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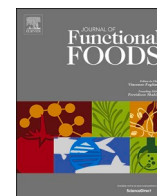
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Fat matters: Fermented whole milk potentiates the anti-colitis effect of *Propionibacterium freudenreichii*

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ABSTRACT

Inflammatory bowel diseases (IBD) constitute a growing concern in western countries. They coincide with gut microbiota dysbiosis, including a loss of immunomodulatory bacteria. Accordingly, probiotic products containing selected immunomodulatory bacterial strains mitigate IBD. Selected strains of *Propionibacterium freudenreichii* display promising modulatory properties and prevent colitis in animal models. Dairy matrices protect propionibacteria immunomodulatory surface antigens during digestive transit. However, the functional role of the dairy matrix components in such fermented dairy products remains unknown. In the present work this study, *P. freudenreichii* CIRM-BIA129, a probiotic strain known for its anti-inflammatory properties, was used to ferment whole milk, skim milk or skim milk ultrafiltrate. The preventive potential of fermented products was tested in DSS-induced mice colitis, in comparison with their unfermented counterparts. *P. freudenreichii*-fermented milk prevented colitis. Dairy fat in the fermented product potentiated the anti-colitis effects of the probiotic. This work opens new perspectives for developing immunomodulatory functional fermented foods.

1. Introduction

1.1. Inflammatory diseases constitute a growing concern.

Many developed countries presently experience an epidemic of chronic inflammatory disease, which can be, at least in part, attributed to a deleterious shift in lifestyle and in diet. Indeed, consumption of Western diet combined with overnutrition and sedentary lifestyle evoke a state of chronic metabolic inflammation, referred to as metaflammation (Christ et al., 2019). This in turn contributes to an increased incidence of non-communicable diseases, which include inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC). This constitutes a rising public health problem with global epidemic dimensions responsible for a growing burden of care. The

causes of such an epidemic are complex. Dietary factors such as overconsumption of fat and protein, yet low intake of fruits and vegetables, are probably involved (Hou et al., 2011). Indeed, high-fiber and fruit intakes are associated with decreased risk of IBD, including CD and UC (Hou et al., 2011). By contrast, a review of clinical evidence associated fermented dairy products with an anti-inflammatory activity. This association was particularly significant in subjects with metabolic disorders (Bordoni et al., 2017). In addition to the diet, dysbiosis of the gut microbiota may also play a key role in such inflammatory ailments. Indeed, exacerbated immune response in IBD involves an interplay between the gut microbiota, host genetic factors, and environmental factors.

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1.2. Dysbiosis coincides with inflammatory ailments.

In many inflammatory diseases, including IBD, the gut microbiota is modified, in comparison to that of healthy volunteers. This modification constitutes dysbiosis, which can be defined as “a disturbance to gut microbiota homeostasis due to an imbalance in the flora itself, changes in their functional composition and metabolic activities, or changes in their local distribution” (DeGruttola et al., 2016). Indeed, dysbiosis of the gut microbiota was described in IBD patients, including in CD (Sokol et al., 2008) (Matsuoka and Kanai, 2015) and in UC (Zhang et al., 2007) (Kostic et al., 2014). Dysbiosis is more pronounced in CD patients than in UC ones, with a more reduced diversity and a less stable microbial community (Pascal et al., 2017). Indeed, increasing human diseases are often associated with a modern western lifestyle and with a reduction in the intestinal microbiota diversity (Mosca et al., 2016). This includes metabolic diseases and cancer, but also immune disorders such as CD, UC, allergy and celiac disease. Loss of microbial diversity in this context is characterized by a reduction of the population of commensal bacteria with a recognized immunomodulatory role. This includes reduced population of *Faecalibacterium prausnitzii*, a butyrate-producing bacterium with immunomodulatory anti-inflammatory properties (Quévraïn et al., 2016); (Touch et al., 2022). This also includes species of Bifidobacteria, also recognized as key immunomodulators (Tojo et al., 2014). In line with this, a meta-analysis of available clinical data indicated that the consumption of a mix of 8 strains of probiotic bacteria, VSL#3, which includes bifidobacteria and lactic acid bacteria, mitigates the symptoms of IBD in humans (Sniffen et al., 2018). This evidences the key importance of a research work aimed at the identification of food-grade bacteria able to down-regulate inflammation in the context of colitis.

1.3. Propionibacteria exhibit promising immunomodulatory properties.

Dairy propionibacteria, including the main species *Propionibacterium freudenreichii*, are generally recognized as safe (GRAS) bacteria, with the qualified presumption of safety (QPS) status, which are consumed both in Swiss-type cheeses and in probiotic food supplements. They constitute promising immunomodulatory probiotic candidates that may be useful to mitigate symptoms of colitis. In a pioneer study, selected strains of *P. freudenreichii* were shown to induce the modulatory IL-10 in human immune cells *ex vivo* (PBMC). These strains, when administered in a preventive way, were further shown to prevent acute colitis induced by TNBS in BALB/c mice (Foligné et al., 2010). A wider screening of *P. freudenreichii* strains then revealed that this ability is highly dependent on the strain, with a great variability of the immunomodulatory properties (Foligné et al., 2013). These last depend on the presence of specific key immunomodulatory surface proteins, and extraction of such proteins suppresses propionibacteria ability to induce IL-10 (Le Marechal et al., 2015). Moreover, mutational inactivation of key genes (*slpB*, *slpE*) encoding such surface layer proteins also suppresses this immunomodulatory ability (Deutsch et al., 2017). The *P. freudenreichii* CIRM-BIA129 was thus selected as the most modulatory one, exposing SlpB and SlpE proteins at its surface. It prevents acute colitis induced by TNBS (Plé et al., 2015), as well as colitis induced by DSS (Rabah, 2020) and mucositis induced by the cancer chemotherapy drug 5-fluorouracil (2018). Inactivation of the *slpB* gene in this strain suppresses adhesion to human intestinal epithelial cells, as well as protective effect towards colitis and mucositis (do Carmo et al., 2017; 2019). In line with this, cloning and expression of this gene in *Lactococcus lactis* confers to this lactic acid bacterium enhanced anti-inflammatory properties (Belo et al., 2021). In humans, a clinical study revealed that propionibacteria were members of the gut microbiota of breast-fed preterm infants, while they were absent of that from formula-fed ones, in which necrotizing enterocolitis (NEC), a leading and intractable cause of mortality in preterm infants, is more frequent (Colliou et al., 2017). Accordingly, these authors isolated a *P. freudenreichii* strain from a healthy infant and described its ability to confer protection against pathogen infection and

to increase intestinal Th17 cells in mice. In line with this, a pilot clinical study indicated that the consumption of whey cultures of *P. freudenreichii* improved the clinical activity index score in patients with active UC (Mitsuyama et al., 2007). These authors suggested that this healing effect was mediated by a secreted compound, found in culture supernatant and referred to as bifidogenic growth stimulator (BGF), produced by *P. freudenreichii* (Suzuki et al., 2006). These data strongly suggest that, in addition to being a GRAS and QPS dairy starter with a long history of safe use, *P. freudenreichii* also constitutes a human symbiont and a promising immunomodulatory probiotic.

1.4. The dairy matrix may play a role in the modulatory effect.

We previously addressed the role of the dairy matrix in the modulation of *P. freudenreichii* probiotic activity. Using an *in vitro* gastrointestinal digestion system, the cheese matrix was shown to protect the immunomodulatory SlpB protein from digestive proteolysis (Rabah, Ménard, et al., 2018). Both the viability of the propionibacterium and the integrity of the SlpB were preserved during digestion of the cheese, by contrast with a liquid culture. In young weaned piglets, the cheese matrix modulated *P. freudenreichii* immunomodulatory properties (Rabah, Ferret-Bernard, et al., 2018). Compared to liquid cultures, cheese containing *P. freudenreichii* enhanced Treg and Th2 cells in piglets PBMC and MLNC. Consumption of such a cheese reduced severity of subsequent DSS-induced colitis in mice, including weight loss, disease activity index, score, as well as induction of Tumor Necrosis Factor α (TNF α), Interferon γ (IFN γ) and Interleukin-17 (IL-17) (Rabah, 2020). Such data indicate the interest of using a fermented dairy product such as cheese for the vectorization of propionibacteria to the gut. It is easy to hypothesize that the dairy constituents, including proteins and lipids, protect these bacteria from the insults of low gastric pH, of digestive enzymes and of bile salts. However, the precise role of these constituents in the overall probiotic effect of the fermented food remains elusive. Do dairy lipids and proteins play a role, *per se*, as immunomodulators? Indeed, bioactive milk proteins reportedly exert anti-inflammatory effects (Chatterton et al., 2013) and so do casein hydrolysates (Chen et al., 2022). Dietary fats, depending on their structure, may exert opposite effects on intestinal inflammation (Basson et al., 2020).

We therefore assessed the question of the respective role of propionibacteria, of milk protein and of milk fat in the anti-colitis effect of *P. freudenreichii*-fermented milk. To do so, whole milk, skim milk and milk ultrafiltration permeate were fermented by *P. freudenreichii* CIRM-BIA129. These fermented products, as well as their unfermented control, were administered to mice prior to colitis induction. The research work indicate that the most protective product is the whole milk fermented by *P. freudenreichii* CIRM-BIA129, suggesting a protective role of milk fat.

2. Materials & methods

2.1. Growth of dairy propionibacteria

The strain *Propionibacterium freudenreichii* CIRM-BIA129, equivalent to ITG P20, was initially isolated from a Swiss-type cheese. It was kindly provided by CNIEL (Centre National Interprofessionnel de l'Économie Laitière) and maintained by the CIRM-BIA microbiological resource center (Centre International de Ressources Microbiennes, Bactéries d'Intérêt Alimentaire, Rennes, France). Starting from a frozen stock, precultures were grown in liquid Yeast Extract Lactate (YEL) broth containing 0.1 M lactate and 10 g.L⁻¹ yeast extract as described (do Carmo et al., 2017; 2019)(Malik et al., 1968). CIRM-BIA129 was incubated at 30 °C during 3 days, without agitation, until stationary phase was reached.

2.2. Preparation of fermented dairy products

Dairy media for propionibacteria growth were prepared as previously described (Cousin et al., 2012). Milk ultrafiltration (UF) permeate was prepared as follows. Raw cow milk was skimmed using a cream separator (Westfalia, Chateau-Thierry, France). It was then separated using an UF pilot equipment (T.I.A., Bollene, France) equipped with an organic spiral membrane with a molecular weight cut-off of 5 kDa (Koch International, Lyon, France). The temperature during the ultrafiltration process was maintained at 55 °C. The collected UF permeate was supplemented with 0.1 M food-grade sodium lactate (Sigma-Aldrich, St. Louis, MO, USA) and 10 g.L⁻¹ food-grade grade casein hydrolysate (Casein Peptone Plus, Organotechnie, La Courneuve, France) prior to sterilization using a 0.2 µm filtration unit (Nalgene, Roskilde, Denmark) and stored at 4 °C. UHT skim milk and whole milk (Agrilait, France) were purchased from a local supermarket and supplemented with 0.1 M food-grade sodium lactate (Sigma-Aldrich) and 10 g.L⁻¹ food-grade grade casein hydrolysate, as was UF permeate. Starting from YEL pre-cultures, UF pre-cultures were grown prior to inoculation of dairy media (UF, skim milk, whole milk). Growth was at 30 °C during 3 days, without agitation, until stationary phase was reached, corresponding to bacterial densities of 10⁹ CFU g.L⁻¹, as described previously (Cousin et al., 2012).

2.3. Animal experiments

C57Bl/6 mice ($n = 80$), male, 6 weeks old, were obtained from the “Biotério central” of the Federal University of Minas Gerais (Belo Horizonte, Minas Gerais, Brazil). They were randomly divided into 8 groups of 10 animals. They were kept in microisolators at a controlled temperature (25 °C $\pm$ 1), with a 12 h/12 h light/dark cycle and *ad libitum* access to food (standard chow pellets – Nuvilab Brazil) and autoclaved drinking water. During 14 days, mice received treatments indicated in Table 1. They were daily gavaged with 200 µL of sterile phosphate buffered saline (PBS), filter-sterilised milk UF, UHT sterile skim milk, UHT sterile whole milk, or with 200 µL of *P. freudenreichii* culture in milk UF, in skim milk, or in whole milk. During the last 7 days, colitis was induced, in all groups except the negative control (NC) group, by replacing drinking water by a 2.5% dextran sulphate sodium (DSS) solution in autoclaved water (DSS 40 kDa, TdB Consultancy, Uppsala, Sweden). On the 15th day, animals were euthanized by anesthetic overdose (Ketamine 270 mg/Kg and xylazine 30 mg/Kg). The Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFGM, Brazil) approved all experimental procedures realized in this work by the protocol no. 148/2020. Total blood was collected for permeability assay. The colon was collected and measured, washed with sterile PBS, and divided into 2 sections for histology and for gene expression analysis.

2.4. Intestinal permeability

Intestinal permeability was assessed as described previously (do Carmo et al., 2019). On the last day, mice were gavaged with 100 µL of a solution of radiolabelled Diethylenetriamine Pentaacetic Acid (DTPA) containing 18.5 MBq of ^{99m}technetium (^{99m}Tc-DTPA). After 4 h, the

Table 1

Groups codes and treatment description.

Group code	Treatment administered	Colitis induction
NC	PBS	No
DSS	PBS	DSS 2.5%
DSS-PUF	Milk Ultra Filtrated	DSS 2.5%
DSS-SM	Skim Milk	DSS 2.5%
DSS-WM	Whole Milk	DSS 2.5%
DSS-UF129	Fermented milk Ultra Filtrated	DSS 2.5%
DSS-SM129	Fermented Skim Milk	DSS 2.5%
DSS-WM129	Fermented Whole Milk	DSS 2.5%

animals were anesthetized as described above and blood was collected. The radioactivity was counted in a gamma radiation counter (Wizard, PerkinElmer) according to the instructions of the manufacturer. Results were expressed as a percentage of dose per g of blood, using the following equation: % dose = (cpm in blood/cpm in administrated dose) $\times$ 100 (cpm: counts per minute) (Generoso et al., 2011).

2.5. Assessment of colitis severity

Food and liquid consumption were daily measured for each isolator and animals were weighed individually. During the last three days, feces were analyzed for consistency and for the presence of blood using the Feca-Cult test, (Inlab, Brazil). The disease activity index (DAI) was determined as indicated in Table 2, according to the 3 main clinical symptoms of colitis: diarrhoea, rectal bleeding, and weight loss, following a scoring previously described (Murthy et al., 1993). The size of PBS-washed colons was measured.

2.6. Histology

Colon sections were gently washed in PBS, rolled in a distal-proximal configuration, fixed in neutral buffered 10%(v/v) formalin solution (Sigma-Aldrich) and processed for histological analysis. Hematoxylin phloxine saffron (HPS) and Alcian Blue stained sections of the paraffin-embedded proximal colonic tissue were used for histological analysis. Tissue damage was determined by two blinded investigators quantifying the destruction of mucosal architecture, the presence and degree of cellular infiltration, the extent of muscle thickening and the goblet cell depletion. The destruction of mucosal architecture was scored as 0–3 (0: none, 1: 1/3 basal, 2: 2/3 basal and 3: loss of crypt and epithelium). Then, presence and degree of cellular infiltration was rated as 0–3 (0: none, 1: infiltrate around crypt basis, 2: extensive infiltration reaching the muscularis mucosae and 3: infiltration of the submucosa). Next, the extent of muscle thickening was scored as 0–3 (0: none, 1: mild, 2: moderate and 3: extensive thickening). Finally, presence or absence of goblet cell depletion was determined as 0: goblet cells presence and 1: loss of goblet cells. An extension factor of 1–4 was implemented when the criteria measured reached 25, 50, 75 and 100% of the tissue evaluated.

Furthermore, the crypt depths and the number of goblet cells per crypt were determined based on histology images. Five pictures from random sections of the colon were collected per animal. For the crypt measurements, 20 crypts were measured from each image and the mean was determined for each animal. The number of goblet cells crypt was counted on 10 crypts per image, and the average was used for the analysis.

2.7. Gene expression analysis

After collection, colon samples were stored in RNAlater RNA-later (Invitrogen, USA) at –20°C until further RNA extraction. The RNA extraction was performed using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. Purified RNA (1 µg) was then used to produce the cDNA with the QSCRIPT cDNA synthesis kit (Quantabio, USA) for a final volume of 20 µL. The cDNA incubation program consisted of 5 min at 22°C, 30 min at 42°C, and 85°C for 5 min.

Table 2

Disease Activity Index.

Score	Weight loss (%)	Stool consistence*	Occult/Gross Bleeding
0	None	Normal	Absent
1	0–5		
2	5–10	Loose	Occult blood
3	10–15		
4	>15	Diarrhea	Gross bleeding

Both the RNA and the produced cDNA were quantified in a nanodrop spectrophotometer (Thermo Scientific, USA). qPCR was carried out in a 16 µL volume containing 15 ng cDNA, specific primers (300 nM), and 8 µL IQTM SYBR Green Supermix (Bio-Rad). Reactions were run on a CFX96 real-time system (Bio-Rad, France) using the following cycling parameters: DNA polymerase activation and DNA denaturation 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 15 s, and extension at 60 °C for 30 s. Melting curve analysis was included to check the amplification of single PCR products. The primers used are listed in Table 3. All genes were analysed in duplicate and the amount of mRNA was normalized by using the geometrical mean value of four reference genes: *actB* (beta-actin), *gapdh* (glyceraldehyde 3-phosphate dehydrogenase), *ppiA* (peptidylprolyl isomerase A) and *tuba* (beta-tubulin). The online tool Ref-FINDER (<https://www.heartcure.com.au/reffinder/>) was used to identify the most stably expressed genes in the experimental conditions tested from a panel of six gene candidates. The values corresponding to the amount of amplified gene compared to the NC control group (2-ΔΔCT) were expressed as mean ± SEM. The differences among the groups were assessed by the Kruskal-Wallis test followed by Dunn's multiple comparisons test performed using GraphPad Prism version 9.5.1 for macOS, GraphPad Software, San Diego, California USA, <https://www.graphpad.com>.

3. Results

3.1. Consumption of *P. freudenreichii*-fermented milk attenuated the clinical signs of DSS-induced colitis.

The animal study is depicted in Fig. 1A. We first explored

Table 3
Primers used in this study.

Gene	Primer Sequence	Reference
<i>ACTB</i> *	Forward	CCTTCTTGGGTATGGAATCCTGT
	Reverse	CAGTGTGTTGGCATAGAGTCTTTAC
<i>GAPDH</i> *	Forward	TGACCTCAACTACATGGTCTACA
	Reverse	CTTCCCACTTCGCGCCTTG
<i>TUBA</i> *	Forward	TGTCTGGACAGGATTCGC
	Reverse	CTCCATCAGCAGGAGGTG
<i>PPIA</i> *	Forward	GGCAATGCTGGACCAAC
	Reverse	CATTCTGGACCAAAACG
<i>TNF</i>	Forward	CTGTAGCCACGTCGTAGC
	Reverse	TTGAGATCCATGCCGTG
<i>IL6</i>	Forward	GAGGATACCACTCCCAACAGACC
	Reverse	AAGTGATCATCGTTGTTTCATACA
<i>IL1B</i>	Forward	GCAACTGTTCTGAACTCAACT
	Reverse	ATCTTTTGGGGTCCGTCAACT
<i>NOS2</i>	Forward	CAGCTGGGCTGTACAAACCTT
	Reverse	CATTGGAAGTGAAGCGTTTCG
<i>PPARG</i>	Forward	CAGGCTTCCACTATGGAGTTC
	Reverse	GGCAGTTAAGATCACACCTATCA
<i>OCLN</i>	Forward	CAGCCTTCTGCTTCATCG
	Reverse	GTCCGGTTCACTCCCATTA
<i>CLDN1</i>	Forward	ATCCATAGGAAAGGCCCTTCAGCA
	Reverse	TACATGTAGGGCAACCAAGTGCCT
<i>CLDN5</i>	Forward	ACGGGAGGAGCGCTTTAC
	Reverse	GTTGGCGAACCAGCAGAG

* Reference genes.

macroscopic markers of DSS-induced colitis, in the presence or in the absence of the administration of the probiotic *P. freudenreichii* CIRM-BIA129 (Fig. 1A). This last was consumed either under the form of a fermented milk ultrafiltrate (DSS-UF129), of a fermented skim milk (DSS-SM129), or of a fermented whole milk (DSS-WM129). In addition, to evaluate the impact of the fermentation by CIRM-BIA129 versus that of the vehicle food matrix, mice were also treated with the unfermented dairy products (DSS-UF, DSS-SM and DSS-WM). As expected, addition of 2.5 % DSS to the drinking water led to clinical signs such as an increased disease activity index (DAI) score, taking into account weight loss, stool consistence and gross bleeding (Fig. 1B), as well as colon shortening (Fig. 1C and D). Consumption of the unfermented sterile dairy products tested here had no significant effect on the clinical signs of DSS-induced colitis. Indeed, DAI remains increased, in the context of colitis, whether mice consumed sterile UF, skim milk, or whole milk. Nevertheless, consumption of the *P. freudenreichii*-fermented dairy product alleviated the clinical signs of DSS-induced colitis, as indicated by a DAI score intermediate between the NC and the DSS, and no significant difference between the DSS-UF129, the DSS-WM129 groups, and the NC groups (Fig. 1B). In the same manner, colon shortening was not modified by the unfermented sterile dairy products tested, but significantly decreased in DSS-UF129 and DSS-SM129 groups when compare to the DSS group (Fig. 1C and D). These data show that consumption of *P. freudenreichii*-fermented dairy products reduced colitis and lowered colon damage.

3.2. Consumption of *P. freudenreichii*-fermented milk attenuated the histological symptoms of DSS-induced colitis.

At a microscopic scale, the histopathological analysis revealed a length-wide destruction of the colonic epithelium, with considerable modification of the mucosal architecture, loss of crypts, cellular infiltration and thickening of the muscular layer, as a result of colitis induction (DSS), when compared to healthy control intestinal tissue (NC) (Fig. 2). As a result of this destruction, the histopathological score (Fig. 3A), based on the mucosal architecture, the presence and degree of cellular infiltration, the extent of muscle and the presence or absence of goblet cells, was increased in the DSS group from 2.04 ± 0.85 in the control NC group to 6.68 ± 3.37 in the DSS group. Accordingly, the depth of the crypts (Fig. 3B) and the number of goblet cells per crypt (Fig. 3C) were significantly reduced as a result of DSS treatment. None of the unfermented dairy products was able to reduce this score significantly, although results indicate a trend towards a reduced score, when compared to the DSS group. However, crypts depth and density of goblet cells remained low. Considering the *P. freudenreichii*-fermented dairy products, only the consumption of the fermented whole milk (DSS-WM129 group) significantly restored a low score, down to 2.2 ± 1.41 (Fig. 2A and B). Furthermore, the crypt depth, which was dramatically reduced by DSS, was partially restored by the consumption of both fermented skim milk (DSS-SM129) and fermented whole milk (DSS-WM129) (Fig. 2C). The number of goblet cells, also drastically reduced in DSS-induced colitis group (9.05 ± 1.08), was partially prevented by the two fermented products SM129 and WM129 (11.04 ± 1.98 and 13.67 ± 2.3 , respectively), although only DSS-WM129 did not differ from the healthy control (14.22 ± 3.29) (Fig. 2D).

3.3. Consumption of *P. freudenreichii*-fermented milk attenuated the pathological increase in intestinal permeability.

We further investigated the permeability of the gut by monitoring the passage of radiolabelled Diethylenetriamine Pentaacetic Acid (DTPA) from the gut content into the blood stream. Gut permeability was drastically increased as a result of DSS treatment (Fig. 2E). Indeed, the DTPA fraction monitored in the blood was $0.33\% \pm 0.1$ in control NC mice, while it was $0.74\% \pm 0.18$ in the DSS one. Consumption of unfermented sterile dairy products resulted in a trend towards reduced gut permeability, especially with whole milk, but this trend failed to reach

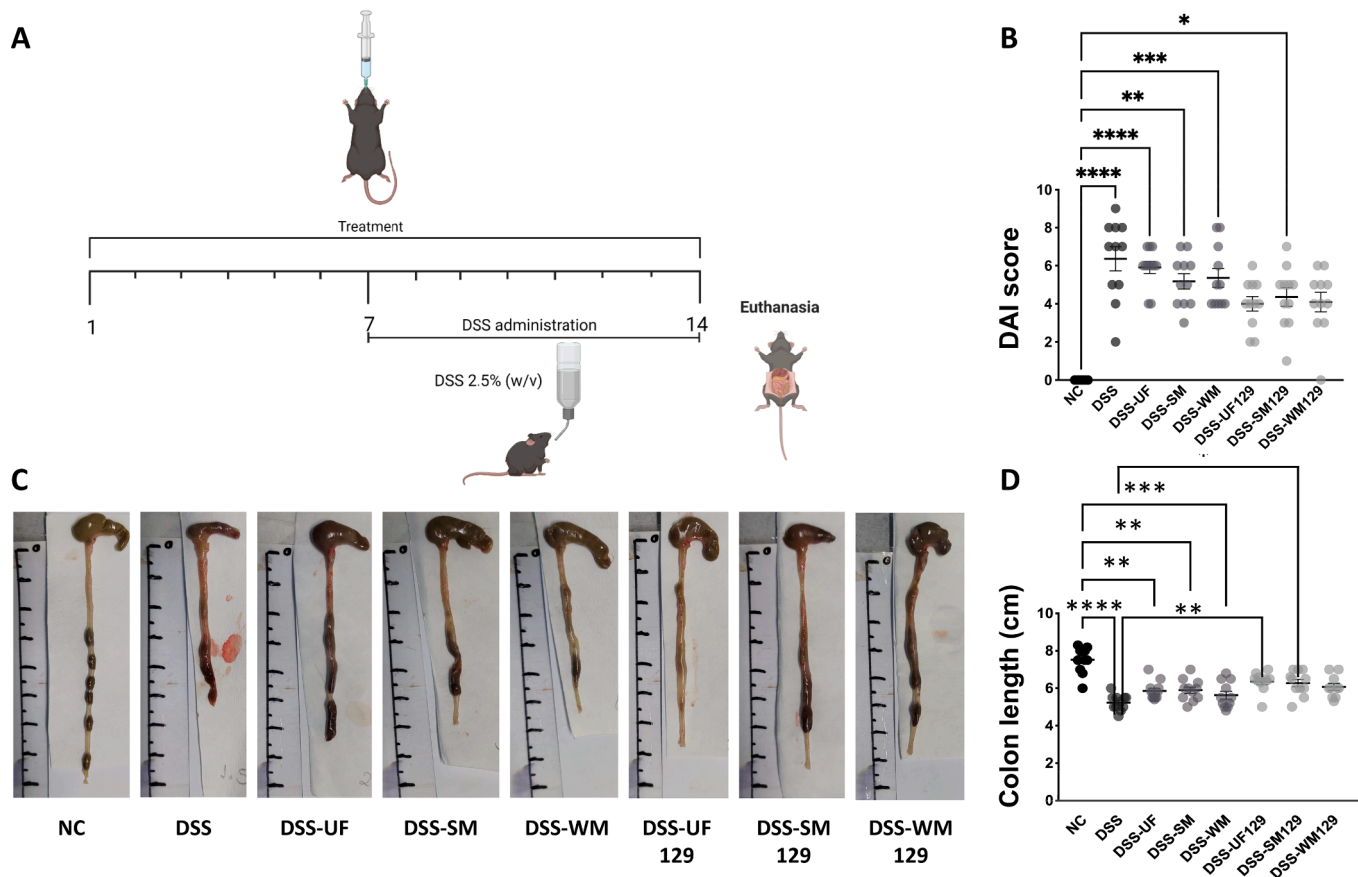


Fig. 1. *P. freudenreichii*-fermented milk alleviated DSS-induced experimental colitis in mice. (A) Design of the experimental protocol to investigate the effect of dairy matrices, fermented or not by *P. freudenreichii* CIRM-BIA129, in an experimental model of colitis in mice. Mice were gavaged during the 14 days of the experiment with 200 μ L of sterile phosphate buffered saline (PBS), sterile milk ultrafiltrate (UF), sterile skim milk (SM), sterile whole milk (WM), or with 200 μ L of *P. freudenreichii* CIRM-BIA129 culture in milk ultrafiltrate (UF129), in skim milk (SM129), or in whole milk (WM129). Mice had access to sterilised drinking water for negative control mice (NC) or to 2.5 % DSS in drinking water. (B) Disease activity index (DAI) was determined according to the 3 main clinical symptoms of colitis: diarrhoea, rectal bleeding, and weight loss. (C) Representative examples of colon morphology. (D) Colon size. Data represent mean $\pm$ SEM of 10 mice per group. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

statistical significance. Consumption of *P. freudenreichii*-fermented dairy products however significantly prevented this increase in intestinal permeability (Fig. 2E) mitigating the permeability back to basal levels. In particular, the fermented whole milk reduced this DTPA fraction to $0.25\% \pm 0.14$.

3.4. Consumption of *P. freudenreichii*-fermented milk attenuate DSS-induced gene expression changes in the colon.

The impact of DSS treatment, of unfermented dairy products, and of *P. freudenreichii*-fermented dairy products was also assessed with respect to gene expression in colonic tissue. As expected, DSS-induced colitis drastically modulated the expression of genes coding for immunomodulatory and barrier related proteins. Indeed, the barrier related genes *ocln*, *cldn1* and *cldn5* were up to 11-fold less expressed in the DSS group than in the NC group (Fig. 4). Likewise, the expression of the inflammatory genes *tnf* and *il1b* increased 37 and 52 times in the DSS group, respectively (Fig. 5). The expression of *il6* was also highly induced by DSS, but changes were not statistically significant against the NC group. We also found that the expression of *ppar γ* , coding for the peroxisome proliferator activated receptor gamma, was down-regulated by DSS, while the abundance of *nos2* coding for the nitric oxide synthase 2 was 80-fold higher in the DSS group than in the NC group. Interestingly, the consumption of fermented whole milk (DSS-WM-129) was able to prevent alterations of the expression of all these genes, except *tnf*. The other fermented preparations also appeared to exhibit similar effects, but in a

lesser extent than DSS-WM-129. Moreover, while DSS-WM-129 prevented the effects of DSS on the expressions of *ocln*, *nos2* and *il1b*, their expressions in the DSS-WM group were similar to those in the DSS group, showing the impact of the fermentation. Altogether, these results showed that DSS treatment resulted in dramatic changes in the expressions of many colonic epithelium genes, changes that were prevented by *P. freudenreichii* fermented preparations, notably fermented whole milk.

4. Discussion

The food matrix containing the probiotic *P. freudenreichii* has been shown to play a determinant role in its probiotic effects. When consumed in a fermented dairy product such as a fermented milk or a cheese, *P. freudenreichii* reaches higher live populations in the gut, when compared to isolated bacteria (Hervé et al., 2007; Rabah, Ferret-Bernard, et al., 2018), and affords protection towards induced colitis (Plé et al., 2015). The structure of the food matrix, resulting from the network involving proteins, lipids, and minerals, clearly plays a protective role towards the probiotic and its key surface molecules involved in the modulatory effects. In the current research work, we sought the respective role of dairy proteins and of dairy fat in the anti-colitis effect of *P. freudenreichii* CIRM-BIA129, a strain known for its immunomodulatory potential. This work indicates that dairy fat plays a beneficial role in the *in vivo* monitored anti-inflammatory effect of *P. freudenreichii*-fermented milk, giving new evidence for the need to consider the impact of the food delivery vehicle on the efficacy of probiotics further arguing

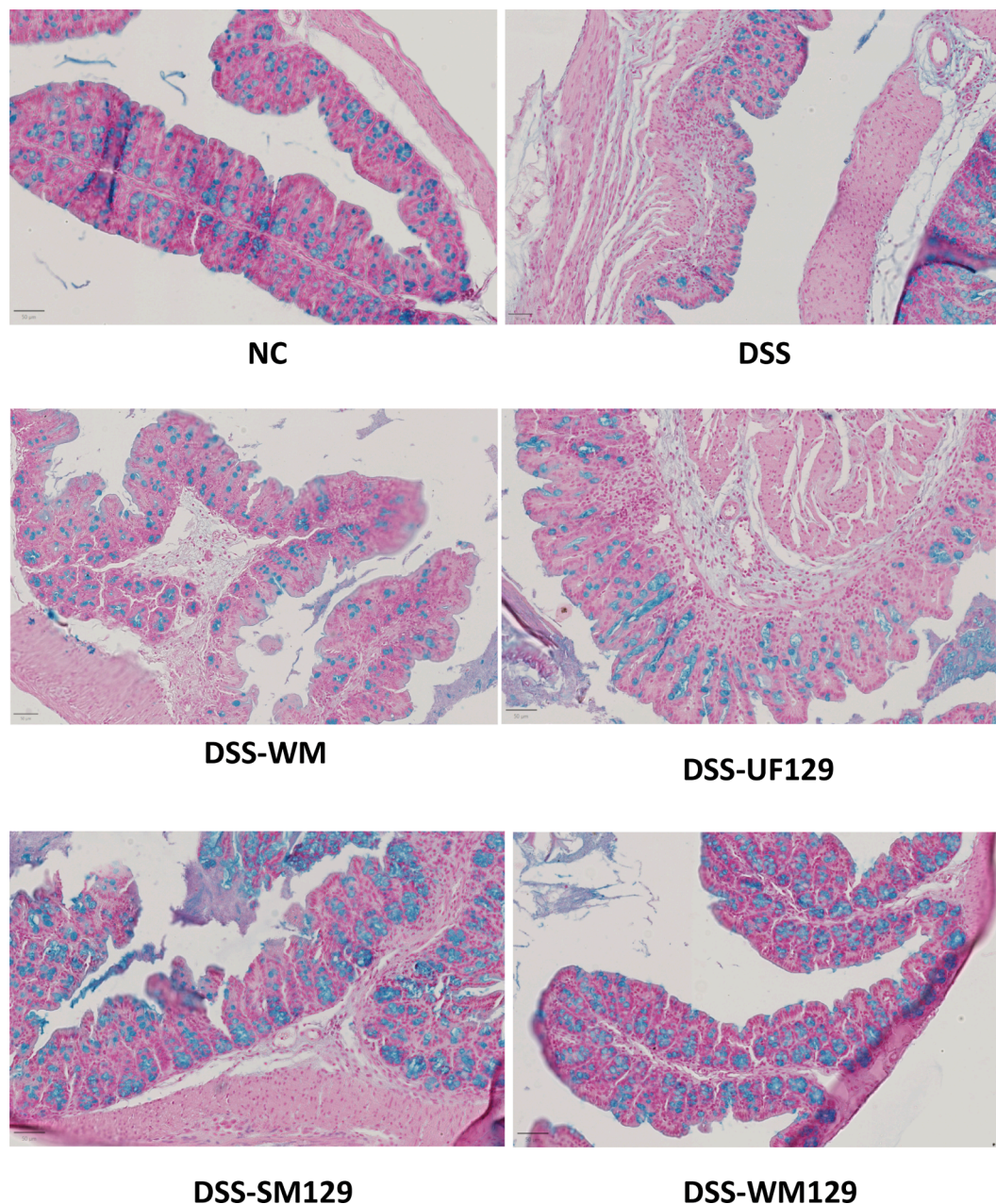


Fig. 2. *P. freudenreichii*-fermented milk alleviated DSS-induced colon histological damages. Representative images of Hematein Phloxin Safran (HPS) and Alcian Blue stained colon tissues (magnification 200 x) illustrate damages caused by DSS, when compared to control animals (NC). Damages were lower in mice consuming *P. freudenreichii*-fermented whole milk (DSS-WM129). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for a focus on how to improve the effectiveness (Lê et al., 2022).

Several strains of *P. freudenreichii* were already described as modulators of inflammation and of chemically induced colitis in mice models. A first study reported a promising protective effect of propionibacteria cultures in mice with TNBS-induced colitis (Foligné et al., 2010). In the same model, cheese containing *P. freudenreichii*, either as a sole bacterium (Plé et al., 2015), or in conjunction with selected immunomodulatory strains of the dairy starters *S. thermophilus* and *L. delbrueckii*, also afforded protection towards TNBS-induced colitis (Plé et al., 2016). Such a cheese furthermore protected mice from DSS-induced colitis (Rabah, 2020). In these studies, consumption of *P. freudenreichii* limited colon shortening and thickening, reduced disease activity index, histopathological score, colonic myeloperoxidase (MPO) activity, as well as pathological increase of gut permeability. In the research work

presented here, consumption of milk fermented by *P. freudenreichii* CIRM-BIA129 was further shown to exert a preventive effect towards colitis induced by DSS. As a macroscopic indication, the shortening of the colon length was prevented by this consumption, whatever the food matrix. However, the disease activity index, which is increased during colitis, was reduced by the consumption of *P. freudenreichii*-fermented milk, yet not of fermented ultrafiltrate, which consists in the aqueous phase of milk, devoid of fat and of proteins. Furthermore, as a microscopic indication, the histopathological score was reduced as a result of fermented whole milk consumption, while the other fermented products failed to do so. Hence, the food matrix components, including dairy fat, play a role in the protective effect of *P. freudenreichii*-fermented products.

In our study, consumption of *P. freudenreichii*-fermented milk

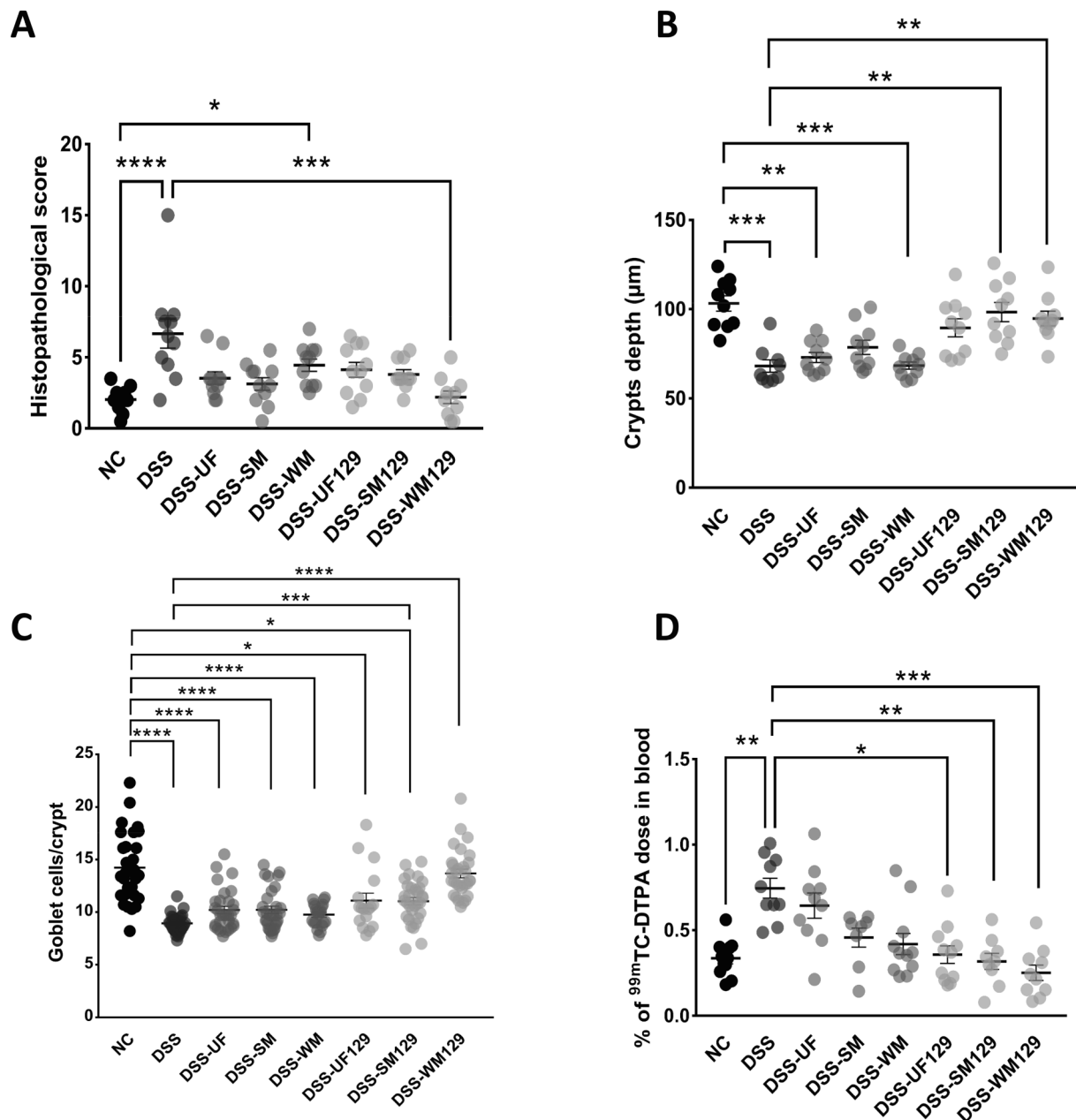


Fig. 3. *P. freudenreichii*-fermented milk alleviated DSS-induced colon histopathological injury and increased permeability. (A) Histological score were evaluated by quantifying the destruction of mucosal architecture, cellular infiltration, muscle thickening and loss of goblet cells. (B) The depth of colon crypts was measured for 50 crypts per animal. The mean value was used for comparison between groups. (C) Goblet cells were measured as the ratio goblet cells/crypt, 50 crypts per animal. The average was used for comparison between groups. (D) On the last day, mice were gavaged with a solution of radiolabelled ^{99m}Tc-DTPA. After 4 h, the animals were anesthetized and radioactivity determined in the blood. Data represent mean $\pm$ SEM of 10 mice per group. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

prevented the pathological increase of gut permeability upon colitis induction by DSS. Indeed, passage of the radiolabelled DTPA to the blood stream, which was drastically increased by DSS-induced colitis, was limited by the consumption of all the *P. freudenreichii*-fermented products. As key actors of gut integrity, intestinal epithelial cells, under the mucus layer, are tightly bound by tight junctions (TJ). TJ proteins, including ZO-1, occludin and claudin, are main actors of the integrity of the gut epithelial barrier and play a crucial role in intestinal homeostasis and tightness. Indeed, reduced expression of TJ proteins coincides with permeability increase in a leaky gut and has been described in animal models of colitis (Camilleri, 2019). We thus investigated the expression of genes encoding TJ proteins. Indeed, expression of the genes encoding occluding, claudin-5 and claudin-1 were repressed in DSS-treated mice

in the present work, which is in line with the observed increased gut permeability. By contrast, consumption of *P. freudenreichii*-fermented whole milk restored expression of genes encoding TJ proteins. This is consistent with the concomitant restoration of intestinal integrity as shown by DTPA monitoring. Damages of the intestinal epithelial TJ result in abnormal increase of gut permeability. Pro-inflammatory metabolites of the gut microbiota, including lipopolysaccharide, in turn translocate through the intestinal barrier and elicit an inflammatory response. This last in turn exacerbates gut barrier defects in a vicious circle. Identifying food components able to restore gut barrier integrity thus constitutes a major quest. Protection of the gut barrier integrity, in the context of DSS-induced colitis, was already reported for probiotic bacteria such as *Limosilactobacillus reuteri* (H. L. (Lee et al., 2022);

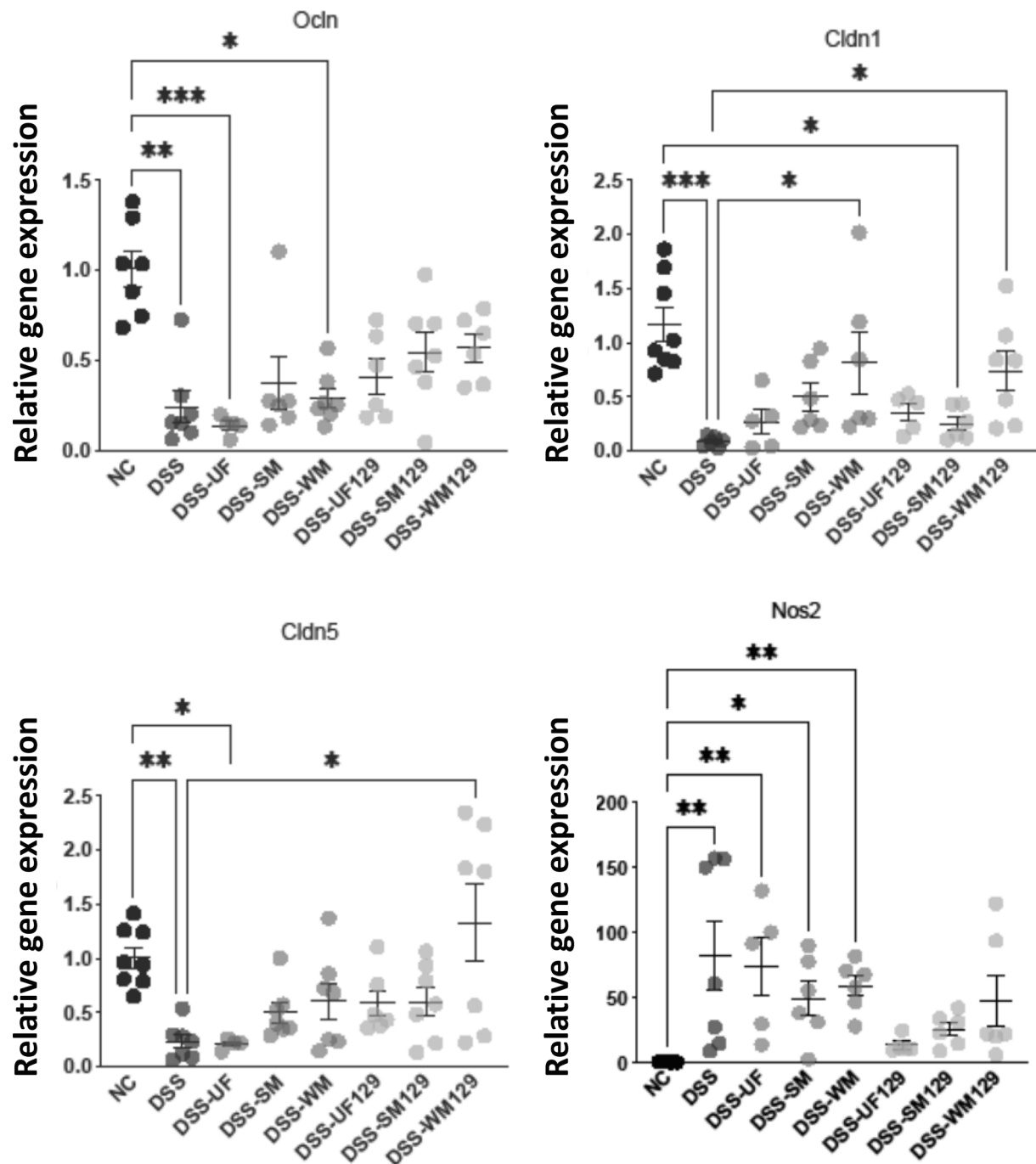


Fig. 4. *P. freudenreichii*-fermented milk modulated expression of key genes in the colon. The expression of key genes was monitored in colon extracts. Data are presented for genes Ocln, Cldn1, Cldn5, Nos2, Il1b, Il6, Tnf and Pparg. Relative expression was determined using Actb, Gapdh and Tuba as reference normalisation genes. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Lacticaseibacillus casei (Zakostelska et al., 2011); *Limosilactobacillus fermentum* (Kaur et al., 2022); *Bifidobacterium breve* (Niu et al., 2022); *B. bifidum* (Shang et al., 2022) and *B. longum* (Y. (Chen et al., 2021)). In these reports, the probiotic bacteria were also shown to restore the expression of tight junction protein genes. Such an effect was evidenced here as a result of *P. freudenreichii*-fermented products. Moreover, we show here that milk components, and in particular dairy fat, potentiate this protective effect. Indeed, the amount of propionibacteria was the same, i.e. 10^9 CFU g.L⁻¹, so that the differential effect of fermented products cannot be attributed to differential population of propionibacteria.

In the different animal studies dealing with probiotics and cited

above, restoration of the gut barrier integrity was intimately linked to the ability of the probiotic bacterium to modulate inflammation. The inflammatory response includes the induction of the production of pro-inflammatory cytokines, which in turn contribute to the progression of colitis. Several probiotic bacteria, including dairy starters, were reported to mitigate the inflammatory response by preventing such inductions (Illikoud et al., 2022) and by repressing the TLR4-NF- κ B pathway (Y. (Chen et al., 2021)). In our study, DSS upregulated the pro-inflammatory cytokine IL-1 β , indicating the induction of gut inflammation. IL-1 β is a key factor in the pathogenesis of colitis and its upregulation is associated with ulcerative colitis (Bergemalm et al., 2021). Such induction exacerbates gut inflammation through the dysregulation

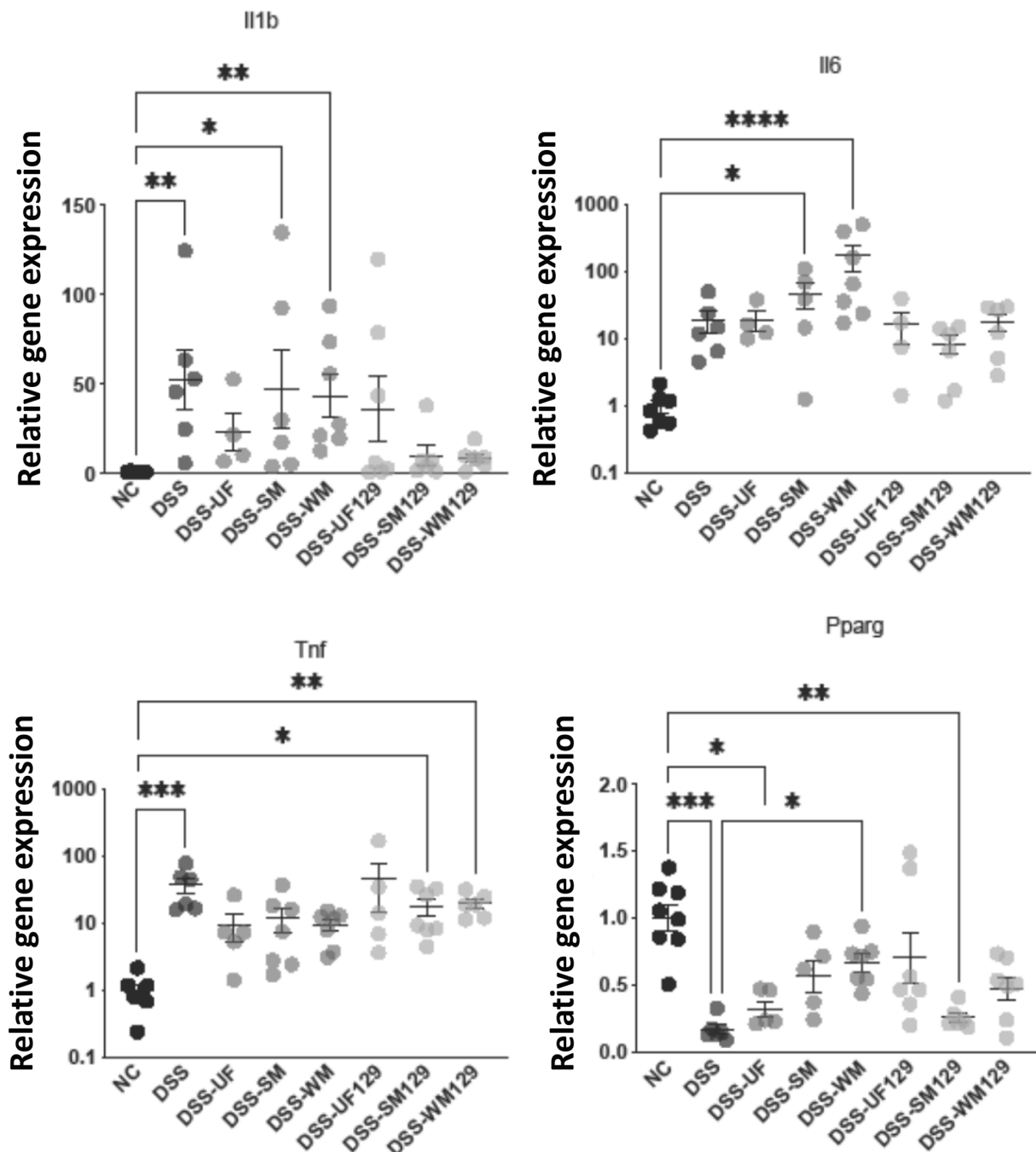


Fig. 5. *P. freudenreichii*-fermented milk modulated expression of key genes related to inflammation in the colon. The expression of key genes was monitored in colon extracts. Data are presented for genes Ocln, Cldn1, Cld5, Nos2, Il1b, Il6, Tnf and Pparg. Relative expression was determined using Actb, Gapdh and Tuba as reference normalisation genes. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.

of macrophage polarization. However, consumption of *P. freudenreichii*-fermented milk prevented this IL-1 β induction, in line with previous probiotic studies. Immunomodulation was accordingly reported in *P. freudenreichii*, including an ability to induce IL-10 production in human immune cells (PBMC), and to prevent induction of pro-inflammatory cytokines *in vivo* in the context of colitis (Foligné et al., 2010) (Plé et al., 2015). Our study further demonstrates that an appropriate food delivery vehicle is necessary for this potential to give rise to protection against colitis. Indeed, protection was significant here only when *P. freudenreichii* was provided within a fermented milk, yet not in a UF culture. Accordingly, fermented whole milk afforded protection, while

unfermented whole milk failed to do so.

Altogether, our results indicate that milk constituents, including dairy proteins and fat, potentiate the anti-colitis effects of the probiotic *P. freudenreichii*. Interestingly, consumption of unfermented whole milk also restored a gut permeability similar to that of healthy control mice, while being less anti-inflammatory than the fermented whole milk. In line with this, a previous report evidenced that the probiotic *L. casei* BL23, when provided within a fermented milk, alleviated symptoms of colitis in mice, while it failed to do so when provided in a nutrient-free buffer (B, (Lee et al., 2015). This raises the question of the potentiating effect of milk constituents in fermented milk anti-colitis effects.

Interestingly, milk phospholipids were shown to alleviate DSS-induced colitis and to prevent goblet cell depletion (Wang et al., 2019). The protective effect of milk polar lipids was then shown to be more pronounced if mice received a western high-fat diet (Garcia et al., 2022). Milk fat globule membranes (MFGM) were shown to maintain the gut mucosal barrier, to inhibit oxidative stress and to attenuate colitis and hepatic injury in DSS-treated mice (Wu et al., 2022). Milk extracellular vesicles (mEVs) are bioactive constituents of milk and play a role in maintaining intestinal health. Their consumption alleviates the symptoms of DSS-induced acute colitis and limits the induction of pro-inflammatory cytokines (Du, Wang, et al., 2022). Furthermore, two subsets of mEVs contribute differently to colitis healing, one by modulating innate immunity and the other by decreasing inflammation (Benmoussa et al., 2019). This protective effect of mEVs was further shown to involve modulation of intestinal gene expression and of the gut microbiota in favour of bifidobacteria (Du, Wang, et al., 2022). Exosomes deriving from both bovine and human milk were further shown to mitigate DSS-induced colitis, to reduce expression of pro-inflammatory cytokines and to provide miRNAs involved in gene expression regulation (Reif et al., 2020). Milk fat globule-EGF factor 8 (MFG-E8) is known to play a role in maintaining the integrity of the gut mucosa. Accordingly, administration of this protein alleviates colitis and promotes mucosal repair (Chogle et al., 2011). Feeding mice with casein was further shown to facilitate recovery from DSS-induced colitis (Yu et al., 2021). Dietary intervention revealed that consumption of milk proteins limited the colon shortening and the IL-1 β levels, while increasing the gut concentration of bifidobacteria, in DSS-induced colitis (Ma et al., 2021). Indeed, several probiotics revealed an anti-colitis effect when provided within a fermented milk, an effect which may be stronger than when the probiotic is consumed without a food matrix (Lee et al., 2015). As a possible explanation, immunomodulation often relies on key surface proteins or glycoproteins acting as MAMPs (microbial associated molecular pattern) and such MAMPs are susceptible to proteolysis during digestion. The dairy matrix protects such molecules from proteolysis, which may enhance their probability to interact with the host immune response. Concerning *P. freudenreichii*, its anti-colitis effect was accordingly particularly strong, in a previous study, when using Emmental cheese as food matrix delivery vehicle, with a significant effect on macroscopic, histological and immunological markers (Rabah, 2020). This cheese, as a Swiss-type (pressed cooked) cheese, is particularly rich in dairy proteins and lipids. Thus, different dairy matrices, with different structures, may potentiate the effects of probiotic bacteria. A special attention should thus be paid to protein and lipid constituent of the food matrix, when developing innovative fermented functional foods.

5. Conclusion

As a conclusion, this work demonstrates the key role of milk, including milk fat, in potentiating the anti-colitis potential of *P. freudenreichii*. This opens new perspectives for the development of fermented functional foods, which should consider the presence of the different components of milk, as well as the food structure, in addition to bacterial fermentation.

Ethics statement

The Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFGM, Brazil) approved all experimental procedures realized in this work by the protocol no. 148/2020.

CRediT authorship contribution statement

Marine Mantel: Investigation. **Tales da Silva:** Investigation. **Rafael Gloria:** Investigation. **Danièle Vassaux:** Investigation. **Kátia Duarte Vital:** Investigation. **Valbert Nascimento Cardoso:** Investigation. **Simone Odília Antunes Fernandes:** Investigation. **Éric Guédon:** Data curation. **Yves Le Loir:**

Supervision. **Ana Maria Caetano Faria:** Supervision. **Malvyn Rolli-Derkindere:** Supervision. **Vasco Azevedo:** Supervision. **Gwénaél Jan:** Conceptualization, Formal analysis, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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