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Immunomodulation properties of multi-species fermented milks

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ABSTRACT

Dairy propionibacteria (PAB) are used as a ripening starter in combination with Lactic acid bacteria (LAB) for dairy products such as Swiss-type cheese. LAB and PAB have also been studied for their probiotic properties but little is still known about their individual and/or synergistic beneficial effects within dairy matrices. In the context of a rising incidence of Inflammatory Bowel Diseases, it has become crucial to evaluate the immunomodulatory potential of bacteria ingested in large numbers *via* dairy products. We therefore selected different strains and combinations of technological LAB and PAB. We determined their immunomodulatory potential by IL-10 and IL-12 induction, in human peripheral blood mononuclear cells, on either single or mixed cultures, grown on laboratory medium or directly in milk. Milk was fermented with selected anti-inflammatory strains of LAB or PAB/LAB mixed cultures and the resulting bacterial fractions were also evaluated for these properties, together with starter viability and optimum technological aspects. The most promising fermented milks were evaluated in the context of TNBS- or DSS-induced colitis in mice. The improvement in inflammatory parameters evidenced an alleviation of colitis symptoms as a result of fermented milk consumption. This effect was clearly strain-dependent and modulated by growth within a fermented dairy product. These findings offer new tools and perspectives for the development of immunomodulatory fermented dairy products for targeted populations.

1. Introduction

The human gastrointestinal tract is a complex ecosystem, in which resident and transiting bacteria co-exist. This microbiota fulfils important physiological and metabolic functions which include maintenance of the gut associated immune system (Purchiaroni et al., 2013). In some cases, dysbiosis, i.e. imbalanced intestinal microbiota, occurs. This leads to impairment of the immune function of the gut and chronic gastrointestinal illness can occur, associated with more or less severe symptoms, referred as “inflammatory bowel disease” (IBD) (Duboc et al., 2013). The potential role for some pathobionts in establishing and/or exacerbating inflammation should not be disregarded (Kamada et al., 2013) and this may occur in the specific context of Crohn's disease-related polymorphisms, as recently confirmed in the case of adherent and enteroinvasive *Escherichia coli* (Nguyen et al., 2014). Nonetheless, reports have suggested that a lack of specific bacteria with anti-inflammatory properties in the dysbiosis accompanying IBD may also be responsible for gut inflammation (Eeckhaut et al., 2013; Kang et al., 2010; Morgan et al., 2012; Sokol et al., 2008). The association between the increased relative abundances of *Faecalibacterium prausnitzii* and extended remission periods in patients with Crohn's disease is obvious. Similarly, data showed that patients with IBD had lower faecal counts of the butyrate-producing bacteria *Butyricoccus pullicaecorum*, *Roseburia hominis* and *F. prausnitzii* (Eeckhaut et al., 2013; Machiels et al., 2014). In addition, representatives from the spore-forming Clostridium clusters IV and XIV are involved in the induction of tolerance responses (T regulatory cells or Treg) in the colon (Atarashi et al., 2011). Targeting these resident bacteria is conceptually attractive as a potential therapy for treatment of these diseases. Indeed, referring to the observations made by Kang et al. (Kang

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et al., 2010), reducing the presence of *Enterococcus* sp., *Clostridium difficile*, *E. coli*, *Shigella flexneri*, and *Listeria* sp. (more abundant in CD patients) while at the same time encouraging species such as *Eubacterium rectale*, *Bacteroides fragilis* group, *B. vulgatus*, *Ruminococcus albus*, *R. callidus*, *R. bromii*, and *Faecalibacterium prausnitzii* (5–10-fold more abundant in healthy subjects than in CD patients) may procure therapeutic benefits. However, these overall strategies focused on achieving an equilibrium of commensals bacteria are currently difficult to implement in patients with IBD, thus raising concerns and technical challenges. In the current context of an increase in IBD in developed countries (Leone et al., 2013) the impact of probiotic bacteria on intestinal health has been studied, the aim being to prevent IBD or improve its treatment (Minocha, 2009; Martín et al., 2013). Dietary changes constitute an effective tool to modulate the gut microbiota, including its structure and activity (David et al., 2014). Because fermented food products form part of our diet, their microbial constituents need to be taken into account, as should the possibility of modulating this major microbial intake.

Lactic acid bacteria (LAB) are Gram positive bacteria that are naturally found in the environment and also present in the gastrointestinal tract of humans and animals. They are widely used for their technological properties as starters in fermented foods, including cereals, bread, milk, vegetables and meat. Some LAB strains have also been the subject of particular study regarding their so-called probiotic properties, with potential health claims that include modulation of enzymatic activities by yoghurt (Savaiano, 2014) and their marked impact on gut inflammation (Shen et al., 2014). In terms of the scientific basis for the discovery of probiotic microorganisms (Papadimitriou et al., 2015), it is necessary to obtain a clear view of the current situation in this field. This includes the industrial and regulatory perspectives that are likely to determine the current and future role of probiotics both scientifically and economically, and how they might affect both patients and healthy consumers (Foligné et al., 2013b; Sanders et al., 2013). *Propionibacterium freudenreichii*, belonging to PAB, is a gram positive bacterium with GRAS (generally recognised as safe) status, that is found in the environment (soil, straw, etc) (Falentin et al., 2010) and is well known for its role as a ripening starter in the cheese industry (e.g. Emmental, Leerdammer). It is also studied for its probiotic activities, such as modulation of the gut microbiota *via* the production of DHNA (Hojo et al., 2002). Fermented foods containing both LAB and *P. freudenreichii* are increasingly being regarded as a source of bacteria that could modulate gut health, and particularly to treat or prevent IBD. Indeed, when these bacterial species are included in dairy products, their populations may reach very high levels (10^9 bacteria/gram in Emmental cheese for example). Furthermore, fermented dairy products provide protective food matrices that are effective in protecting bacteria against digestive stresses (Leverrier et al., 2005; Ranadheera et al., 2010). However, the immunomodulatory properties of these dietary bacteria have mainly been studied *in vitro* after growth in laboratory medium and as a pure culture. These studies showed that the immunomodulatory properties could vary markedly within the same species, as observed in *Lactobacillus delbrueckii bulgaricus* (Santos Rocha et al., 2012), *Lactobacillus plantarum* (Van Hemert et al., 2010) and *P. freudenreichii* (Foligné et al., 2010), suggesting that strain characterization is very important. However, in many dairy fermented products, populations of different LAB are sometimes mixed with other species (e.g. bifidobacteria, propionibacteria). To date insufficient knowledge has been obtained regarding their interactions in terms of immunomodulatory potential and possible synergistic or antagonist effects (Timmerman et al., 2004). During the present study, our aim was to evaluate the immunostimulatory potential of these species that are ingested in large quantities *via* the consumption of dairy products. For this purpose, we selected different species of LAB and different strains of *Propionibacterium freudenrechii* and tested *in vitro* their anti-inflammatory potential by measuring IL-10 induction in human PBMC (peripheral blood mononuclear cells). *In vivo*, different experimental models of colitis in mice have been shown to induce various aspects of IBD, although they do not completely mimic the human diseases (DSS colitis and TNBS colitis). These methods are complementary to evaluate the immunomodulation achieved, either by micro-organisms alone or included in fermented products. Consequently, in a second step, some of our selected fermented milks were evaluated *in vivo* in a context on either TNBS- or DSS-induced experimental colitis in mice in order to clarify the effects of starter strains grown in dairy products on gut inflammation.

2. Material & methods

2.1. Strains and media

The *Lactococcus lactis* ssp *lactis* CB 460 and *Lactococcus lactis* ssp *cremoris* CB 461 strains were obtained from the CIRM-BIA collection (CB) (INRA, STLO, RENNES, FRANCE). They were isolated from a traditional fermented milk (named “gros lait”, made in Brittany, France), and were stored at −80 °C in M17 (Terzaghi and Sandine, 1975) supplemented with 15% glycerol. Before their use in M17, they were sub-cultured twice from frozen glycerol stocks on M17 (30 °C), using a 2% inoculum. For use in milk, the strains were first sub-cultured twice in UHT commercial skimmed milk (2%). In some cases, *L. lactis* CB 460 and CB 461 were grown as a mixed culture and referred to as Mix1: they were co-cultured (2% of each strain) in M17 or milk and subcultured once before use (2%). Two commercial starters (Standa Industries, Caen, France) were used (Table 1): PAL Tex, referred to as Mix2 and consisting in a mixture of *Lactococcus lactis* and *Streptococcus thermophilus* strains, and PAL YOG, referred to as Mix3 and consisting of *S. thermophilus* strains. Mix2 and Mix3 were received as lyophilizates, and resuspended in 100 ml UHT commercial skimmed milk before freezing. Before they were used, Mix2 and Mix3 were sub-cultured once in milk (1%) at 30 °C.

The *Propionibacterium freudenreichii* CB 129 strain (*P. f* CB 129), also known as ITG P20 (Actalia, Rennes, France), was stored at −80 °C in YEL medium (Malik et al., 1968). It was sub-cultured twice from frozen stock using a 2% inoculum. When specified, the strain was grown on milk ultrafiltrate obtained as previously

Table 1
Bacterial species and strains.

Name	Other name	Species & strain(s)
CB[a]129	ITG[b] P20	*Propionibacterium freudenreichii* CB129
Mix1		*Lactococcus lactis* ssp *lactis* CB460 *Lactococcus lactis* ssp *cremoris* CB461
Mix2	Pal Tex[c]	*Streptococcus thermophilus* *Lactococcus lactis*
Mix3	Pal Yog[c]	*Streptococcus thermophilus*
Mix1-*Pf*		Mix1 *Propionibacterium freudenreichii* CB129
Mix2-*Pf*		Mix2 *Propionibacterium freudenreichii* CB129
Mix3-*Pf*		Mix3 *Propionibacterium freudenreichii* CB129

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