthy" food microbes are recommended.

Although it is widely acknowledged that the intake of so-called probiotic microorganisms is able to influence the host's immune responses (van Baarlen, Wells, & Kleerebezem, 2013; Marteau, et al., 2004; Foligné, Daniel, & Pot, 2013) epidemiological data on IBD and fermented foods (especially cheeses) are scarce. Some case-control studies have indicated that a high cheese intake is associated with CD (Maconi et al., 2010), whereas others have shown that patients with IBD eat cheese less frequently (Zvirbliene, Kiudelis, Zalinkevicius, & Kupcinskas, 2006). Meta-analyses of the impact of dairy products and cheese on diseases are inconsistent, since no particular cheese types or cheese-derived core microbial ecosystems have been clarified (Aune et al., 2012; Labonte, Couture, Richard, Desroches, & Lamarche, 2013). Although cheese-enriched diet was found to enhance anti-inflammatory and immune regulatory responses in normal mice and in a dextran sulfate sodium (DSS) colitis mouse model (Hosoya, Ogawa, Sakai, & Kadooka, 2012), the beneficial outcomes were not attributed to specific microorganisms (whether as live forms or inactivated). In addition to potentially bioactive peptides (Beermann, & Hartung, 2013; Korhonen, 2009) fatty acids and amines in milk, dominant cheese microbiota should also be considered, (Stanton, Ross, Fitzgerald, & Van

Sinderen, 2005). Indeed, foodborne bacteria and fungi from cheese are detectable in the human distal colon, where they have been shown to be metabolically active and capable of altering the resident microbiota (David et al., 2014). Moreover, many microbial-derived antigens, secreted compounds, surface molecules and cell-wall components (such as peptidoglycan, exopolysaccharides, teichoic acids, and mannans) have immunomodulatory properties (Lebeer, Vanderleyden, & De Keersmaecker, 2010). Whereas the type of immune-related response clearly depends on the intrinsic characteristics of each microbe from defined genera and species (*e.g.* Gram-positive or Gram-negative bacteria, yeasts and fungi), precise immune tuning will be strain-specific (as demonstrated *in vitro* for probiotic strains of lactic acid bacteria (LAB) such as lactobacilli (Nova et al., 2007, Foligne et al., 2007) and bifidobacteria (López, Gueimonde, Margolles, & Suárez, 2010; Riedel et al., 2006; Hoarau et al., 2008) and yeasts (Foligné, Dewulf, Vandekerckove, Pignède, & Pot, 2010; Romanin et al., 2010; Maccaferri, Klinder, Brigidi, Cavina, & Costabile, 2012). Previous research has demonstrated the major role of bacterially induced IL-10 *in vitro* and the associated potential for relieving experimental colitis *in vivo* (Foligne et al., 2007; López, Gueimonde, Margolles, & Suárez 2010; Riedel et al., 2006; Hoarau, et al., 2008; Foligné, Dewulf, Vandekerckove, Pignède, & Pot, 2010; Romanin et al., 2010; Maccaferri, Klinder, Brigidi, Cavina, & Costabile, 2012; Peran et al., 2005). However, attempts to characterize the immune patterns induced by strains from cheese-ripening ecosystems are rare (Rahman et al., 2013) and have been restricted to non-starter LAB.

We have previously studied the diverse immunomodulatory properties of various microbial strains used in the preparation of dairy products (Adouard, unpublished data). By considering neutral, immuno-enhancing and immune-regulatory bacteria and yeasts, we defined distinct starter mixtures with opposite properties and used them to produce experimental cheeses likely to have opposite immune effects. Given that the health effects of probiotics are strain-specific, we postulated that multiple-strain preparations would be more effective than single-strain preparations (Timmerman, Koning, Mulder, Rombouts, & Beynen, 2004). In the context of gut inflammation, multiple-strain preparations have never been assessed in pre-clinical or clinical trials. There is increasing evidence to suggest that murine colitis may be prevented by administering several mixtures (Rachmilewitz et al., 2004; Roselli et al., 2009; Drouault-Holowacz et al., 2006; Hart et al., 2004; Gionchetti et al., 2012), although the efficacy varies with the composition of the mixture (Roselli et al., 2009). Similarly, the immune and health properties of multiple-strain and multiple-species probiotic mixes in a cheese ecosystem cannot be deduced from their respective components (Timmerman et al., 2007; Chapman, Gibson, & Rowland, 2011). Thus, we decided to screen various mixtures for their immuno-regulatory potential, with the objective of selecting optimal strains as tools for further cheese-making experiments (*i.e.* custom ripened dairy products with either neutral, anti- or pro-inflammatory-like dominant ecosystems). Indeed, prototype cheeses may be useful in

evaluating the effects of immune intervention in preclinical models of immune-dysregulated diseases (such as colitis).

This type of tool would be of great interest for better dietary management in immune-related disorders and could open up new perspectives in cheese-making. For example, a custom-designed cheese could form part of an appropriate diet in a specific at-risk population or patient population.

The objective of the present study was thus to evaluate the potential impact of fermented dairy products (based on the immune determinants of their microbial components), rather than to design a "probiotic cheese" *per se*.

2. Material and Methods

2.1. Microorganisms, growth conditions and mixtures

In the present study, 14 strains (10 bacteria, 3 yeasts and 1 fungus isolated from a dairy environment) were considered. Furthermore, five reference strains (*Bifidobacterium longum* Bb536, *Escherichia coli* TG1, *Lactobacillus acidophilus* NCFM, *Lactobacillus salivarius* Ls33 and *Lactococcus lactis* MG1363, Table S1) were used for immune cell stimulation, as previously described (Foligné et al., 2010). All strains used in this study were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Microflex Technology, Bruker Daltonics, Fremont, CA, USA), as previously described (Blondiaux, Gaillot, & Courcol, 2010). All growth media were purchased from Difco (Pessac, France). With the exception of *Hafnia alvei* (grown in Luria Bertani medium), bacterial cheese isolates were grown in 100 mL of brain heart infusion broth in 500 ml Erlenmeyer flasks at 25°C and were placed on a shaking table (200 rpm). *Lactococcus lactis* was cultivated in M17 medium supplemented with 0.5% glucose in 100 mL Schott bottles at 30°C. Lactobacilli were grown at 37°C with limited aeration in de Man, Rogosa, Sharpe (MRS) broth in 100 mL Schott flasks while *E. coli* was grown at 37°C in LB medium and *B. longum* was grown anaerobically in MRS broth supplemented with 0.05% L-cysteine-hydrochloride (Sigma, St Quentin Fallavier, France). Yeasts and fungi were grown in 100 mL of potato dextrose broth in 500 mL Erlenmeyer flask, at 25°C and shaken at 200 rpm. All strains were grown until the late stationary phase (as measured in preliminary growth experiments; data not shown). The reference strain preparations were prepared as follows: stationary-phase microbes were washed twice in PBS and resuspended in PBS containing 20% glycerol to adjust the cell density to McFarland 3 using a portable photometer (Densimat, bioMérieux, Craponne, France) (Araujo, Rodrigues, & Pina-Vaz, 2004).

In order to mimic complex multiple-strain cheese ecosystems as closely as possible, six distinct mixtures of ten or so bacteria and yeasts were prepared (Table 1). Each of the mixtures had a bacteria/yeast ratio of 1. Hence, the component volumes (in a total volume of 200 µl) were as follows: *Lactococcus lactis* 25 µl, *Brevibacterium aurantiacum* 15 µl, *Arthrobacter arilaitensis* 15 µl, *Hafnia alvei* 15 µl, *Staphylococcus equorum* 15 µl, *Corynebacterium casei* 15 µl, *Debaryomyces hansenii* 33 µl, *Kluyveromyces lactis* 33 µl, *Geotrichum candidum* 33 µl.

Table 1. Composition of the various mixtures of microbial strains used in the present study.
The most IL-10-inducer strains (so-called-anti-inflammatory) are specified in bold while those which are more associated with a pro-inflammatory response are indicated as underlined.

Strains	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix6
Lc. lactis	S3	S3	S3	S3	S3	S3
B. aurantiacum	ATCC 9174	ATCC 9174	ATCC 9174	ATCC 9174	ATCC 9174	ATCC 9174
A. arilaitensis	Re 117^T	Re 117^T	Re 117^T	Re 117^T	Re 117^T	Re 117^T
H. alvei	**GB01**	Type 2 n°920	**GB01**	---	**GB01**	**GB01**
S. equorum	**1265/GM16**	Mu206	**1265/GM16**	**1265/GM16**	**1265/GM16**	**1265/GM16**
C. casei	**DPC S298^T**	1-3b	**DPC S298^T**	**DPC S298^T**	**DPC S298^T**	**DPC S298^T**
D. hansenii	CLIB 623	CLIB 623	CLIB 623	CLIB 623	CLIB 623	CLIB 623
K. lactis	CLIB 683	CLIB 683	CLIB 683	CLIB 683	CLIB 683	CLIB 683
G. candidum	**UCMA 103**	ATCC 204307	---	**UCMA 103**	**UCMA 103**	ATCC 204307
P. camemberti	---	---	---	---	FM 13	---

2.2. Chemicals

Chemicals and reagents were purchased from Sigma-Aldrich Chemical, St Quentin Fallavier, France, unless otherwise stated.

2.3. Peripheral mononuclear blood cell (PBMC) assay

Peripheral mononuclear blood cells ($2x10^6$/ml) isolated from three healthy donors were seeded in RPMI 1640 medium (Life Technologies, Paisley, United Kingdom) in 24-well tissue culture plates (Corning, New York, NY, USA), as previously described (Foligne et al., 2007). Ten microliters of thawed suspensions of the mixtures or individual reference strains to be tested were added. After 24 h of stimulation, culture supernatants were collected and assayed for cytokine levels using ELISAs for human IL-10, IFNγ and IL-12p70 (BD Biosciences, San Jose, CA, USA) and human IL-6 and TNFα (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendations. Data were first generated in $pg.L^{-1}$ for each donor and then expressed as the mean for all three donors as a percentage of the value measured with a reference strain (*i.e. B. longum* Bb536 for IL-10 and *Lc. lactis* MG1363 for IL-12, TNFα and IFNγ). This normalization against internal standard strains has been validated as an accurate and reproducible way of comparing large numbers of strains (Foligne et al., 2007).

2.4. Experimental/prototype cheeses and the feeding procedure

Pilot-scale Livarot-type cheese was produced (via coagulation, cutting, draining, and molding of the curd) under aseptic conditions in a 14 liter tank. The pasteurized milk used (12 liters) was standardized at a fat concentration of 29 g/l fat by mixing semi-skimmed and full-fat milk (Alsace Lait, Hœrdt, France). Ten milliliters of a filter-sterilized 100 $g.L^{-1}$ $CaCl_2$ solution was added to the milk and the mix was pre-heated at 34°C prior adding *Lc. Lactis* to obtain a final concentration of 10^6 cfu/ml. After the pH of the milk had reached 6.3, a mix of the appropriate selected ripening bacteria and yeast strains was added at concentrations of each microorganism at 10^6 and 10^4 cfu/ml, respectively, for both Cheese A (CheA) and Cheese B (CheB). Filter-sterilized rennet (Naturen, Chr. Hansen, Arpajon, France) containing 520 mg/l of active chymosin was then added (0.3 ml/l of milk). The coagulation time was 20 min, and the curd was cut after 30 min of hardening. The curd was then stirred for 5 min at a rate of 10 $stirs.min^{-1}$. After the curd had stood for 15 min, 6.5 liters of whey were removed. Two cheeses were shaped in 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). After 24 h, the cheeses were removed from the mold. After another two hours, the cheeses were cut into smaller pieces (diameter: 50 mm; thickness: 15 mm; weight: 20 g). These were placed in a sterile environment at 14 °C with a relative humidity of 97% for a 28-day ripening period. In order to compare the cheeses with an analogous matrix (MTX), solidified milk was prepared daily and treated with the filter-sterilized rennet and the $CaCl_2$.

For both the DSS and trinitrobenzene sulphonic acid (TNBS) colitis experiments, groups of 10 mice were given either 1 ml of phosphate buffer (the control group), 1 g of the dairy matrix (the MTX group) or 1 g of the corresponding cheeses (the CheA and CheB groups) via the intragastric route. Treatments were given daily (in the late afternoon) for five and ten consecutive days for the TNBS and DSS experiments, respectively.

2.5. Experimental colitis

Animal care and ethical aspects

Ninety female BALB/c mice (aged 7 weeks on arrival) were obtained from Charles River (Saint-Germain-sur-l'Arbresle, France). The animals were randomly divided into groups of five or ten and 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 in compliance 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 current 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 Nord-Pas-de-Calais region's Ethics and Welfare Committee for Animal Experiments (Lille, France; authorization numbers: 19/2009R and 21/2009R).

DSS-induced colitis and the assessment of inflammation

Acute colitis was induced in BALB/c mice on day 7 by the administration of DSS (molecular weight 36000-50000 Da, MP Biomedicals, Illkirch, France) dissolved at a concentration of 5% v/v in the animals' drinking water for 7 consecutive days (Foligne et al., 2007b). Groups of 10 mice were used, and a healthy control group (*i.e*. not exposed to DSS) was given tap water. Animals were weighed daily. After the mice had been sacrificed, colon lengths and weights were scored before tissue processing for microscopic analysis. Briefly, cross-sectional rings of the mid-colon were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections (thickness: 5 µm) were stained with May-Grünwald-Giemsa reagent and scored as described by Hartmann et al. (Hartmann et al., 2000).

TNBS-induced colitis and inflammation scoring

Acute colitis was triggered on day 5 by the intra-rectal administration of 50 µl to reach 100 mg/kg bodyweight TNBS (Sigma-Aldrich Chemical) in 0.9% NaCl/ethanol (50/50 v/v) (Drouault-Holowacz et al., 2006). The animals were monitored daily for bodyweight loss. Three days after the induction of colitis, mice were sacrificed and blood samples were collected immediately. The serum was separated and frozen (-20°C). After dissection, two independent observers blindly scored the macroscopic inflammation of the colon on the Wallace scale (Wallace, MacNaughton, Morris, & Beck, 1989). Two-centimeter-long fragments of the distal colon were collected and frozen at –80°C.

Samples for histologic analysis were processed for paraffin-embedded in 5-µm sections and stained with May Grünwald-Giemsa reagent, as described above.

Following examination under the microscope, tissue lesions were scored according to the Ameho criteria (Ameho et al., 1997). Lastly, the degree of polymorphonuclear neutrophil infiltration in the distal colon was assessed by quantifying myeloperoxidase (MPO) activity, as described previously (Bradley, Priebat, Christensen, & Rothstein, 1982).

Cytokine and serum amyloid protein A (SAA) assays

Murine IL-6 and SAA levels were measured using commercial ELISA kits from BD Pharmingen (San Diego, CA, USA) and Biosource International (Camarillo, CA, USA), respectively, with a lower limit of detection of 15 pg/ml for IL-6 and 30 ng/ml for SAA.

Real-time quantitative PCR and gene expression analysis

Murine intestinal tissue (0.5 cm lengths of the colon) was processed with RNA stabilization solution (RNA-later, Ambion, Life Technologies) and stored at -80°C. After homogenization of samples using the FastPrep instrument (MP Biomedicals, Illkirch-Graffenstaden, France), total RNA was isolated using RNA spin columns (Macherey-Nagel, Hoerdt, France). Reverse transcription and real-time PCR were performed with reaction kits (the High-Capacity cDNA RT Kit) and reagents (Universal PCR Master Mix) from Applied Biosystems (Courtaboeuf, France), according to the manufacturer's instructions and published procedures. The PCR reactions were performed with a MX3005P Stratagene machine (Agilent Technologies, Massy, France). For the *Tnfa, Il1-b, Nos2, Cox-2, Gpx2,* and *IL6* target genes, a custom gene expression assay (TaqMan, Applied Biosystems) was used with commercially designed and validated primers (Table S2). The housekeeping gene beta-actin was run as an internal control. The recorded data were analyzed using the $2^{\Delta\Delta}$Ct calculating method and expressed as a fold-increase over the control group's values.

2.6. Statistical analysis

Results are expressed as the mean ± standard error of the mean. Statistical analysis was performed with a two-tailed Student's t-test or a non-parametric one–way analysis of variance (the Mann Whitney U-test) when appropriate. The threshold for statistical significant was set to $p < 0.05$.

3. Results

3.1. Immunomodulatory profiles of microorganism ecosystems

The immunomodulatory effects of the fourteen strains used in the present work had been previously investigated in a larger study of the diversity of cheese-ripening microorganisms as pure cultures (Adouard, unpublished data). Thus, the release of cytokines by human PBMCs from healthy donors allowed us to determine whether these strains exhibit an anti-inflammatory (*i.e.* high IL-10 inducers and low release of IFNγ and IL-12) or pro-inflammatory (i.e. low IL-10 inducers and higher inducers of IFNγ and IL-12) based on *in vitro* profile (relative to the reference strains) In this context, some strains appear to induce responses with intermediary levels of cytokines, then characterized to be more or less neutral, as shown in Table 1. Based on the strain ranking, six different mixtures of strains were reconstituted in order to mimic the bacterial and yeast diversity encountered in prototype ripened-cheese ecosystems (Mounier et al., 2005). We selected no more than 10 microorganisms per mixture and maintained the 1:1 ratio of bacteria to yeast. Using a core set of common strains used in cheese-ripening (including *Lc. lactis*, *B. auranticum*, *A. arilaitensis*, *D. hansenii* and *K. lactis*), Mix 1 was completed with four anti-inflammatory strains of *H. alvei*, *S. equorum*, *C. casei* and *G. candidum*. In theory, Mix 1 was the most anti-inflammatory blend. In contrast, Mix 2 was supplemented with four strains showing a pro-inflammatory profile, making it (in theory) the least anti-inflammatory mixture. To estimate the role/importance of an individual strain, four other mixtures were designed by removing, adding or substituting a single species. When compared with Mix 1, Mix 3 lacked an anti-inflammatory strain of *Geotrichum* spp, whereas a pro-inflammatory *G. candidum* was added to the Mix 6. Lastly, Mix 4 lacked an anti-inflammatory strain of *Hafnia* and Mix 5 comprised a strain of *Penicilium camemberti* (Table 2).

Table 2: Final microbial counts reached in the two experimental cheeses

Microorganisms	Microbial concentration at 28 days of ripening (log CFU.g^{-1})	
	Cheese A (Mix2)	Cheese B (Mix1)
Bacteria		
Lc. Lactis S3	7.52 ± 0.12	6.25 ± 0.44
B. aurantiacum ATCC9174	6.55 ± 0.12	6.07 ± 0.61
C. casei 1-3b	9.00 ± 0.50	-
DPC S298T	-	8.37 ± 0.32
H. alvei GB01	-	6.90 ± 0.25
Type 2 n°920	7.09 ± 0.18	
A. arilaitensis Re117^{T}	6.17 ± 0.18	5.55 ± 0.15
S. equorum 1265	-	6.32 ± 0.12
Mu206	6.80 ± 0.17	-
Yeasts		
D. hansenii CLIB 623	6.41 ± 0.25	6.85 ± 0.29
G. candidum UCMA 103	-	7.81 ± 0.23
ATCC 204307	6.80 ± 0.26	-
K. lactis CLIB 683	6.90 ± 0.18	7.08 ± 0.15

The six mixtures' ability to induce PBMCs to release cytokines into the supernatant was compared with the most strongly inducing reference strain, i.e. *B. longum* Bb536 for IL-6 and IL-10 and *Lc. lactis* MG1363 for the pro-inflammatory mediators IL-12, TNFα and IFNγ. All mixes were weak inducers of IL-12 and IFNγ and moderate inducers of TNFα; this is the profile typically observed for mixtures of eukaryotic organisms (Fig. 1A-D). Although just half the biomass was composed of yeasts, the low observed stimulatory ability suggests that yeasts were preponderant in the overall immune response. It may thus be of value to add eukaryotic strains to bacterial mixes that induce pro-inflammatory mediators (IL12, TNFα and IFNγ. In contrast, the presence of yeasts did not influence the bacteria's propensity to induce substantial amounts of IL-6 and IL-10.

Figure 1

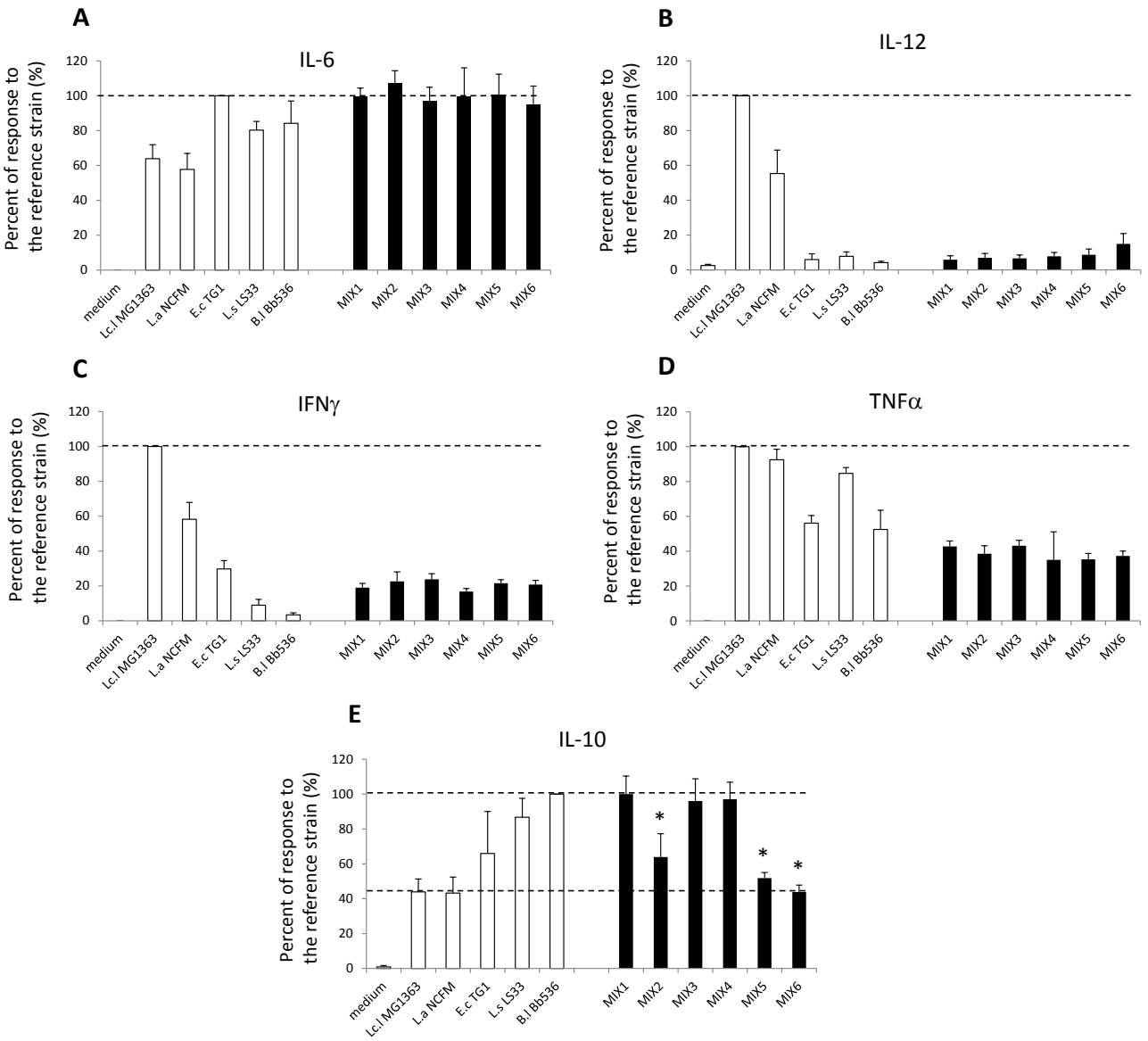

Figure. 1. Production of various cytokines in the supernatants of cultured human PBMCs (three donors) after 24 h of stimulation with an equivalent mass of reference strains (white bars) or six distinct, reconstituted mixtures of ripened cheese microorganisms. (A) IL-6, (B) IL-12, (C) IFNγ, (D) TNFα and (E) IL-10. Data are expressed as the mean ± SEM response as a percentage of a high-inducer reference strains (*B. longum* for IL-10 and *Lc. Lactis* for the other mediators).

The six distinct mixtures stimulated similar amounts of IL-6, IL-12, TNFα and IFNγ suggesting that the PBMCs sensed the various consortia of microorganisms in much the same way (Fig. 1A-D). However, the mixtures differed significantly in terms of secretion of IL-10 (Fig. 1E). It is noteworthy that Mix 1 induced anti-inflammatory factors to the same extent as the *B. longum* reference strain, whereas (as expected) Mix 2 was significantly less able to induce IL-10 ($p<0.05$). Compared with Mix 1, the absence of the anti-inflammatory strains *Geotrichum candidum* UCMA 103 or *Hafnia alvei* GB01 (in Mix 3 and Mix 4, respectively) did not modify the net anti-inflammatory balance. In contrast, replacing *G. candidum* UCMA 103 by the neutral strain *G. candidum* ATCC 204307 (in Mix 6) was enough to reverse the mixture's anti-inflammatory characteristics by nearly 50% ($p<0.05$).
Hence, Mix 6 had a low IL-10-stimulating capacity (close to that of the *Lc. lactis* and *Lb. acidophilus* reference strains). Lastly, addition of the fungus *Penicilium camemberti* (Mix 5) to the anti-inflammatory consortium was also associated with a 50% reduction ($p<0.05$) in the *in vitro* anti-inflammatory potential.

3.2. The impact of two distinct, designed, ripened cheeses on healthy mice

In view of the different *in vitro* immune potentials of Mix 1 and Mix 2, we decided to inoculate milk with these cocktails in order to produce cheese-like dairy products. The two “tailored” prototype cheeses were prepared simultaneously. After four weeks of ripening, the two cheeses had similar final counts for all microorganisms (Table 2).

CheA and CheB (based on fermentation with a poorly anti-inflammatory Mix 2 and the anti-inflammatory Mix 1) were evaluated (along with MTX) in healthy mice. Feeding healthy mice for 8 days with the different diets did not induce any significant changes in body weight or in colonic transcription levels of the tested genes (*i.e. Tnf, Il1b, Il6, Il10, Ifng, Zo1, Nos2*, *Ptgs2* and *Hmox1*, with fold-increases or –decrease of no more than 1.5 *vs.* controls). Only a small (2-fold) increase in expression of *Pparg* was measured in the CheB group (data not shown). Blood levels of SAA and IL-6 were undetectable, suggesting that all treatments with dairy products were safe and well-tolerated under basal, non-inflamed conditions.

3.3. The impact of two distinct, designed, ripened cheeses in two murine models of experimental colitis

As expected, seven days of DSS administration induced severe colitis in mice, with an average bodyweight loss of 15%, significant colon shortening, and elevated macroscopic and histological scores (Fig. 2A-C and data not shown), relative to healthy control mice. Although MTX was associated with a slightly lower weight loss, the MTX, CheA and CheB diets were not associated with any significant protective effects in terms of the inflammatory parameters investigated (data not shown). CheA and CheB were associated with non-significant trends toward the intensification of certain factors (such as macroscopic injury; Fig. 2B, 2C and 2D). There were no significant differences between CheA and CheB.

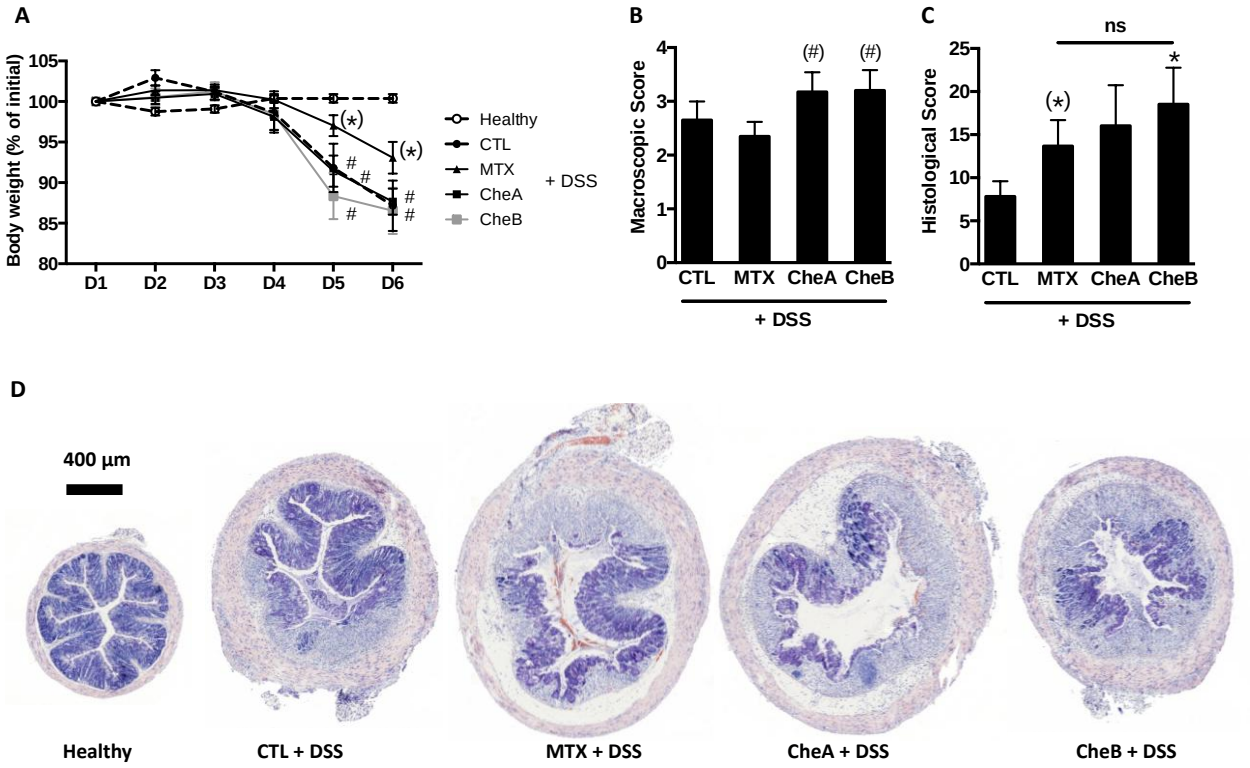

Figure 2. Impact of dietary cheeses on seven-day DSS colitis in mice. (A) Body weight loss (as a percentage of the initial weight) in the healthy, PBS control (CTL), milk matrix (MTX), cheese A (CheA) and cheese B (CheB) groups. (B) Macroscopic scores. (C) Microscopic scores. The data represent the mean (± SD) of 10 mice per group. *p < 0.05 vs. the control group; (*) 0.05 <p <0.1 vs. the control group; # p < 0.05 vs. the MTX group; (#) 0.05 < p <0.1 vs. the MTX group). (D) Representative histologic preparations of the colon (May-Grünwald-Giemsa staining) from mice from the various groups. Scale bars: 400 µm.

In the TNBS colitis model, the symptoms in the control, MTX and CheB groups were quite similar, with body weight loss of up to 15% and similar macroscopic and histological scores (Fig. 3A-C) Transcriptional analysis of the inflammatory marker genes *Cox2, Nos2*, *Il6, Hmox1, Tnf and Il1b,* and the measurement of MPO levels and blood SAA levels showed that the degree of induction was similar for all three conditions (Fig. 3D-F). These findings suggest that CheB (the cheese containing the most anti-inflammatory bacterial and yeast strains) had no specific effect on the symptoms of colitis. In contrast, mice fed for five consecutive days with CheA (the cheese containing an overall more neutral/less anti-inflammatory consortium of strains) showed exacerbated signs of colitis. This was evidenced by a significant ($p<0.05$) increase in weight loss (by up to 20%), a significantly higher disease score, greater colonic MPO activity, greater expression of inflammatory genes (Fig. 3D-F) and more pronounced pathological features (Fig. 3G). Hence, the rate of progression of colitis and the intensity of inflammation were significantly higher in the CheA group than in CheB group ($p<0.05$).

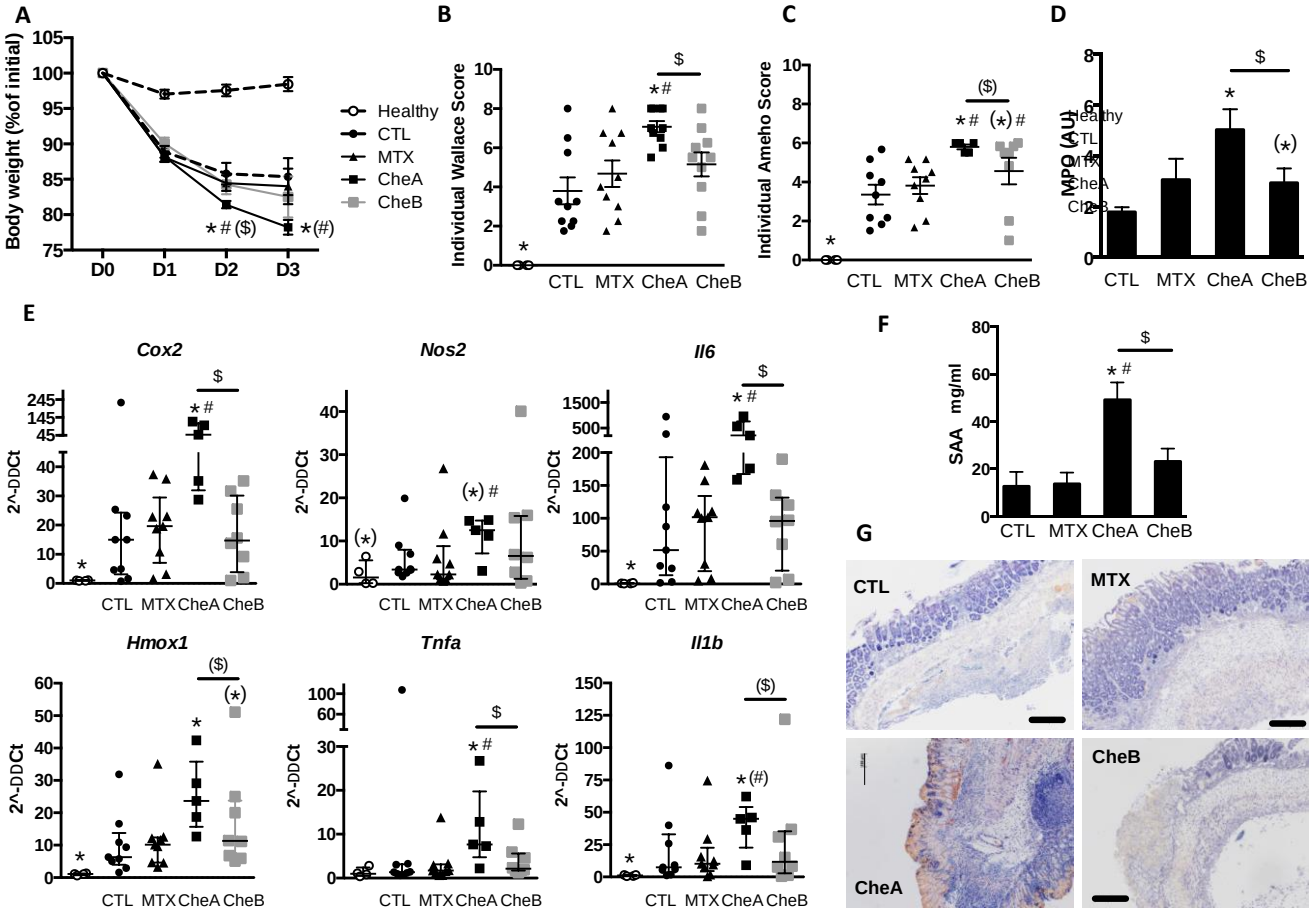

Figure. 3. Impact of dietary cheeses on three-day TNBS colitis in mice. (A) Body weight loss (as a percentage of the initial weight) in the healthy, PBS control (CTL), milk matrix (MTX), cheese A (CheA) and cheese B (CheB) groups. (B) Wallace macroscopic scores. (C) Ameho histological scores. (D) Myeloperoxidase activity (MPO). (E) Transcription of inflammatory genes in the colon. (F) Serum Amyloid A protein (SAA) levels. The data represent the mean (± SD) of 10 mice per group, (*$p < 0.05$ vs. the control group; (*) $0.05 <p <0.1$ vs. the control group; # $p < 0.05$ vs. the MTX group; (#) $0.05 < p <0.1$ vs. the MTX group; $ $p < 0.05$ vs. the CheA group; ($) $0.05 < p <0.1$ *vs*. the MTX group. (G) Representative histologic preparations of the colon (May-Grünwald-Giemsa staining) from mice from the various groups. Scale bars: 200 µm.

4. Discussion

In the present study, we addressed the immunostimulatory diversity among microorganism mixes and thus the potential impact of the widely consumed, live, food-borne microbes found in cheese-ripening ecosystems. Our results clearly showed that bacterial and yeast consortia are not immunologically inert *in vitro*. Based on the immunomodulatory profiles of individual microbial strains, the immune response associated with the reconstituted mixtures could be predicted to some extent (at least for the IL-10 read-out). Although removing an anti-inflammatory strain from an anti-inflammatory cocktail may appear empiric and cannot necessarily be generalized to other situations, this approach seems to preserve the overall anti-inflammatory balance. Adding or changing an immune-enhancing strain may reverse or lower the immunomodulatory response.

As previously shown by our group (Foligne et al., 2007), screening for probiotics with PBMCs (based on the IL-10/IL-12 ratio) is mostly predictive of *in vivo* anti-inflammatory efficacy, especially in the TNBS model of colitis. This correlation was less clear in the DSS mouse model of chemically induced experimental colitis. Although these models mimic some (but not all) of the aspects of human IBD and thus must be interpreted with caution, they are useful for studying probiotic-host interactions (Claes, De Keersmaecker, Vanderleyden, & Lebeer, 2011). The acute DSS colitis model appears to be more appropriate for assessing epithelial barrier function and microbial translocation. In contrast, immunocompetent T and B cells are required for the onset of hapten-induced colitis in the TNBS model. Furthermore, the DSS model also evokes features associated with Th2-like colitis and UC. Thus, it is not surprising that some treatments or nutritional intervention(s) may be protective in one model but not in another (as observed for smoking risk factors in CD *vs.* UC and in TNBS *vs.* DSS models (Montbarbon et al., 2013). We also observed that some yeasts might be able to alleviate colitis even though they did not induce high cytokine levels *in vitro*; this suggests that the yeast's impact on inflammation was correlated with epithelial cell signaling (Foligné, Dewulf, Vandekerckove, Pignède, & Pot, 2010). Our present results have clearly established that *in vitro* immunoprofiling of human PBMCs is a powerful tool for screening food-borne bacteria and foodborne ecosystems containing high proportions of fungi, Gram-positive bacteria and Gram-negative bacteria, extending previous data from pure cultures to mixtures.

The present study's main objective was to determine whether the microbial composition of a fermented diet modified the inflammation in murine models. Given that the various microbial consortia had similar cell counts, metabolic pathways and survival aptitudes in the gut, the main phenotypic differences were related to immune determinants. However, the impact of the two

"opposite" cheeses on the resident microbiota needs be explored further, in order to characterize and understand the role(s) of cheese-derived microorganisms in IBD. Furthermore, models of T-cell-mediated colitis based on the adoptive transfer of T cells in immunodeficient mice (a Th1-type cytokine-mediated hyperresponse model known to be modulated by the gut microbiota and probiotics (Philippe et al., 2011) are likely to provide valuable information prior to clinical studies. In a view to consider the design of functional foods, the whole physiology of the various strains, considering the survival of strains in the product (Tripathi, & Giri, 2014), adhesion to epithelial cells (Monteagudo-Mera et al., 2012), immune profile and regulatory mechanisms of microorganisms (Ashraf, Vasiljevic, Day, Smith, & Donkor, 2014), alone and in combination, have to be taken into account, together with metabolites and the occurrence of milk-derived peptides, that can be either anti- (Korhonen, 2009) or pro-inflammatory (Ul Haq, Kapila, & Saliganti, 2014). Then the final product can be evaluated in reliable models for disease's susceptibility.

In conclusion, the present results further improve our knowledge of the biological activities of cheese-ripening microflora in IBD. Extrapolation of the results of nutritional and inflammatory studies in mice to humans is problematic (Leist, & Hartung, 2013; Seok et al., 2013), and further robust epidemiological and clinical studies in humans are required. However, in view of the massive intake of microorganisms through cheese consumption, one should not neglect the microorganisms' impact on the immune balance and overall health. Pre-illness dietary habits should also be studied. On this basis, one can seek to design cheeses with anti-inflammatory properties for the prevention of colitis (either by maintaining remissions or limiting relapses). This type of ecological, microbiota-targeted therapy would complement other (often expensive) treatments. Furthermore, cheeses with immune-enhancing properties could be of value for specific application such boosting vaccine responses. Taken as a whole, our present results suggest that there are technological opportunities for cheese making in diet management, nutritional advice and food design for further benefits (Rajasekaran, & Kalaivani, 2013). Seksik et al. have questioned whether dietary probiotics, prebiotics or synbiotics have a role in the treatment of patients with IBD (Seksik, Dray, Sokol, & Marteau, 2008); the same question can be asked of cheeses. The answer is "yes" but greatly depends on the individual patient and the specific etiological, immunological and microbiotic features of IBD (Colombel, Watson, & Neurath, 2008). This echoes Hippocrates' advice more than two thousand years ago: "let your food be your medicine and your medicine be your food".

Acknowledgments

We thank Dr. Nicolas Blondiaux (Institut de Microbiologie, Lille University Medical Center, Lille, France) for managing the mass spectrometry analysis. We also thank Katharina Mann-Baërt and Michael Enthal for fruitful advice.

Conflict of Interest Statement: The authors have no conflicts of interest to disclose.

Supplementary data

Table S1. List of the microbial strains and condition for growth used in the present study

Micro-organism species	Strain	Origin	Media	Growth conditions, °C ; rpm
Lactococcus lactis	S3	Cheese	M17	30°C - static
Brevibacterium aurantiacum	ATCC 9174	Cheese (Romadur)	BHI	25°C – 250 rpm
Arthrobacter arilaitensis	Re 117^T	Cheese(Reblochon)	BHI	25°C – 200 rpm
Corynebacterium casei	1-3b	Cheese (Livarot)	BHI	25°C – 200 rpm
Corynebacterium casei	DPC S298^T	Cheese (Gubbeen)	BHI	25°C – 200 rpm
Hafnia alvei	GB001		BHI	25°C – 200 rpm
Hafnia alvei	Type 2 n°920	Dairy product	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	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
Geotrichum candidum	UCMA 103	Cheese	PDB	25°C – 200 rpm
Penicillium camemberti	FM 13	Cheese	PDB	25°C – 200 rpm
Lactococcus lactis	MG1363	Commercial strain	M17	30°C - 200 rpm
Escherichia coli	TG1	Laboratory strain		37 °C - static
Lactobacillus acidophilus	NCFM	Commercial strain	MRS	37 °C - static
Lactobacillus salivarius	Ls 33	Commercial strain	MRS	37 °C - static
Bifidobacterium longum	Bb536	Commercial strain	MRS-cysteine	37 °C - static

Table S2: list of targeted genes and corresponding primer accession numbers

Gene name	Abbreviation	Commercial reference
Actin beta	*ActB*	Mm 01205647_g1
Nitric oxide synthase 2 (inducible)	*Nos2*	Mm 00440502_m1
Heme oxidase 1	*Hmox1*	Mm 00516004_m1
Tumor necrosis factor	*Tnf*	Mm 00443258_m1
Interleukin 1 beta	*Il1b*	Mm 01336189_m1
Prostaglandin synthase 2	*Ptgs2* (*Cox2*)	Mm 00478374_m1
Interleukin 6	*Il6*	Mm 00439614_m1

References

Ameho, C. K., Adjei, A. A., Harrison, E. K., Takeshita K., Morioka, T., Arakaki, Y., Ito, E., Suzuki, I., Kulkarni, A. D., Kawajiri, A., & Yamamoto, S. (1997). Prophylactic effect of dietary glutamine supplementation on interleukin 8 and tumor necrosis factor alpha production in trinitrobenzene sulphonic acid induced colitis. *Gut*, 41, 487-493.

Araujo, R., Rodrigues, A. G., & Pina-Vaz, C., (2004). A fast, practical and reproducible procedure for the standardization of the cell density of an *Aspergillus* suspension: *Journal of Medical Microbiology*, 53, 783-786.

Ashraf, R., Vasiljevic, T., Day, S.L., Smith, S.C., Donkor, O.N. (2014). Lactic acid bacteria and probiotic organisms induce different cytokine profile and regulatory T cells mechanisms.
Journal of Functional Foods, 6, 395-409.

Aune, D., Lau, R., Chan, D. S., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2012). Dairy products and colorectal cancer risk: a systematic review and meta-analysis of cohort studies. *Annals of Oncology*, 23, 37-45.

Bach, J. F. (2002). The effect of infections on susceptibility to autoimmune and allergic diseases. *New England Journal of Medicine*, 347, 911-920.

Beermann, C., & Hartung, J. (2013). Physiological properties of milk ingredients released by fermentation. *Food & Function*, 4, 185-199

Birrenbach, T., & Bocker, U. (2004). Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. *Inflammatory Bowel Diseases*, 10, 848-859.

Blondiaux, N., Gaillot, O., & Courcol, R. J., (2010). MALDI-TOF mass spectrometry to identify clinical bacterial isolates: evaluation in a teaching hospital in Lille. *Pathologie Biologie (Paris)*, 58, 55-57.
Bradley, P. P., Priebat, D. A., Christensen, R. D., & Rothstein, G. (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *Journal of Investigative Dermatology*, 78, 206-209.

Chapman, C. M., Gibson, G. R., & Rowland, I. (2011). Health benefits of probiotics: are mixtures more effective than single strains? *European Journal of Nutrition*, 50, 1-17.

Claes, I. J., De Keersmaecker, S. C., Vanderleyden, J., & Lebeer, S. (2011). Lessons from probiotic-host interaction studies in murine models of experimental colitis. *Molecular Nutrition and Food Research*, 55, 1441-1453.

Clarke, J. O., & Mullin, G. E. (2008). A review of complementary and alternative approaches to immunomodulation. *Nutrition in Clinical Practice*, 23, 49-62.

Colombel, J. F., Watson, A. J., & Neurath, M. F. (2008). The 10 remaining mysteries of inflammatory bowel disease. *Gut*, 57, 429-433.

Cosnes, J. (2004). Tobacco and IBD: relevance in the understanding of disease mechanisms and clinical practice. *Best Practice & Research. Clinical Gastroenterology,* 18, 481-496.

David, L. A., Maurice, C. F., Carmody, R. N, Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S.B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, 505, 559-563.

Drouault-Holowacz, S., Foligné, B., Dennin, V., Goudercourt, D., Terpend, K., Burckel, A., & Pot, B. (2006). Anti-inflammatory potential of the probiotic dietary supplement Lactibiane Tolérance: *in vitro* and *in vivo* considerations. *Clinical Nutrition*, 25, 994-1003.

Foligné, B., Daniel, C., & Pot, B. (2013). Probiotics from research to market: the possibilities, risks and challenges. *Current Opinion in Microbiology*, 16, 284-292.

Foligne, B., Dessein, R., Marceau, M., Poiret, S., Chamaillard, M., Pot, B., Simonet, M., & Daniel, C. (2007). Prevention and treatment of colitis with *Lactococcus lactis* secreting the immunomodulatory *Yersinia* LcrV protein. *Gastroenterology*, 133, 862-874.

Foligné, B., Dewulf, J., Vandekerckove, P., Pignède, G., & Pot, B. (2010). Probiotic yeasts: anti-inflammatory potential of various non-pathogenic strains in experimental colitis in mice. *World Journal of Gastroenterol*ogy, 16, 2134-2145.

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D, Poiret, S., Dewulf, J., Brassart, D., Mercenier, A., & Pot, B. (2007). Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. *World Journal of Gastroenterol*ogy, 13, 236-243.

Gentschew, L., & Ferguson, L. R. (2012). Role of nutrition and microbiota in susceptibility to inflammatory bowel diseases. *Molecular Nutrition and Food Research,* 56, 524-535.

Gionchetti, P., Calafiore, A., Pratico, C., Laureti, S., Vitali, G., Poggioli, G., Campieri, M., & Rizzello, F. (2012). Randomized controlled trials in pouchitis. *Reviews on Recent Clinical Trials*, 7, 303-306.

Hart, A. L., Lammers K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., Campieri, M., Kamm, M. A., Knight, S. C., & Stagg, A. J. (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut*, 53, 1602-1609.

Hartmann, G., Bidlingmaier, C., Siegmund, B., Albrich, S., Schulze, J., Tschoep, K., Eigler, A., Lehr, H. A., & Endres, S. (2000). Specific type IV phosphodiesterase inhibitor rolipram mitigates experimental colitis in mice. *Journal of Pharmacology and Experimental Therapeutics*, 292, 22-30.

Hoarau, C., Martin, L., Faugaret, D., Baron, C., Dauba, A., Aubert-Jacquin, C., Velge-Roussel, F., & Lebranchu, Y. (2008). Supernatant from bifidobacterium differentially modulates transduction signaling pathways for biological functions of human dendritic cells. *Plos One*, 3, e2753.

Hosoya, T., Ogawa, A., Sakai, F., & Kadooka, Y. (2012). A cheese-containing diet modulates immune responses and alleviates dextran sodium sulfate-induced colitis in mice. *Journal of Dairy Science*, 95, 2810-2818.

Khosravi, A., Yáñez, A., Price, J. G., Chow, A., Merad, M., Goodridge, H. S., & Mazmanian, S. K. (2014). Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host & Microbe*, 15, 374-381.

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods,* 1, 177-187

Labonte, M. E., Couture, P., Richard, C., Desroches, S., & Lamarche B. (2013). Impact of dairy products on biomarkers of inflammation: a systematic review of randomized controlled nutritional intervention studies in overweight and obese adults. *American Journal of Clinical Nutrition*, 97, 706-717.

Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nature Reviews in Microbiol*ogy, 8, 171-184.

Leist, M., & Hartung, T. (2013). Inflammatory findings on species extrapolations: humans are definitely no 70-kg mice. *Archives of Toxico*logy, 87, 563-567.

López, P., Gueimonde, M., Margolles, A., & Suárez A. (2010). Distinct Bifidobacterium strains drive different immune responses *in vitro*. *International Journal of Food Microbiology*, 138, 157-165.

Maccaferri, S., Klinder, A., Brigidi, P., Cavina, P., & Costabile, A. (2012). Potential probiotic Kluyveromyces marxianus B0399 modulates the immune response in Caco-2 cells and peripheral blood mononuclear cells and impacts the human gut microbiota in an in vitro colonic model system. *Applied and Environmental Microbiol*ogy, 8, 956-964.

Maconi, G., Ardizzone, S., Cucino, C., Bezzio, C., Russo, A. G., & Bianchi Porro, G. (2010). Pre-illness changes in dietary habits and diet as a risk factor for inflammatory bowel disease: a case-control study. *World Journal of Gastroenterology*, 16, 4297-4304.

Marteau, P., Lepage, P., Mangin, I., Suau, A., Doré, J., Pochart, P., & Seksik, P. (2004). Review article: gut flora and inflammatory bowel disease. *Alimentary Pharmacology* & Therapeutics, 20, 18-23.

Montbarbon, M., Pichavant, M., Langlois, A., Erdual, E., Maggiotto, F., Neut, C., Mallevaey, T., Dharancy, S., Dubuquoy, L., Trottein, F., Cortot, A., Desreumaux, P., Gosset, P., & Bertin B. (2013). Colonic inflammation in mice is improved by cigarette smoke through iNKT cells recruitment. *Plos One*, 8, e62208.

Monteagudo-Mera, A., Rodríguez-Aparicio, L., Rúa, J., Martinez-Blanco, H., Navasa, N., García-Armesto M. R., & Ferrero M. A. (2012). *In vitro* evaluation of physiological probiotic properties of different lactic acid bacteria strains of dairy and human origin. *Journal of Functional Foods*, 4, 531-541

Mounier, J., Gelsomino, R., Goerges, S., Vancanney, M., Vandemeulebroecke, K., Hoste, B., Scherer, S., Swings, J., Fitzgerald, G. F., & Cogan, T. M. (2005). Surface microflora of four smear-ripened cheeses. *Applied and Environmental Microbiology*, 71, 6489-6500.