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Development of innovative fermented products by exploiting the diversity of immunomodulatory properties and fermentative activity of lactic and propionic acid bacteria

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ABSTRACT

Many consumers nowadays demand plant-based milk analogs for reasons related to lifestyle, health, diet and sustainability. This has led to the increasing development of new products, fermented or not. The objective of the present study was to develop a plant-based fermented product (based on soy milk analog or on hemp milk analog), as well as mixes, using lactic acid bacteria (LAB) and propionic acid bacteria (PAB) strains, as well as consortia thereof. We screened a collection of 104 strains, from nine LAB species and two PAB species, based on their ability to ferment plant or milk carbohydrates, to acidify goat milk, soy milk analog and hemp milk analog, as well as to hydrolyze proteins isolated from these three products. Strains were also screened for their immunomodulatory ability to induce secretion of two interleukins, *i.e.*, IL-10 and IL-12, in human Peripheral Blood Mononuclear Cells. We selected five strains: *Lactobacillus delbrueckii* subsp. *lactis* Bioprox1585, *Lactobacillus acidophilus* Bioprox6307, *Lactococcus lactis* Bioprox7116, *Streptococcus thermophilus* CIRM-BIA251, and *Acidipropionibacterium acidipropionici* CIRM-BIA2003. We then assembled them in 26 different bacterial consortia. Goat milk and soy milk analog fermented by each of the five strains or by the 26 consortia were tested *in vitro*, for their ability to modulate inflammation in cultured Human Epithelial Intestinal Cells (HEIC) stimulated by pro-inflammatory Lipopolysaccharides (LPS) from *Escherichia coli*. Plant-based milk analogs, fermented by one consortium composed of *L. delbrueckii* subsp. *lactis* Bioprox1585, *Lc. lactis* Bioprox7116, and *A. acidipropionici* CIRM-BIA2003, reduced the secretion of the proinflammatory cytokine IL-8 in HIECs. Such innovative fermented vegetable products thus open perspectives as functional foods targeting gut inflammation.

1. Introduction

There is a growing awareness of Western consumers to re-balance their diet by decreasing the animal part in favor of plant-based alternatives, entirely or partially, to enlarge the food offer, as recommended by the EAT-Lancet report (Willett et al., 2019). This is in tune with changes in lifestyle, and greater consideration of animal welfare, minimal processing and sustainability (Harper et al., 2022; Henn et al., 2022). This is also related to a seek for a healthy diet decreasing risk of lactose intolerance, of allergy to animal proteins such as milk, egg and shellfishes, or alleviating irritable bowel syndrome, inflammatory bowel diseases and other civilization-related concerns including diabetes, metabolic syndrome and cardiovascular diseases) (Gwee, 2005; Harper et al., 2022; Kopp, 2019). It should be noted that plant-based proteins sources such as soy may also, however, be the target of allergy, although less frequently than egg and cow's milk proteins (Nwaru et al., 2014).

Diversification of food resources is therefore sought, as well as the development of new plant-based products, of mixes, including animal (dairy)-plant or plant-plant. While it is possible to standardize the milk composition, plant composition is highly variable in terms of protein, carbohydrate and lipid content (Craig & Fresán, 2021; Grasso et al., 2020). Plant may contain deleterious components such as alkaloids, phytate, tryptic inhibitors and carbohydrates responsible for bloating, or may develop greeny flavor not really appreciated by consumers (Guillon & Champ, 2002; Harper et al., 2022). Plant products may also be part of the list of allergens. A mild transformation of these new food resources,

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leading to desired final qualities, *i.e.* nutritional, organoleptic and health-benefits, may rely on fermentation, one of the most convenient processes that can be used. Most of the raw materials edible for food, under a solid or liquid form, may indeed be fermented by numerous microorganisms. This leads to a huge diversity of fermented food products worldwide (Tamang et al., 2016, 2020). Relevant plant resources include cereals (barley, rice, wheat, oat, rye, sorghum, millet), legumes (soy, bean, pea, faba bean, lupin, lentils) as well as other vegetables (cabbage, small cucumber, carrot, beet, onions, cassava, manioc).

Among used microorganisms, lactic acid bacteria (LAB) are highly prone to transform various carbohydrates into acids, in a wide range of animal and plant-based fermented products, such as yogurt, cheese, salami, sauerkraut, and kimchi (Tamang et al., 2016). They indeed produce various enzymes, including β-galactosidases, α-galactosidases and α-glucosidases, able to hydrolyze notably lactose present in milk, raffino-oligosaccharides in legumes, and sucrose in numerous vegetables or fruits, respectively (Gänzle & Follador, 2012; Teuber, 1995). Fermentation of these carbohydrates, and the resulting acidification, also confers an increased shelf life to the fermented products by limiting the growth of pathogen and spoilage microorganisms. LAB furthermore provide enzymes involved in the production of peptides, amino acids and volatile compounds that are essential for the final texture and flavor of the fermented products (Liu et al., 2010; Rodriguez-Serrano et al., 2018; Thierry et al., 2015).

Propionic acid bacteria (PAB) were initially isolated from Swiss-type cheeses, in which they contribute to the specific aroma, texture, and opening. They are responsible for the nutty and sweet flavors of the cheese, and the carbon dioxide by-product leads to the opening of the eyes in Swiss-type cheeses (Thierry et al., 2011).They also produce flavor compounds via the degradation of amino acids (Thierry et al., 2004) and the lipolysis of triglycerides (Abeijón Mukdsi et al., 2014). Propionic fermentation in cheese uses lactate as a preferred carbon source, to produce acetate and propionate. However, carbohydrates (including lactose and esculin), as well as polyols (including glycerol and erythritol) may also be used to sustain propionic fermentation. As lactose users, strains of PAB express β-galactosidase activity, while α-galactosidase activity is not documented in these bacteria. They are found in milk and in some cheeses, as well as in other fermented products such as silage (Thierry et al., 2011). Their complex metabolism relies on oxidation of substrates into pyruvate, followed by reduction of pyruvate via the Wood-Werkman cycle, which involves biotin- and cobalamin-dependent enzymes. Fermentation of foods by PAB is known to increase shelf life via the production of bacteriocins and short chain fatty acids. It is also reported to enhance nutritional value of food via the release of B vitamins (including cobalamin and folic acid) (Rabah et al., 2017).

In addition to enhanced organoleptic and nutritional properties of foods, it is generally accepted that fermentation contributes to maintaining human health, via specific probiotic effects. The most recognized probiotic effect is most probably the alleviation of lactose intolerance symptoms in humans via the consumption of LAB starters contained in yogurt (Deng et al., 2015). Consumption of yogurt is furthermore associated with reduction in adiposity factors including body mass index and waist circumference (Cormier et al., 2016), type 2 diabetes mellitus (Guo et al., 2019) and cardiovascular diseases (Fernandez et al., 2017). Moreover, randomized control trials also investigated the effects of kefir (Pražnikar et al., 2020), kimchi (Kim & Park, 2018), sauerkraut (Nielsen et al., 2018), natto (Araki et al., 2020), vinegar (Zhu et al., 2019) and sourdough bread (Marco et al., 2021; Rizzello et al., 2019). Concerning the mechanistic basis such of beneficial effects, the microbial activity during food fermentation may reduce the concentration of high-calorie carbohydrates, improve carbohydrate tolerance, increase digestion of carbohydrates and of proteins, reduce concentration of toxics and of anti-nutritive factors, increase bioavailability of bioactive compounds, vitamins, amino acids, organic acids and cofactors (Marco et al., 2021). Bioactive compounds may include flavonoids, tannins, ɣ-aminobutyrate, conjugated linoleic acid or angiotensin-converting enzymes inhibitors. Moreover, 70% of the human immune system is located close to the gastro-intestinal tract and bacteria found in fermented foods are likely to play an immunomodulatory role. This is in line with the effect of fermented foods on reduced risk of childhood allergies (Alm et al., 1999; Nicklaus et al., 2019).

Usually, bacteria starters are selected on the basis of their ability to ferment raw materials. In contrast, their probiotic properties are barely known. In a context of food quality and health improvement, there is a need to combine the different aspects of bacteria potential, and to select them according to their technological and probiotic properties. However, these criteria are mostly strain-dependent. The objective of the present study was to develop a specific strategy to select and associate LAB and PAB within consortia which would be able at the same time to ferment plant-based milk analogs which are appreciated as alternative sources of proteins and other nutrients (Paul et al., 2020) and to have anti-inflammatory properties. Mixes of goat milk and plant-based milk analogs were also considered.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of 104 strains from 9 lactic acid bacteria (LAB) and 2 propionic acid bacteria (PAB) species were included in this study. They were provided by the International Centre for Microbial Resources-Food Associated Bacteria (CIRM BIA, Centre International de Resources Microbiennes, bactéries d'intérêt alimentaire, Rennes, France, https://collection-cirmbia.fr/) (N = 71) and by Bioprox (Bioprox Pure Culture, Noyant, France) (N = 33). The strains were 12 *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum* as reclassified by Zheng et al., (2020)), 12 *Lactobacillus helveticus*, 11 *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus)*, 10 *Lactobacillus delbrueckii* subsp. *lactis*, 10 *Streptococcus thermophilus*, 10 *Propionibacterium freudenreichii*, 10 *Acidipropionibacterium acidipropionici*, 9 *Lactococcus lactis* subsp. *lactis*, 8 *L. delbrueckii* subsp. *bulgaricus*, 8 *Limosilactobacillus fermentum* (formerly *Lactobacillus fermentum*), 3 *Lactobacillus acidophilus*, and 1 *Lactobacillus johnsonii*. In addition, *L. acidophilus* NCFM (Gilliland et al., 1975), *L. lactis* subsp. *cremoris* MG1363, *Lc. lactis* subsp. *lactis* NCDO2118, *L. rhamnosus* GG (ATCC 53103), *Bifidobacterium longum* Bb536, *Lacticaseibacillus casei* (formerly *Lactobacillus casei*) CIRM-BIA667, *Lactiplantibacillus pentosus* (formerly *Lactobacillus pentosus*) CIRM-BIA660, and *P. freudenreichii* CIRM-BIA129 were used as controls for various purposes.

LAB and PAB strains were first reactivated from frozen (−80 °C) glycerol stocks, then routinely cultivated in a laboratory broth medium for 24 h and 48 h, respectively. They were inoculated at 1% v/v in Yeast Extract Lactate (YEL, made as described by Malik et al., (1968)) medium for propionibacteria, in M17-glucose broth (Difco™, Becton Dickinson, MD, USA) for *Streptococcus* and *Lactococcus* strains and in Man, Rogosa and Sharpe (MRS, Difco™) broth for *Lactobacillus* strains. Cultures were incubated at 30 °C or 43 °C according to the species affiliation (see Supplementary Table S1). *B. longum* Bb536 was precultured in MRS added with 0.5 g/L cysteine at 37 °C for 48 h under anaerobiosis using anaerobiosis generators (ATCO Biocult, ATCO, Bretteville sur Odon, France) in the presence of an oxygen indicator (Anaerotest®, Merck, Germany).

For this study, two subcultures of the microbial strains were performed on appropriate laboratory culture media, prior to their cultivation on cow milk ultrafiltrate (MUF), goat milk, hemp milk analog or soy milk analog. MUF was prepared by an ultrafiltration process on the dedicated STLO dairy technology platform as previously described (Cousin et al., 2012; Michalski et al., 2006). Briefly, raw cow milk was skimmed using a GEA Westfalia separator (Chateau-Thierry, France). Skimmed milk was ultrafiltered at 25 °C through tubular ceramic membranes (total membrane area of 2 × 0.36 m^2; molecular weight cut-

off 8 kDa, Membralox, EP1960, Pall, Bazet, France) set on a TIA/Pall 1.08 m pilot (Bollène, France). The collected MUF was supplemented with 5 g/L of casein hydrolysate (Organo-technie, La Courneuve, France) as nitrogen source for the growth of LAB strains and additionally supplemented with 50 mM of sodium L-lactate (galaflow SL60, Société Arnaud, Paris, France) for the cultivation of PAB strains. MUF media were sterilized by 0.2 μm filtration (Nalgene, Roskilde, Denmark) and stored at 4 °C. The overall composition of MUF was previously described by Tarnaud et al. (2020). It was as follows: carbohydrate 5% (w/w); non-protein nitrogen 0.28% (w/w), minerals 0.75% (w/w) and dry matter 6.14% (w/w).

UHT skimmed goat milk (Lactel, Vitré, France) was purchased from a local supermarket. UHT soy milk analog and hemp milk analog were provided by a local company (Sojasun Technologies Triballat Noyal, France).

LAB and PAB populations were quantified by colony forming unit (CFU) counting (serial dilutions and plating), in each of food-based culture media cited above. PAB strains were enumerated on YEL-agar (Malik et al., 1968) after 6 days of anaerobic incubation at 30 °C using anaerobiosis generators (ATCO Biocult) in the presence of an oxygen indicator (Anaerotest®, Merck). *Lactobacillus* strains were enumerated on MRS-agar (Difco^{TM}) after two days of anaerobic incubation (ATCO Biocult) at 30 °C or 43 °C. *Lactococcus* and *Streptococcus* strains were enumerated on M17-glucose-agar (Difco^{TM}) after two days of incubation, without anaerobiosis, at 30 °C or 43 °C for *Lactococcus* and *Streptococcus* strains, respectively.

2.2. Carbohydrate utilization assay

Bacterial cultures, performed in appropriate broth media, were centrifuged (8,000 × *g*, 10 min, 20 °C) and resuspended either in sterile API 50 CHL medium (API systems, BioMérieux, Marcy-l'Étoile, France) for LAB strains or in sterile API 50 CHP, an adapted medium for the PAB strains. The composition of CHP was, per liter: tryptone 5 g (Biokar Diagnostics, Beauvais, France), Yeast extract 2.5 g (Biokar Diagnostics), K_2HPO_4 0.328 g, $MnSO_4$ 0.056 g, bromocresol purple 0.15 g, pH 7. These bacterial suspensions, after vortex mixing, were transferred into each of the 50 wells on the API 50 CH strips according to the supplier instructions. The ability to hydrolyze stachyose was additionally tested, under the same conditions, in API 50 CHL or API 50 CHP media supplemented with 6 g/L of stachyose (Sigma Aldrich, Saint Quentin Fallavier, France), for LAB and PAB strains, respectively. This was done for all isolates and reference strains. The API strips were incubated at the various temperatures indicated above, depending on the species. Results were determined after 24 h (LAB) or 48 h (PAB) of incubation and confirmed after 48 h (LAB) or 96 h (PAB) of incubation.

2.3. Protein hydrolysis

Bacterial cells were harvested from precultures in appropriate broth media in triplicate (8,000 × *g*, 10 min, 20 °C), then resuspended at 10 % (v/v) in a modified API 50 CHL and API 50 CHP media for LAB and PAB strains, respectively.

API 50 CHL and API 50 CHP were supplemented with glucose 6 g/L used as the sole carbon source, the yeast extract was diminished to 0.2 g/L to limit the supply in nitrogen compounds that were supplied by 5 g/L of either homemade goat caseinate, and hemp protein isolate (see the preparation of both protein isolates in the paragraph below) or commercial soy protein isolate purchased from https://www.bulk.com/fr/, or tryptone 5 g/L (BIOKAR Diagnostics, Beauvais, France). This latter was used as a positive control of bacterial growth. The sterile modified API 50 CHL and API 50 CHP media were used as a control to estimate the changes in the nitrogen compounds, peptides and free amino acids released by protein hydrolysis during the incubation period. LAB and PAB cells were incubated in these media at the optimal growth temperature for 48 h and 96 h, respectively. All isolates and reference strains were tested.

The changes in the amount of nitrogen compounds, present in each medium, were measured in triplicates using the o-phthalaldehyde (OPA) method described by Church et al. (1983) and adapted to microplate. The proteins were first precipitated by half-diluting samples with 2% (w/w) trichloroacetic acid final concentration. This allows the free NH_2 groups present at the N-terminal extremity of the peptides and amino acids to be preferentially detected by the OPA. The ability of one strain to hydrolyze the proteins in the medium was determined by the ΔNH_2. ΔNH_2 = Conc. eq Met (sample) - Conc. eq Met (non-inoculated control). The results were expressed as mM equivalent Methionine, which was used as a standard for the calibration curve. Positive values mean that strains released nitrogen compounds by proteolysis, and negative values mean that strains consumed nitrogen compounds initially present and/or produced in the medium.

2.4. Preparation of goat caseinate and hemp protein isolates for protein hydrolysis assays

Goat caseinate and hemp protein isolates were prepared by isoelectric precipitation from full-fat goat milk powder (Biocoop, Rennes, France) and from concentrated hemp milk analog provided by a local company (Sojasun Technologies Triballat Noyal, France), respectively. The goat milk powder was first resuspended at 10 % (w/v) in reverse-osmosis purified water and heated at 60 °C for 2 h under stirring and cooled down to 20 °C prior to casein precipitation to pH 4.6 using HCl 1 M. Precipitated caseins were recovered by centrifugation (3,500 × *g*, 20 min, 4°C). After two washes with pH 4.6 reverse-osmosis purified water, the precipitates were solubilized in pH 7.5 reverse-osmosis purified water overnight at 4 °C, freeze-dried, and stored at 4 °C. The concentrated hemp milk analog was first adjusted to pH 10 with NaOH 5 M and stirred 2 h at 35 °C to improve protein solubilization. Residual fat and insoluble materials were removed by centrifugation (8,500 × *g*, 20 min, 20 °C). The hemp proteins contained in the supernatant were precipitated at pH 4.6 using HCl 1 M. The following steps were the same as those used for the caseinate preparation.

2.5. PBMCs stimulation

First, a standardized bacterial stock in terms of biomass (1.10^9 CFU/mL) was constituted as previously described by Deutsch et al., (2017). Briefly, LAB and PAB were harvested from MUF cultures by centrifugation (12,000 × *g*, 5 min, 4 °C), washed twice with phosphate buffer salt (PBS), and resuspended in PBS containing 20 % glycerol. These standardized bacterial preparations were stored at − 80 °C until further use.

Human peripheral blood mononuclear cells (PBMC) were purchased from Stemcell Technologies (Vancouver, BC, Canada). There were isolated from blood of a single healthy donor. The criteria were as follows: donor from 25 to 50 years old, not obese (body mass index between 20 and 30), with no inflammatory diseases and non-smoker. Human PBMCs were first washed in RPMI 1640 medium (Roswell Park Memorial Institute medium, Stemcell technologies, Vancouver, BC, Canada) and adjusted to 1×10^6 cells/mL in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 % fetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany). PBMC (1×10^6 cells/mL) were seeded in 24-well tissue culture plates (Corning, NY, US). Ten microliters of the thawed bacterial suspensions, prepared as described above were added. This resulted in a bacteria-to-cell ratio of 10:1. Two controls were included: *B. longum* Bb 536 as a positive IL-10-inducing positive control and PBS containing 20 % glycerol as a negative (non-stimulating) control. All controls and samples were tested in triplicate. After 24 h of coculture at 37 °C in air with 5 % CO_2, culture supernatants were collected and clarified by centrifugation (800 × *g*, 8 min, 20 °C). The supernatant was collected and transferred to 96-well plates containing 5 μL/well of protease inhibitor (Sigma-Fast). The 96-

A

	Number of strains	Lactose +	Sucrose +	Raffinose +	Stachyose +	lac-, suc-, raf-, sta-	lac+, suc+, raf+, sta+
P. freudenreichii	10	8	2	1	0	2	0
A. acidipropionici	10	10	10	9	7	0	6
L. delbrueckii subsp. *bulgaricus*	8	8	0	1	1	0	0
L. delbrueckii subsp. *lactis*	10	10	8	2	2	0	2
L. plantarum	12	12	12	12	5	0	6
L. rhamnosus	11	7	5	1	0	4	0
S. thermophilus	10	9	10	0	0	0	0
L. fermentum	8	7	8	7	2	0	1
L. helveticus	12	10	1	0	0	1	0
L. acidophilus / L. johnsonii	4	3	3	3	3	1	3
Lc. Lactis	9	9	7	0	0	0	0
Total number	104	93	66	36	20	8	18
%	100.0	89.4	63.5	34.6	19.2	7.7	17.3

B

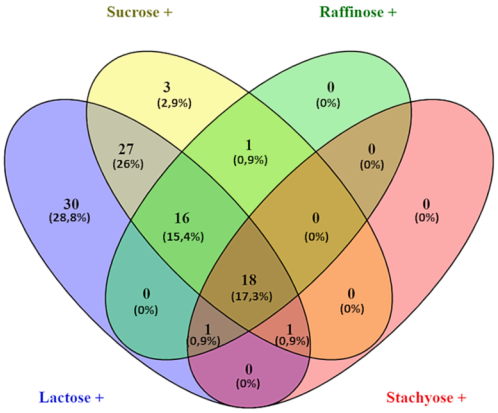

Fig. 1. Variability of carbohydrates utilization among LAB and PAB starters. A. Table summering utilization of lactose, sucrose, raffinose, and stachyose utilization. **B.** Venn diagram representing the distribution of 104 strains (84 LAB and 20 PAB) based on their ability to utilize lactose, sucrose, raffinose and stachyose. The 18 strains that utilize the 4 carbohydrates are *A. acidipropionici* (N = 6), *L. acidophilus* (N = 3), *L. plantarum* (N = 6), *L. delbrueckii* subsp. *lactis* (N = 2), and *L. fermentum* (N = 1). Eight strains are unable to utilize none of these four carbohydrates: *P. freudenreichii* (N = 2), *L. johnsonii* (N = 1), *L. rhamnosus* (N = 4), and *L. helveticus* (N = 1). Venn diagram was generated by Venny 2.1.0 (Oliveros, 2007).

well plates were immediately transferred to −20 °C and then to −80 °C until ELISA assays.

2.6. HT-29 cell challenging

HT-29 cells were first cultivated in T-25 flasks in complete Dulbecco modified Eagle medium (DMEMc, Dominique Dutscher, Brumath, France) supplemented with 10 % (v/v) fetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin sulphate) at 37 °C with 5 % CO_2. Trypsin (0.05 %)/EDTA (0.2 %) (Gibco, Saint Aubin, France) was used to release adherent cells for subculturing. HT-29 cells were seeded in 24-well microtiter plates at a density of 10^5 cells/well in volume of 1 mL. The growth medium was changed every two days until complete confluence, *i.e.* 10^6 cells per well in 1 mL volume. Twenty-four hours before cell challenging, the culture medium was discarded, and the cells were washed with PBS pH 7.4 (Dominique Dutscher). One milliliter of fresh DMEMc containing 1 μg/mL of LPS from *Escherichia coli* 0111: B4 (Sigma Aldrich, Saint Quentin Fallavier, France) was added to cells composing the inflammatory group. For the non-inflammatory group, fresh DMEMc containing sterile distillated water was added. The two groups of cells were incubated in the presence of each fermented matrix described above for 24 h in 5 % CO_2 at 37 °C. Inflammatory and non-inflammatory controls, with non-fermented matrix, were included. All samples and controls were performed in triplicate. The percentage of HT-29 cell viability after the different stimulation conditions was checked by trypan blue staining, and the cell viability was not affected. After 24 h of HT-29 cell stimulation, the plates were centrifuged (800 × *g*, 4 °C, 10 min), the supernatant was collected and transferred to 96-well plates containing 5 μL/well of protease inhibitor (Sigma-Fast). The 96-well plates were immediately transferred to −20 °C and then to −80 °C for ELISA assays.

2.7. Cytokines quantification

Levels of IL-10, IL-12, in the PBMC cell supernatants and IL-8 cytokine production in the HIEC cell supernatants were quantified in 96 well plates (Nunc Immuno MaxiSorp, Thermo Electron LED) using commercially available ELISA kits (BD Biosciences, Pont de Claix, France) in accordance with the manufacturer's instructions. Absorbance was read at 450 nm using microplate spectrophotometer system (Bio teck instruments, Inc, Winooski, USA). Quantified data presented is the total concentration (pg/mL) of a given cytokine produced. All quantifications were performed in triplicate.

2.8. Clustering of LAB and PAB strains with similar phenotypes

Eighty-four LAB and 20 PAB strains were clustered by considering 11 phenotypic traits evaluated after *in vitro* analyses: four coded as a binary trait for lactose, sucrose, raffinose, and stachyose hydrolysis, and 7 as continuous traits including the hydrolysis of caseins, soy and hemp proteins, the acidification abilities of goat milk, soy milk analog and hemp milk analog, and the IL-10/IL-12 ratio. Indeed, it has been shown that this ratio could predict the *in vivo* protective capacity of bacterial strains (Foligné et al., 2007). These authors suggest that the potential probiotic strains can be pre-screened *in vitro* for their immunomodulating potential, before animal and clinical investigations.

The data were normalized to give equal weights to all traits before computing Euclidian distances between the phenotypes. The distance matrix was then used to compute a hierarchical classification, using the IBM SPSS Statistics26 software (Armonk, USA) and construct the clusters from that classification. The number of clusters (twenty clusters for LAB and eight for PAB) was chosen to ensure that strains had very similar profiles in each cluster.

2.9. Statistical analysis

The results are expressed as Mean ± SD (standard deviation). The analysis of variance (ANOVA) was used to analyze the experimental data. Statistical significance was set at $p < 0.05$ (Bartlett's test). Calculations were performed using GraphPad Prism Software (Prism 9 for Windows).

3. Results

3.1. Techno-functional screening

3.1.1. Ability of 104 LAB and PAB strains to utilize carbohydrates

A total of 104 strains belonging to 9 LAB and two PAB species was tested *in vitro* to investigate their ability to utilize 50 carbohydrates (Supplementary Table S1). Strains were mainly compared with respect to their ability to utilize the four main carbohydrates found in the studied matrices (goat milk, soy milk analog and hemp milk analog), namely, lactose, sucrose, raffinose and stachyose (Fig. 1). Lactose was utilized by 89.4% of the tested strains (93/104). Sucrose was utilized by 63.5% of the tested strains (66/104), with a variable number of strains per species: all studied *S. thermophilus* and *A. acidipropionici* strains, while none of *L. delbrueckii* subsp. *bulgaricus* strains, were able to utilize

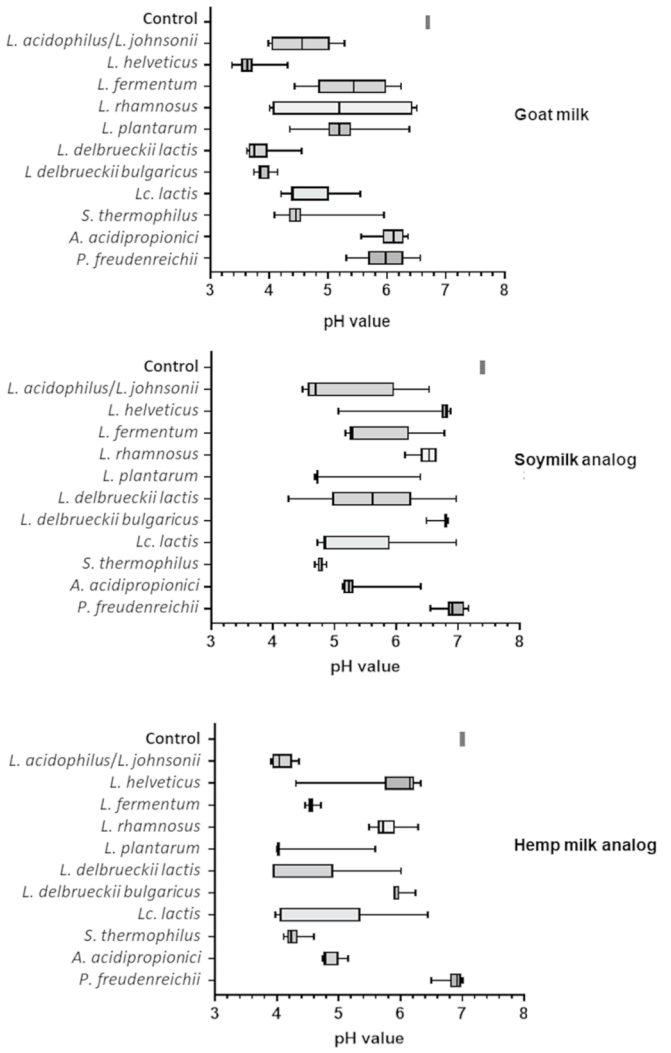

Fig. 2. Variability of acidification abilities among LAB and PAB starters. Box-Plot representing the ability of LAB and PAB strains to acidify goat milk, soy milk analog, and hemp milk analog. Data are grouped and represented per species. The pH was measured at the end of culture at optimal growth temperature, 24 h for LAB and 48 h for PAB.

sucrose.

Raffinose and stachyose were utilized by a smaller fraction of the tested strains, *i.e.*, 34.6% (36/104) and 19.2% (20/104), respectively. Raffinose was utilized by a very high number of strains per species: all *L. plantarum* strains, 9 out of 10 *A. acidipropionici*, 7 out of 8 *L. fermentum*, and all *L. acidophilus*, or only by a few strains: 1 out of 8 *L. delbrueckii* subsp. *bulgaricus*, 2 out of 10 *L. delbrueckii* subsp. *lactis*, and 1 out of 11 *L. rhamnosus*, or even by none of tested the strains of *S. thermophilus*, *L. helveticus*, and *Lc. lactis*. Stachyose was utilized by 7 out of 10 *A. acidipropionici*, 5 out of 12 *L. plantarum*, all *L. acidophilus* strains, 2 out of 8 *L. fermentum*, 1 out of 8 *L. delbrueckii* subsp. *bulgaricus*, and 2 out of 10 *L. delbrueckii* subsp. *lactis*.

Among the 104 strains, only 17.3% (18/104) were able to use the four carbohydrates within 48 h or 96 h of fermentation (Fig. 1). These strains belong to *A. acidipropionici* (N = 6), *L. acidophilus* (N = 3), *L. plantarum* (N = 6), *L. delbrueckii* subsp. *lactis* (N = 2), and *L. fermentum* (N = 1). A fewer number of strains, 7.7% (8/104) were unable to use any of the four sugars: *P. freudenreichii* (N = 2), *L. johnsonii* (N = 1), *L. rhamnosus* (N = 4), and *L. helveticus* (N = 1), while they used monosaccharides such as glucose (Supplementary Table S1).

3.1.2. Ability of 104 LAB and PAB strains to acidify goat milk and vegetable analogs

Among the 104 tested strains, 71.1% (N = 74) acidified goat milk to pH values below 5.5, which triggered milk coagulation. 51.4% (N = 54) acidified soy milk analog, while 61.5% (N = 62) acidified hemp milk analog. This was reached within 24 h for LAB or 48 h for PAB, (Fig. 2 and Supplementary Table S1). This acidifying ability appeared to be species-, strain-, and matrix-dependent. Indeed, only 39 out of 104 tested strains acidified the three matrices. These strains belong to *L. delbrueckii* subsp. *lactis* (4/10), *L. plantarum* (11/12), *S. thermophilus* (9/10), *L. fermentum* (4/8), *L. helveticus* (1/12), *L. acidophilus* (3/3), *L. johnsonii* (1/1), and *Lc. lactis* (6/9).

Strains of *Lc. lactis* and *S. thermophilus* acidified the three substrates, while *P. freudenreichii* strains slightly acidified goat milk but not soy and hemp milk analogs (Fig. 2 and Supplementary Table S1). The average pH values for *Lc. lactis* strains were 4.62 ± 0.45, 5.32 ± 0.86 and 4.60 ± 0.98 in goat milk, soy milk analog and hemp milk analog, respectively. The average pH values for *S. thermophilus* strains were 4.56 ± 0.51, 4.78 ± 0.06, and 4.28 ± 0.16 in goat milk, soy milk analog, and hemp milk analog, respectively. The average pH values for *P. freudenreichii* were 5.97 ± 0.40, 6.94 ± 0.19, and 6.87 ± 0,16 in goat milk, soy milk analog, and hemp milk analog, respectively (Fig. 2 and Supplementary Table S1). Further, *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* strains strongly acidified goat milk to average pH values of 3.86 ± 0.30, 3.90 ± 0.13, and 3. 70 ± 0.28, respectively. Conversely, *A. acidipropionici* strains strongly acidified soy and hemp milk analogs with a higher efficacy than goat milk. The average pH values were 6.07 ± 0.26, 5.34 ± 0.38, and 4.87 ± 0.16 in goat milk, soy milk analog, and hemp milk analog, respectively. The acidification abilities were also strain-dependent. For example, the first quartile of *L. rhamnosus* strains acidified goat milk to pH values below 4.5 and the fourth quartile acidified it to values above 5.5 (Fig. 2 and Supplementary Table S1).

3.1.3. Ability of 104 LAB and PAB strains to hydrolyze proteins from goat milk, or from soy or hemp resources

The ability of the strains to hydrolyze goat caseins or proteins isolated from soy and hemp was evaluated through the resultant free amine groups present in the medium after fermentation (ΔNH_2). Positive values of ΔNH_2 mean that the content of peptides and free amino acids increased during fermentation, in comparison to the initial content of the control medium. The corresponding strains were thus considered as proteolytic strains. Conversely, negative values of ΔNH_2 indicate consumption of peptides and free amino acids initially present in the medium. The corresponding strains were thus considered as non-proteolytic strains.

The ability of the strains to hydrolyze proteins varied over a very large range depending on the bacterial strains, species, and on the nature of the protein source (Fig. 3 and Supplementary Table S1). ΔNH_2 values were from −2.01 (non-proteolytic strains) to + 4.41 (highly proteolytic strains) mM of methionine used as a standard (mM eq. Met) on goat caseins, from −2.63 to 1.76 mM eq. Met on soy proteins, and −3.58 to 3.70 mM eq. Met on hemp proteins. *A. acidipropionici* and *P. freudenreichii* strains were non-proteolytic, whatever the protein source, whereas *L. acidophilus*, *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* strains were able to hydrolyze proteins isolated from the three matrices (Fig. 3).

3.2. Screening of the immunomodulatory bacterial potential

The *in vitro* immuno-stimulation of PBMCs by the 104 bacterial strains revealed distinct patterns of cytokine induction, depending on the bacterial species and strains (Fig. 4). Variations in concentrations of the anti-inflammatory cytokine IL-10 were substantial, with values ranging between 100 and 700 pg/mL depending on the strain (Fig. 4). *B. longum* BB536 and *P. freudenreichii* CIRM-BIA129, used as reference

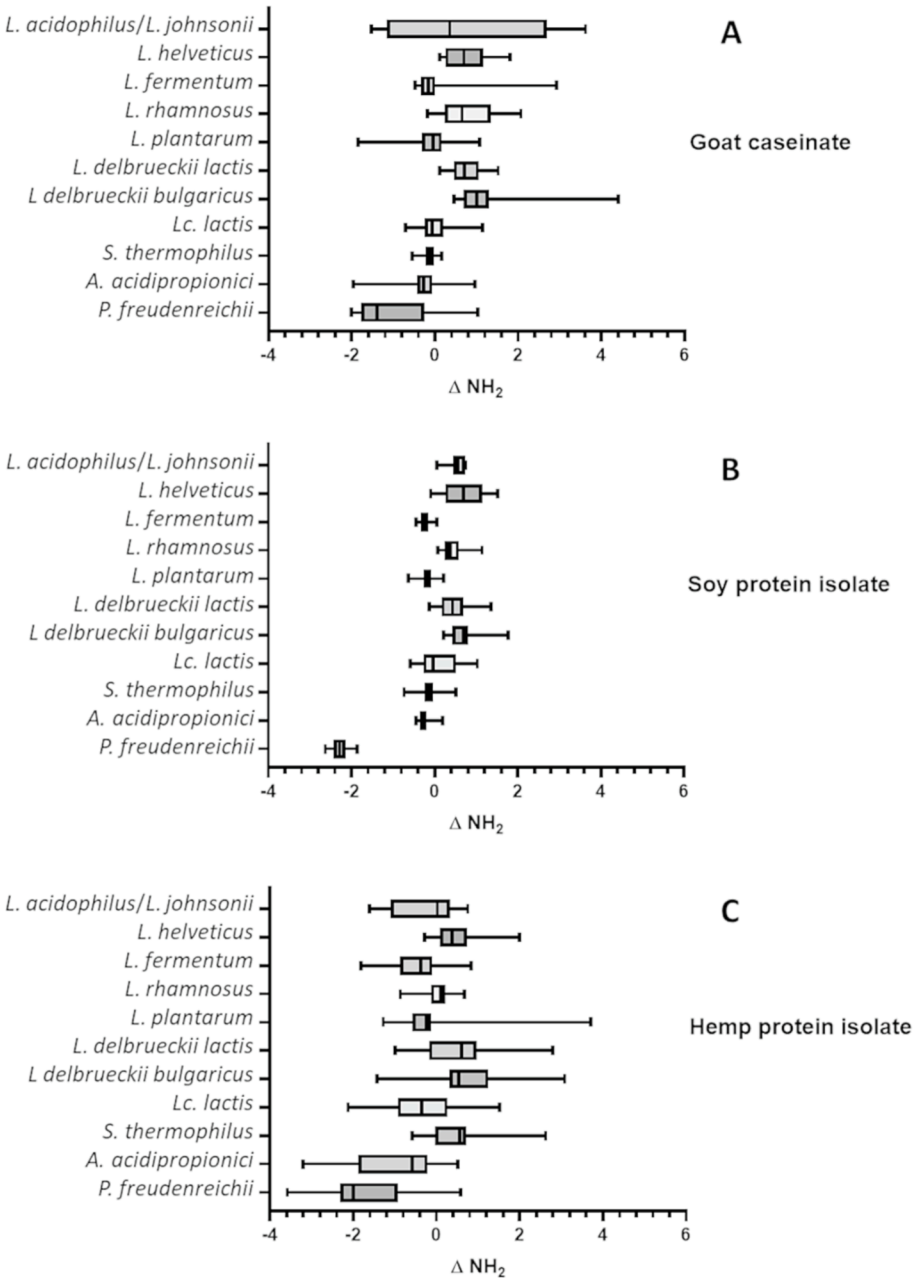

Fig. 3. Variability of proteins utilization among LAB and PAB starters. Box-Plot representing the ability of LAB and PAB strains to hydrolyze goat caseinate (A), soy protein isolate (B) or hemp protein isolate (C). Data are grouped and represented per species. ΔNH_2 = Conc. eq Met (inoculated sample) - Conc. eq Met (non-inoculated control). The results were expressed as mM equivalent methionine, used as the standard.

immunomodulatory strains, induced 650 and 483 pg/ml of IL-10, respectively. The 10*P. freudenreichii* tested strains induced lower levels of IL-10 than the two reference strains, with levels averaging 248 ± 108,84 pg/mL. However, *A. acidipropionici* strains induced higher levels of IL-10 than *P. freudenreichii* CIRM-BIA129, with an efficacy close to that of *B. longum* Bb536. The strains belonging to the species *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *L. rhamnosus*, and *L. acidophilus* induced lower levels of IL-10 than the two reference strains, whereas some *L. helveticus* were as inducers as *P. freudenreichii* CIRM-BIA129.

For the pro-inflammatory cytokine, IL-12, we also observed significant variations between species (Fig. 4), covering a large range of cytokine levels: from undetectable level for *P. freudenreichii*, *A. acidipropionici*, *L. helveticus, L. fermentum, L. rhamnosus, L. plantarum, L. delbrueckii* subsp. *bulgaricus, and L. delbrueckii* subsp. *lactis* strains to 650 pg/mL for the *Lc. lactis strains*. These latter strains induced higher levels of IL-12, *i.e.* 450.5 ± 57.8 pg/mL, than the reference strain *L. lactis* MG1363, *i.e.* 276.8 ± 7.8 pg/mL.

As IL-10 and IL-12 appeared to be the most discriminative cytokines, we used the IL-10/IL-12 ratio (Fig. 4) to distinguish between strains exhibiting a "pro-" *versus* "anti-inflammatory" profile, corresponding to low *versus* high IL-10/IL-12 ratio, respectively. This ratio was described to be useful in identifying strains with marked opposite profiles (Foligné et al., 2007). However, it did not allow discrimination of strains with median cytokine ratios. Among the strains screened, *A. acidipropionici* strains showed the highest IL-10/IL-12 ratio.

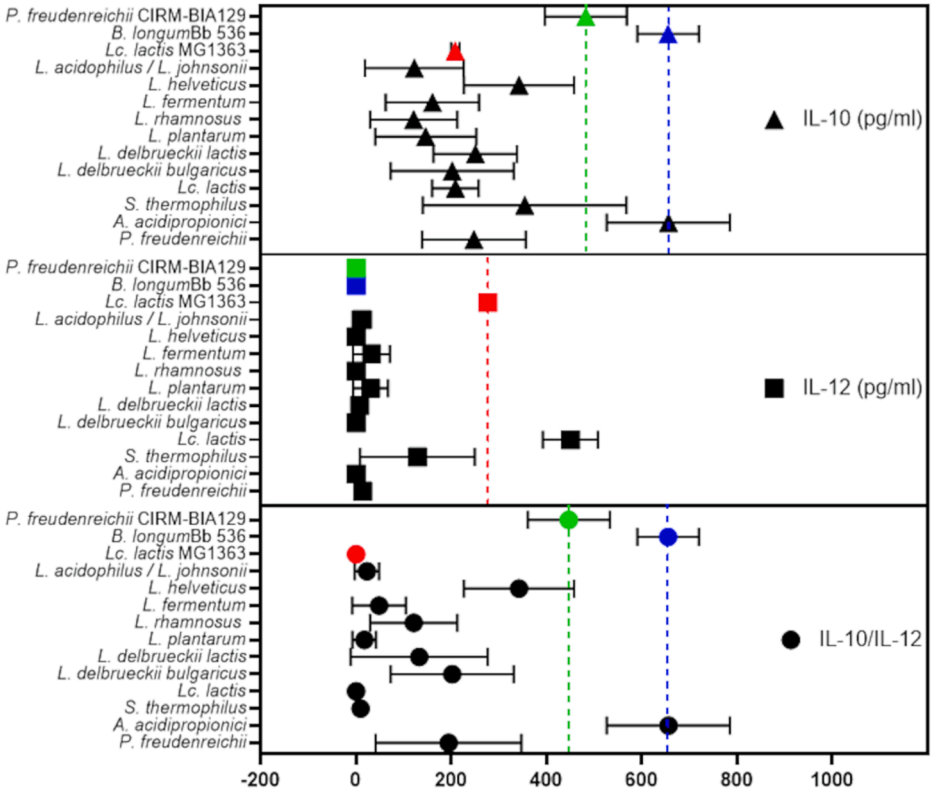

Fig. 4. Variability of immunomodulatory properties among LAB and PAB starters. Summary data (mean and standard deviation) representing the release of IL-10 (▲) and IL-12 (■) cytokines by PBMCs stimulated for 24 h by LAB and PAB strains. Data are grouped and represented per species. The IL-10/IL-12 ratio (●) was calculated. *B. longum* BBb536 (blue), *P. freudenreichii* CIRM-BIA129 (green), and *L. lactis* MG1363 (red) were included as reference strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Clustering the LAB and PAB strains with similar phenotypes, selection of the strains of interest, and bacterial consortia construction

LAB and PAB strains were clustered based on their phenotypes. This included carbohydrate utilization, acidification of the three food matrices, proteolysis of the three protein isolates, *i.e.* goat caseinate, soy and hemp protein isolates, as well as the IL-10/IL-12 ratio induced in human PBMCs.

The LAB strains were grouped into 20 different clusters (Fig. 5). Three clusters, LC1, LC3, and LC4, which contained 21, 22, and 22 strains respectively, encompassed about 77% of the LAB strains. While other clusters contained only one to four strains (Fig. 5). Regarding carbohydrate utilization, most of the LAB clusters, 16 clusters out of the 20, contained strains able to utilize lactose (Lac) or sucrose (Suc). Only four clusters, LC2, LC4, LC5, and LC19 contained strains able to utilize raffinose (Raf) and stachyose (Sta). And three clusters, LC8, LC14, and LC16, contained the strains that were unable to utilize any of the four carbohydrates. Regarding the proteolytic ability of the LAB strains, the clusters LC3 and LC4 encompassed strains with heterogenous proteolytic profiles, compared to the other ones. Conversely, the clusters LC1, LC2, LC5, LC14, LC16, and LC17 contained strains that were able to hydrolyze the three protein isolates. Similarly, for the acidification ability, the clusters LC3, LC4 and LC5 contained strains that acidified goat milk, soy milk analog, and hemp milk analog. Concerning the immune profile (IL-10/IL-12 ratio), even if it was a continuous feature, from 0.31 to 582.89, distinct bacterial profiles appeared. The LC1 cluster was the only one with almost all strains showing a high IL-10/IL-12 ratio. Some other clusters showed very low ratio values and can be considered as having the less promising immunomodulatory properties, *i.e.* especially the mono-strain clusters (LC6, LC11, LC12, LC13, LC16, LC17, and LC20).

The PAB were grouped into eight different clusters (Fig. 6). Only the PC6 cluster encompassed strains that were almost all able to utilize the four carbohydrates, to acidify goat milk, soymilk analog, and hemp milk analog, to proteolyze the proteins issued from these three matrices, and to have a high IL-10/IL-12 ratio. The other clusters contain strains that have various abilities in terms of carbohydrate utilization, and proteolytic activity.

Therefore, the screening gave rise to the selection of five selected strains, including four LAB and one PAB. Thus, among all LAB strains, four strains were selected according to their distinct phenotypes that were the most representative of the different technological and probiotic criteria: *L. delbrueckii* subsp. *lactis* Bioprox1585, *L. lactis* Bioprox7116, *S. thermophilus* CIRM-BIA257, and *L. acidophilus* Bioprox6307 (Fig. 5). The strain *A. acidipropionici* CIRM-BIA2003 was selected for PAB strains. These five strains were assembled in bacterial consortia composed of 2, 3, 4, or 5 strains, as shown in Table 1. This generated 26 bacterial consortia on which immunomodulatory properties were tested in human epithelial cells, as shown below, to select the most promising combination.

3.4. Effect of the food products, fermented by selected bacterial strains and consortia, on IL-8 secretion in response to LPS in human intestinal epithelial cells (HIEC)

We investigated the anti-inflammatory potential of the different fermented food products, fermented either by the selected bacterial individual strains, or by the designed consortia. Human Intestinal Epithelial Cells (HIEC), both in the presence and in the absence of proinflammatory lipopolysaccharide (LPS) from *E. coli*, were exposed to the fermented goat milk and soy milk analog (48 h at 37 °C), prepared using each of the five monocultures, or each of the 26 bacterial consortia. We measured the concentrations of IL-8, involved in the inflammatory process (Fig. 7).

Unstimulated HIECs secreted 115.1 ± 10.8 pg/mg of IL-8 in the absence of LPS (Fig. 7A). Most of the data indicate very low induction of IL-8 in presence of goat milk and in almost all monocultures and consortia that grew either on goat milk analog or soy milk analog, as in the HIEC alone. However, non-inoculated soy milk analog (control) increased IL-8 production by unstimulated cells up to 597.2 ± 77.6 pg/mL) (Fig. 7A). Similar observation was made with soy milk analog

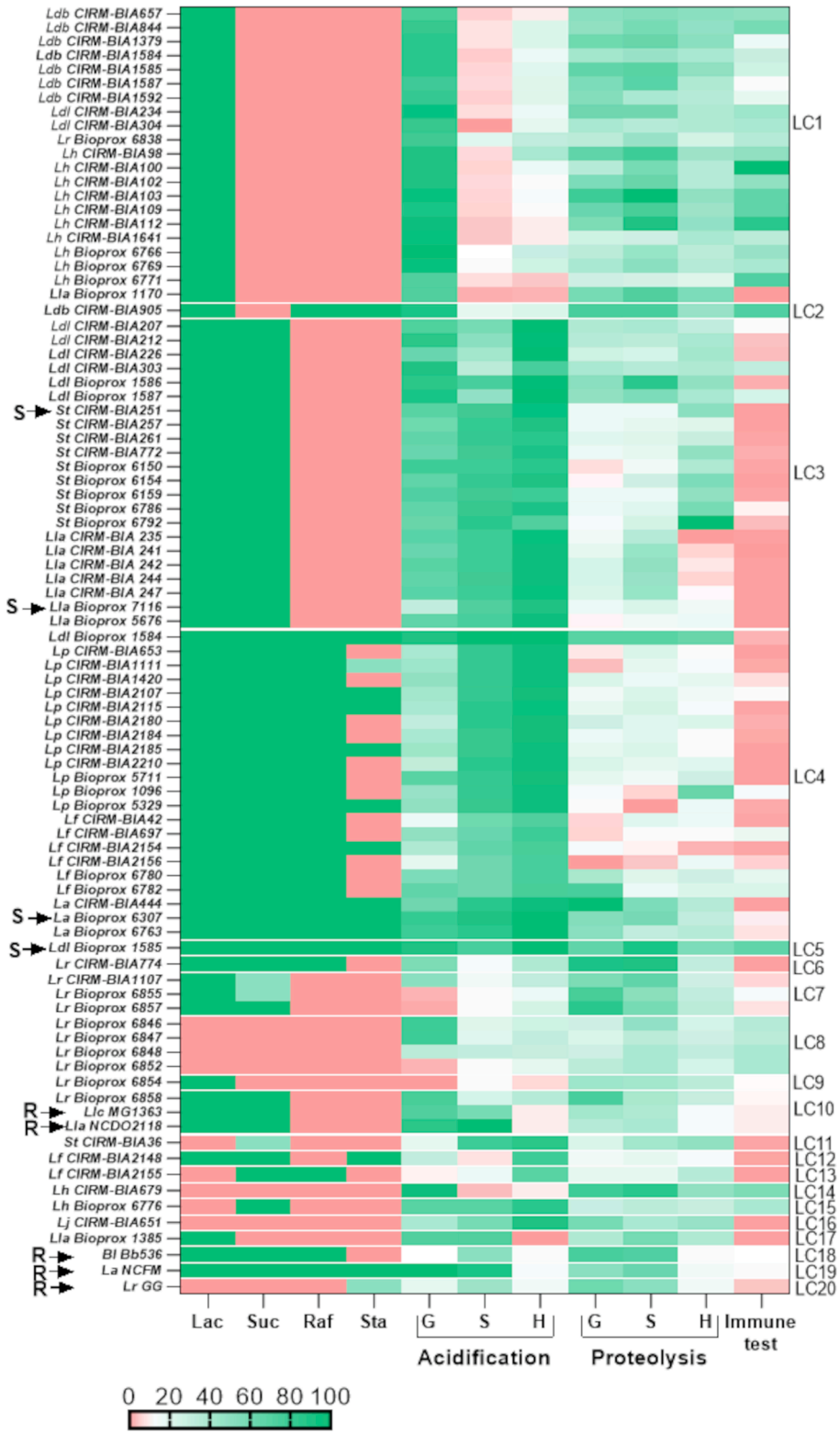

Fig. 5. Clustering of LAB starter strains summarizing technological and probiotic abilities. The heat map shows the distribution of strains according to their phenotypic profiles in terms of carbohydrate fermentation, proteolysis, acidification capacity, and immunomodulatory properties. Lac, lactose; Raf, raffinose; Sta, stachyose; Suc, sucrose; G, goat caseinate; S, soy protein isolate; H, hemp protein isolate. Immune test consists in the IL-10/IL-12 ratio. S and R refer to selected and reference strains, respectively. LC1 to LC20 refer to LAB clusters. Ldb, *L. delbrueckii* subsp. *bulgaricus*; Ldl, *L. delbrueckii* subsp. *lactis*; Lh, *L. helveticus; Lla, Lc. lactis* subsp. *lactis;* St, *S. thermophilus;* Lp, *L. plantarum;* Lf, *L. fermentum,* La, *L. acidophilus;* Lr, *L. rhamnosus;* Llc, *Lc. lactis* subsp. *cremoris.*

fermented by *A. acidipropionici* CIRM-BIA 2003, *Lc. lactis* Bioprox7116 and *S. thermophilus* CIRM-BIA257, either in monocultures or in consortia. These later increased IL-8 production by unstimulated cells, to 419.8 ± 50.5, 733.0 ± 395.2, and 591.0 ± 131.8 pg/mL, respectively. The soy milk analog fermented with consortium 17, composed by these three strains, reached IL-8 levels of 570.3 ± 160.8 pg/mL.

The LPS proinflammatory stimulus, as expected, increased the basal level of inflammation as shown by the high IL-8 secretion of LPS-stimulated HIECs, 1050.0 ± 68.9 pg/mL (Fig. 7B). Co-treatment with soy milk analog further increased IL-8, while goat milk did not. Goat milk and milk analogs fermented by monocultures diversely affected this proinflammatory response without inducing significant modulatory effect. By contrast, milks fermented by the bacterial consortia decreased the IL-8 secretion, compared with those fermented by monocultures.

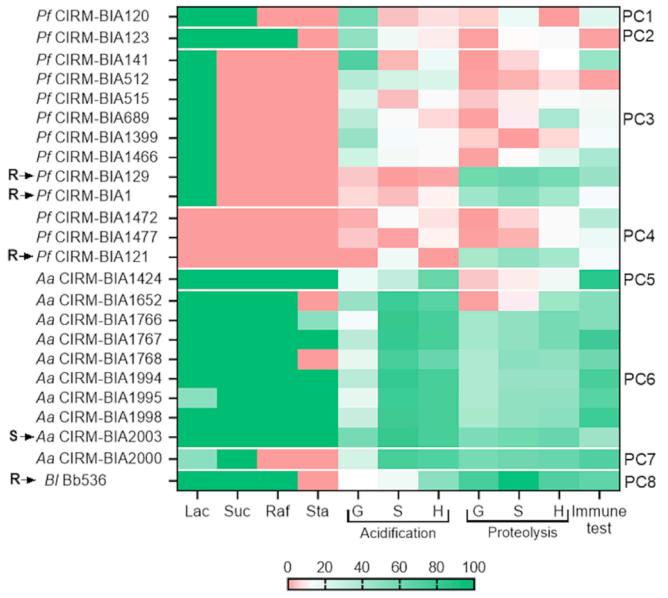

Fig. 6. Clustering of PAB starter strains summarizing technological and probiotic abilities. The heat map shows the distribution of strains according to their phenotypic profiles in terms of carbohydrate fermentation, proteolysis, acidification capacity, and immunomodulatory properties. Lac, lactose; Raf, raffinose; Sta, stachyose; Suc, sucrose; G, goat caseinate; S, soy protein isolate; H, hemp protein isolate. Immune test consists on the IL-10/IL-12 ratio. S and R refer to selected and reference strains, respectively. PC1 to PC8 refer to PAB clusters. Pf, *P. freudenreichii*; Aa, *A. acidipropionici.*

Table 1
Bacterial consortia design.

Bacterial strains and consortia	Description
A. acidipropionici CIRM-BIA2003	Aa CB2003
L. delbrueckii ssp. *lactis* Bioprox1585	Ldl B1585
L. acidophilus Bioprox6307	La B6307
Lc. lactis Bioprox7116	Lla B7116
S. thermophilus CIRM-BIA257	St CB257
Consortium-1	Aa CB2003, Ldl B1585
Consortium-2	Aa CB2003, La B6307
Consortium-3	Aa CB2003, Lla B7116
Consortium-4	Aa CB2003, St CB257
Consortium-5	Ldl B1585, La B6307
Consortium-6	Ldl B1585, Lla B7116
Consortium-7	Ldl B1585, St CB257
Consortium-8	La B6307, Lla B7116
Consortium-9	La B6307, St CB257
Consortium-10	Lla B7116, St CB257
Consortium-11	Aa CB2003, Ldl B1585, La B6307
Consortium-12	Aa CB2003, Ldl B1585, Lla B7116
Consortium-13	Aa CB2003, Ldl B1585, St CB257
Consortium-14	Aa CB2003, La B6307, Lla B7116
Consortium-15	Aa CB2003, La B6307, St CB257
Consortium-16	Ldl B1585, La B6307, Lla B7116
Consortium-17	Aa CB2003, Lla B7116, St CB257
Consortium-18	Ldl B1585, La B6307, St CB257
Consortium-19	Ldl B1585, Lla B7116, St CB257
Consortium-20	La B6307, Lla B7116, St CB257
Consortium-21	Aa CB2003, Ldl B1585, La B6307, Lla B7116
Consortium-22	Aa CB2003, Ldl B1585, La B6307, St CB257
Consortium-23	Aa CB2003, Ldl B1585, Lla B7116, St CB257
Consortium-24	Aa CB2003, La B6307, Lla B7116, St CB257
Consortium-25	Ldl B1585, La B6307, Lla B7116, St CB257
Consortium-26	Aa CB2003, Ldl B1585, La B6307, Lla B7116, St CB257

Seven bacterial consortia, namely Cons-5, Cons-6, Cons-11, Cons-12, Cons-16, Cons-22, and Cons-25, were particularly efficient in reducing the proinflammatory response induced by LPS. Indeed, these consortia, either cultivated in goat milk or in soy milk analog, decreased the IL-8 secretion. This was particularly true concerning the Cons-5, which decreased the initial level of LPS-stimulated HIECs down to 266.0 ± 67.6 pg/ml and 362.3 ± 64.8 pg/mL of IL-8 cytokine, when exposed to goat milk and soy milk analog, respectively. The seven consortia were selected to be tested in two other mixed matrices: goat milk: soy milk analog (50:50) and soy milk analog: hemp milk analog (75:25).

HIECs were also exposed both to the five bacterial individual strains and to the seven bacterial consortia, in the presence or absence of LPS, in soy milk analog, mixed with goat or hemp milk analog (Fig. 8 A and B). Soy milk analog, mixed with goat or hemp milk analog, induced a higher production of the IL-8 cytokine (Fig. 8A) which was dependent on the soy content in the mix and in agreement with the soy milk analog alone (Fig. 7). Moreover, as already observed in goat milk and soy milk analog, the bacterial consortia decreased the IL-8 secretion, when compared to the individual strains in the four tested matrices. Among the consortia tested, the consortium Cons-12 was particularly efficient at reducing IL-8 production, whatever the food matrix, and was thus the most promising one. This consortium was composed of *A. acidipropionici* CIRM-BIA2003*, Lb. delbrueckii* subsp. *lactis* Bioprox1585, and *Lactococcus lactis* Bioprox7116 (Table 1).

4. Discussion

Developed countries presently experience a food transition aiming at a more sustainable production of food products, which should be minimally processed, address ethical concerns and have a reduced negative impact on the environment. Moreover, food products should be safe, healthy and good in order to reach customers' demands. In line with this, less animal-sourced products, and more plant-based ones, are used. Indeed, many Western countries experience a decrease in the consumption of dairy products, yet an increase in that of plant-based products (Islam et al., 2021). Fermentation of plant-based dairy substitutes offers a promising perspective, as fermentation is well known to confer such traits, including hedonic, hygienic and probiotic properties, to fermented dairy products. Dairy products fermentation by lactic acid bacteria, including proteolysis of caseins into peptides, free amino acids and flavor compounds, as well as utilization of lactose to generate lactic acid and exopolysaccharides, is now well understood. By contrast, little is known about such fermentation process in plant-based alternatives to cheeses, yogurts and fermented milks (Harper et al., 2022).

The development of innovative fermented plant-based products that meet current consumers' expectations involves the understanding of how fermentation of plant-based dairy substitutes occurs and of how this fermentation drives health properties, safety, flavor and texture. In this aim, we screened 104 potent starter strains, including lactic acid bacteria (LAB) and propionic acid bacteria (PAB), for their ability (i) to utilize soy milk analog, hemp milk analog or goat milk as a substrate, as well as mixes of soy milk analog / goat milk, and mixes of soy milk analog / hemp milk analog, and (ii) to confer probiotic properties to the obtained fermented product.

First of all, there is a need to consider the biochemical composition of the food matrices to seek for starter bacteria able to ferment them, and to adapt at best the screening in order to select the most efficient bacteria. Thus, when using lactic acid bacteria as starter, conversion of available carbohydrates into lactic acid is a key requisite to the development of fermented dairy analogues. In the case of well-known fermented dairy products, the main carbohydrate, lactose, a β-galactoside, is hydrolyzed by β-galactosidase, which is provided by lactic acid bacteria. The resulting glucose is then converted into lactic acid. In plant-based milk analogs, carbohydrates, other than lactose, are present and other enzymes are needed (Canon, Mariadassou et al., 2020). The ability of lactic acid bacteria to ferment the main carbohydrates of soy, sucrose, as well

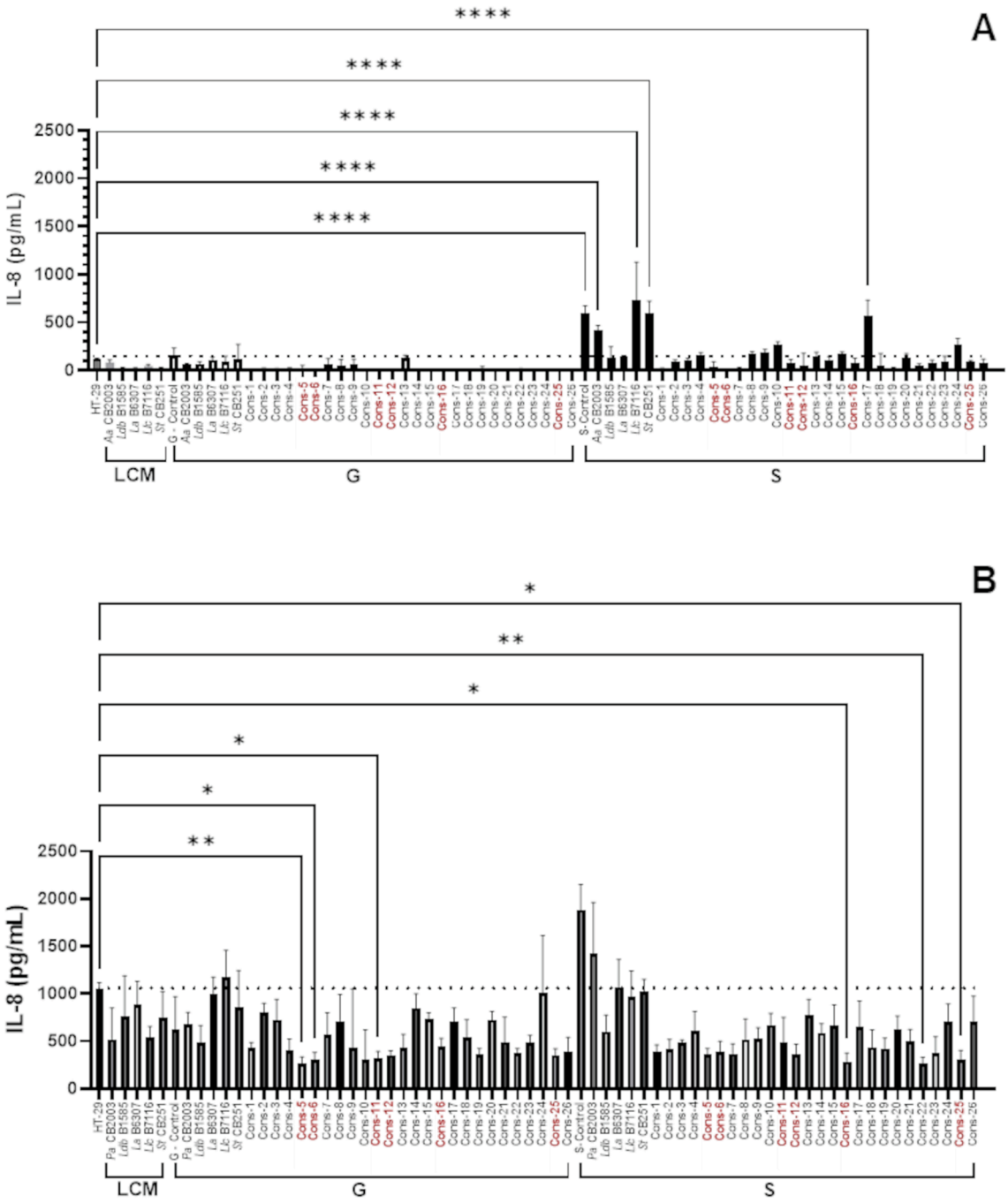

Fig. 7. Effect of goat milk and soy milk analog fermented by bacterial strains and consortia on IL-8 secretion by HIEC. A. Non-inflammatory HIEC control group. **B.** Inflammatory group, HIEC stimulated by LPS (1 μg/ml)**.** HIEC were treated for 24 h with the indicated products. IL-8 concentrations (mean ± SD) were measured in cell supernatants using an ELISA technique. These results are expressed as mean ± SD of triplicate determinations. LCM, laboratory culture medium; G, goat milk; S, soy milk analog. The bacterial consortia, in red, were selected for further analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as α-galactosides such as raffinose and stachyose, which may cause discomfort and flatulence, was shown to be highly variable among bacterial species, and even among strains, within each species (Canon, Mariadassou et al., 2020; Harlé et al., 2020). In our study, this was also observed since only 18, out of the 104 strains screened, were able to hydrolyze the four carbohydrates present in plant-based as well as dairy-plant mixes, *i.e.* lactose, sucrose, raffinose and stachyose, while eight strains used none of them but were able to use at least glucose (Fig. 1 and Supplementary Table S1). Among the most efficient species, this study revealed lactobacilli, and among them some strains of *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum* and *Lactobacillus delbrueckii* subsp. *lactis,* while several strains of *L. delbrueckii* subsp. *bulgaricus* were unable to do so (Jan et al., 2022; Wang et al., 1974). Lactococci and streptococci mainly fermented lactose and sucrose, in line with their ability to adapt to numerous animal and plant niches (Canon, Mariadassou et al., 2020; Harlé et al., 2020). This was also the case in our study for the PAB and more specially the species *A. acidipropionici*, able to utilize at least three out of the four α- and β-galactosides present. There was a great discrepancy between the two propionibacteria species tested since the strains of *P. freudenreichii* used

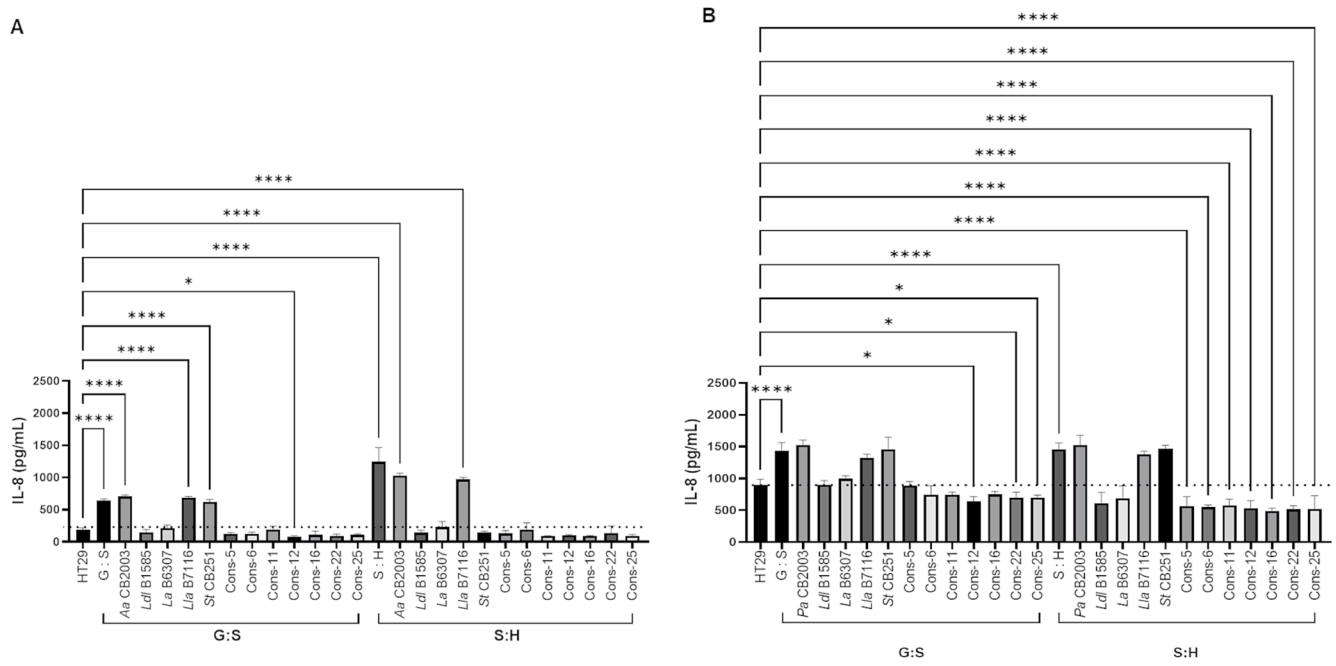

Fig. 8. Effect of mixes fermented by bacterial strains and consortia on IL-8 secretion by HIEC. A. Non-inflammatory HIEC control group. **B.** Inflammatory group, HIEC stimulated by LPS (1 μg/ml)**.** HIEC were treated for 24 h with the indicated products. IL-8 concentrations (mean ± SD) were measured in cell supernatants using an ELISA technique. These results are expressed as mean ± SD of triplicate determinations. G:S, mix of goat milk and soy milk analog; S:H mix of soymilk analog and hemp milk analog.

in this study were able to use mainly lactose (11 out of 13 strains), in agreement with Bücher et al (2021), while only two strains utilized sucrose, and none the α-galactosides, in agreement with Loux et al (2015). To our knowledge, the ability of PAB to use α-galactosides was hardly studied and offers potentiality, notably for *A. acidipropionici*, to be used as starters in legume-based products. In line with this, analysis of *A. acidipropionici* genome indicated the presence of a potent raffinose α-galactosidase enzyme (K7RUE9_ACIA4) (Parizzi et al., 2012).

Such a screening on the various osides can be enlarged to plant species that contain other types of sugars that are not present either in soy or hemp, as for example starch (see supplementary Table S1). This major plant carbohydrate, can actually be used by all *A. acidipropionici* strains, yet no *P. freudenreichii* strain, within the PAB group. It was used only by specific *Lc. lactis* subsp. *lactis* strains, within the LAB group. It should be noted that wild-type strains of this species, originating from plants, can hydrolyze starch, and that this is an important functional feature discriminating plant from dairy lactococci (Parapouli et al., 2013). Accordingly, *P. freudenreichii* is considered a typical dairy PAB species, isolated from milk and cheese, while *A. acidipropionici* is found in more diverse environments including soil, hay and wastewater (Turgay et al., 2022).

Similarly, the ability to hydrolyze proteins has to be tested according to the presence of different types of proteins in milk and in plant. Even if the LAB possess a complex proteolytic system to supply their numerous auxotrophies (Liu et al., 2010), some strains are not able to hydrolyze the proteins, whatever their origin, and rely on the presence of required peptides and free amino acids in the medium or on the presence of other proteolytic species able to provide them. Our work revealed a high diversity in the proteolytic profiles, with as expected the highest activity for the *Lactobacillus helveticus* strains (Liu et al., 2010; Sadat-Mekmene et al., 2011), as well as for *L. delbrueckii* subsp. *lactis* or *bulgaricus*. They were able to hydrolyze not only the goat caseins but also the soy and hemp protein isolates. The other LAB had, in contrast, lower or even no proteolytic activity, depending on the strains and on the substrate used. Regarding PAB, *P. freudenreichii* has very low proteolytic activity, as confirmed in our study (11 out of 13 strains with no proteolytic activity on any of the three types of proteins). This is consistent with the observation that this species is systematically associated in Swiss-type cheese with proteolytic LAB (Gagnaire et al., 2001). In contrast, *A. acidipropionici* showed the ability to hydrolyze the goat, soy and hemp proteins. Knowing which species is proteolytic or not is indeed a key tool to assemble complementary LAB and PAB strains (Canon, Nidelet et al., 2020).

To our knowledge, the immunomodulatory properties are hardly considered as a screening criterium for strain selection in complement to the technological one, in order to select strains both as a starter and as a probiotic. As for the above-mentioned technological criteria, the immunological screening revealed a great strain diversity. Indeed, the IL10/IL12 ratio induced in human immune cells (PBMCs) was highly dependent on the strain. However, some selected strains induced a high ratio, suggesting a great potential as immunomodulatory candidates. This was particularly true for strains of *L. helveticus*, *L. delbrueckii* and of *A. acidipropionici*. This opens new avenues for the development of immunomodulatory fermented foods, which may be relevant in the context of compromised intestinal epithelial barrier (IEB).

Our strategy consisted in taking advantage of the phenotypic complementarity between starter strains in order to design specific bacterial consortia. These consortia were then used to develop fermented products which combine both technological and probiotic abilities. Indeed, the selected bacterial consortia were shown here to counteract the pro-inflammatory effects of *Escherichia coli* LPS in cultured HT-29 HIECs. In a previous screening of probiotic bacteria, the immunomodulatory properties of bacterial strains, as revealed *in vitro*, correlated with their protective effect towards induced colitis in mice (Foligné et al., 2007). In line with this, we have previously shown that the association of immunomodulatory strains of *P. freudenreichii*, *S. thermophilus* and *L. delbrueckii* allows the production of Emmental cheese which protects mice from induced colitis (Rabah et al., 2020). Hence, plant-based as well as mixed dairy and plant-based products fermented by the selected bacterial consortium deserve being tested in such *in vivo* models of injured intestinal barrier.

5. Conclusion

Consumers nowadays seek new healthy, natural, safe and functional food products, with minimal processing, enhanced sustainability and beneficial effects on health. In this work, we have selected lactic and propionic acid bacteria strains for their ability to ferment different food matrices and their ability to modulate the immune response. Taking into consideration the recent demand for vegetarian products, we focused on their ability to utilize both proteins and carbohydrates present in vegetable milk alternatives (soy, hemp), in comparison with those present in goat milk. Taking into consideration the growing incidence of ailments involving inflammation, either acute (IBD) or low-grade (IBS), we also sought their ability to modulate inflammation, both in immune and in intestinal epithelial cells. Taking advantage of phenotypic complementarity between strains, we designed bacterial consortia able to transform such food matrices into functional fermented food products. Such products were then shown to reduce inflammation in intestinal cells which were stressed by pro-inflammatory LPS. Using the most promising selected consortia opens new avenues for the development of fermented foods adapted to populations, presently increasing in western countries, which suffer from various ailments correlated to inflammation of the digestive tract. Such functional food products should be further evaluated in preclinical studies of digestive disorders. They will furthermore give an alternative to presently widely consumed fermented dairy and plant-based products for vegetarian and flexitarian consumers.

CRediT authorship contribution statement

Nassima Illikoud: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Fillipe Luiz Rosa do Carmo:** Conceptualization, Methodology, Investigation, Validation. **Nathalie Daniel:** Investigation. **Gwénaël Jan:** Conceptualization, Validation, Writing – review & editing, Supervision. **Valérie Gagnaire:** Conceptualization, Validation, Writing – review & editing, Supervision.

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

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112557.

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