Mixed dairy and plant-based yogurt alternatives: Improving their physical and sensorial properties through formulation and lactic acid bacteria cocultures

Fanny Canon, Marie-Bernadette Maillard, Marie-Hélène Famelart, Anne Thierry, Valérie Gagnaire

UMR STLO, INRAE, Institut Agro, F35000, Rennes, France

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ABSTRACT

Food transition requires incorporating more plant-based ingredients in our diet, thus leading to the development of new plant-based products, such as yogurt alternatives (YAs). This study aimed at evaluating the impact of lactic acid bacteria (LAB) cocultures and formulation on the physico-chemical and sensory properties of YAs. YAs were made by emulsifying anhydrous milk fat (AMF) or coconut oil in milk and lupin protein suspensions. The starters used, in mono- and cocultures, were the strains Lactococcus lactis NCDO2125, Enterococcus faecalis CIRM-BIA2412 and Lactiplantibacillus plantarum CIRM-BIA1524. Textural properties and metabolites of YAs were evaluated and their sensory properties compared using a sorting task. Some cocultures led to higher firmness, viscosity, and water holding capacity of YAs, compared to monocultures. AMF and a milk:lupin protein ratio of 67:33 gave firmer and more viscous YAs. YAs were sensorially discriminated on the basis of protein ratio and fat type, but not of starters. The cocultures exhibited more diverse functional outputs, such as texturing, production of flavour compounds, proteolysis, when the strains associated in coculture had distinct capacities. Appropriate associations of LAB and formulation offer interesting solutions to improve the perception of YAs, and ultimately, encourage their consumption.

1. Introduction

The current food transition compels us to investigate alternatives to accompany the decrease in the consumption of animal-sourced products, such as dairy and meat, and tend towards a more plant-based diet. When referring to food transition, proteins are mainly targeted, and pulses, which are among the most protein-rich plants, are ideal candidates to substitute animal-sourced products or ingredients (Boye et al., 2010). Pulse consumption is strongly encouraged by the Food and Agriculture Organization due to adequate nutritional composition, relatively low prices, and benefits for maintaining soil health maintenance (Calles et al., 2019). For that purpose, soya has been in the spotlight for many years but soya-based products do not satisfy all palates and has been raising environmental and health concerns (Boeck et al., 2021). In Eastern Europe, where yogurts are massively consumed, soja has been used to prepare yogurt alternatives (YAs). Some pulses also have high protein contents and are technologically similar to soya, rendering them the new target to help substituting dairy proteins. Lupin could offer a solution, as it matches the protein content of soya, contains less antinutritional compounds and fat and is a common crop in Europe and Australia. Several drawbacks remain, such as the flavour and texture of pulses-based products including lupin-based ones, which are still limiting their consumption (Guyomarc’h et al., 2021). However, the substitution of only a part of milk with pulses could alleviate these concerns (Guyomarc’h et al., 2021). The ratio between milk and pulses is of great importance for the acceptability of the dairy alternatives, in terms of texture as well as other organoleptic properties (Ben-Harb et al., 2019, 2020). Another solution to compensate for the weaker structure of gels prepared with pulses is the addition of fat in YAs (Shaker et al., 2000), which can be dairy-based as milk cream or plant-based as coconut oil (Hickisch et al., 2016). Finally, fermentation by lactic acid bacteria (LAB) can also improve the organoleptic and physical properties of products (Marco et al., 2017), particularly pulses such as peas (Shi et al., 2021). However, a unique LAB strain capable of using the different carbohydrates present in mixes, hydrolysing proteins, producing the targeted aroma compounds, and giving a good texture is certainly

* Corresponding author.
E-mail address: valerie.gagnaire@inrae.fr (V. Gagnaire).

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difficult to find, if it exists. Consequently, in the presence of diverse fermentable substrates, it is also relevant to choose appropriate LAB strains, capable for example to ferment milk and lupin substrates in coculture (Canon et al., 2020a). When associated, bacterial strains are known to interact with one another. It is thus important to make sure that the chosen strains interact positively to have better outputs (Canon et al., 2020b). In a previous study of Canon et al. (2021), LAB strains known to interact with one another. It is thus important to make sure that the impact of LAB cocultures, on the physico-chemical and sensory properties of mixed dairy and plant-based YAs.

2. Material and methods

2.1. Bacterial collection and pre-cultures conditions

Three mesophilic LAB strains were tested in the following experiments: Lactiplantibacillus plantarum CIRM-BIA1524 (P) and Enterococcus faecalis CIRM-BIA2412 (F) belong to the collection of the International Centre for Microbial Resources dedicated to bacteria of food interest (CIRM BIA, INRAE Rennes, France, https://www6.rennes.inrae.fr/stilo.end), and Lactococcus lactis NCD02125 (L) to the National Collection of Food Bacteria (formerly National Collection of Dairy Organisms), (Berkshire, UK). The strains were selected because positive interactions were identified in coculture in a chemically defined medium; the two proteolytic strains F and L were able to stimulate the nonproteolytic strain P, while the latter was selected for its ability to hydrolyse galactooligosaccharides (GOS) and produce volatile compounds (Canon et al., 2021).

LAB strains were stored in cryotubes at –80 °C. A cryotube was used for each replicate culture. Bacteria were cultured twice in a rich medium, M17 for lactococci and enterococci (Terzaghi and Sandine, 1975), and de Man Rogosa and Sharpe broth (De Man et al., 1960). Then, they were inoculated in the different YAs as described in the 2.2 section.

2.2. Manufacture of set-type YA

Four recipes of set-type YAs were prepared by varying: i) the ratios of milk:lupin proteins set to 50:50 and 67:33 with a total protein concentration of 6.6% (w/w) and ii) the nature of the fat used: AMF (Eurial, Nantes, France) or COCO (E. Leclerc, Ivry-Sur-Seine, France), at a concentration of 1.5% (w/w). Skim milk powder (medium heat, Euriat, Nantes, France) was used to reconstitute milk at 3.3% (w/w) of proteins and a whey protein isolate (Ingredia, Arras, France) was added to the mixture to reach 6.6% (w/w) of proteins with the milk:lupin protein ratio of 67:33 (Table 1 and Fig. 1). A lupin protein isolate (Prolupin GmbH., Grimmen, Germany) was used in the four recipes.

The preparation steps were the same for the four recipes (Table 1 and Fig. 1). All ingredients were weighted and stirred at 60 °C at 500 rpm for 1 h in the Thermomix T55 bowl (Vorwerk, Wuppertal, Germany). The mix was prohibenogenized using an ultra-turrax at 24,000 rpm for 10 min in a water-bath at 65 °C, then homogenized at 250/50 bar (× 105) to convert in Pa (PandaPlus 2000 homogenizer, GEA, Düsseldorf, Germany). The resulting emulsion was immediately pasteurized at 95 °C for 10 min in the Thermomix T55 and cooled down at 4 °C overnight, prior to bacterial inoculation.

The three LAB strains were harvested by centrifugation at 10,000 g × 5 min at 20 °C, resuspended in sterile distilled water, and centrifuged again before inoculation. Five cultures were used to ferment the YAs: F, L, and P monocultures, and F × P (FP) and L × P (LP) cocultures. The

Table 1

| Composition of the ingredients | Composition of the YAs (g/kg) |
|-------------------------------|-------------------------------|
| Proteins (%) | Carbohydrates (%) | Lipids (%) | AMFMilk:lupin 50:50 (AMF50) | AMFMilk:lupin 67:33 (AMF67) | COCOMilk:lupin 50:50 (COCO50) | COCOMilk:lupin 67:33 (COCO67) |
| Skim milk powder | 32.6 | 55.4 | 0.5 | 101 | 101 | 101 |
| Whey protein isolate | 85.1 | 5.5 | 1.0 | 0 | 13 | 0 | 13 |
| Lupin protein isolate | 88.7 | 0.5 | 3.0 | 37 | 25 | 37 | 25 |
| Coconut oil | 0 | 100 | 99.8 | 15 | 15 | 0 | 0 |
| Anhydrous milk fat | 0.1 | 0 | 0 | 100 | 0 | 0 | 15 |
| Sucrose | 0 | 100 | 0 | 0 | 10 | 10 | 10 |
| Distilled water | - | - | - | - | - | - | - |

<sup>a</sup> Eurial, Nantes, France.
<sup>b</sup> Ingredia, Arras, France.
<sup>c</sup> Prolupin GmbH., Grimmen, Germany.
<sup>d</sup> E. Leclere, Irvy-Sur-Seine, France. The composition of the raw material was given by the suppliers.
inoculation level was high to ensure fast acidification, i.e. at 5.10^7 colony-forming units (cfu)/mL for the proteolytic strains F and L, 10^6 cfu/mL for the nonproteolytic strain P, and for FP and LP, in which P was set to account for 75%. The YAs were incubated at 30 °C until the pH reached 4.7 ± 0.2, then stored for less than 48 h at 4 °C before analyses. A total of 20 different YAs were prepared (Fig. 1), in triplicates.

2.3. Monitoring of acidification and of bacterial growth

Acidification kinetics were established using a wireless iCINAC (AMS, Frépillon, France), to estimate the maximal acidification rates, the slope between pH 5.5 and pH 5 was calculated.

Culturable bacterial counts were determined with appropriate dilutions of samples in 1 g/L tryptone +8.5 g/L NaCl solution in microplates (Baron et al., 2006). L and F were incubated for 24–48 h under aerobic conditions in M17-lactose agar, and P for 48 h anaerobically using CO₂ generators (BD Biosciences, San Jose, USA) in MRS pH 5.4 agar, both at 30 °C.

2.4. Proteolysis

2.4.1. Free amino group dosage

Peptides and free amino acids present in the YAs before and after fermentation were measured in triplicates using the o-phthaldialdehyde (OPA) method of Church et al. (1983) adapted to microplate as described by Canon et al. (2021). The results were expressed as mg of free NH₃ groups/mL with methionine used as a standard.

2.4.2. SDS-PAGE analysis of total protein content

Proteins contained in the different YAs were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) with a molecular weight marker (PrecisionPlus Protein Standards, 250,000, Bio-Rad) was used.

2.5. Physical characterisation of yogurt alternatives

For rheological and textural analyses, YAs were directly prepared in a 54 mm × 70 mm plastic container filled with 40 mL and characterized after incubation overnight at 4 °C. Analyses were performed at 4 °C. The mean of three technical repetitions was taken into account. For WHC measurements, 15 mL centrifuge tubes (Falcon®) were filled with 10 mL and characterized after 30 h at 4 °C.

2.5.1. Rheological properties

YAs were characterized with DHR-2 rheometer, equipped with a plate/plate geometry (DHR2, Stainless Steel, 50 mm Plate, TA Instruments France, 78280 Guynancourt France) with a gap of 1 mm. A stirring step was performed before 6 mL of YA was deposited. Flow curves were obtained at 4 °C prior to a conditioning step of 1 min. The shear rate increased with a linear ramp from 0 to 100 s⁻¹ within 3 min (upward flow curve), was maintained at 100 s⁻¹ for 5 min of holding time, and then linearly decreased from 100 to 0 s⁻¹ within 3 min (downward flow curve). The apparent viscosity at 50 s⁻¹ during the upward flow step was calculated with the TRIOS Software (version #4.1.1.3307, TA Instruments). To determine the level of structure degradation, thixotropy, which is a time-dependent flow behaviour, was determined by calculating the area between the upward and downward flow curves (strain as a function of shear rate) referred to as the hysteresis loop (Mezger, 2006).

2.5.2. Textural analysis

Texture evaluation was performed with a TA1 texture analyzer (Lloyd Instrument, Bognor Regis, England) equipped with a 100 N load cell and a 12 mm cylindrical probe. The depth of immersion was 15 mm at a constant speed of 1 mm s⁻¹. The compression was carried out one time using a trigger force of 0.03 N. Force-time curves were recorded and firmness was calculated with NexigenPlus software (version 3.0, Lloyd Instruments) as the maximum force that occurs when the gel initially breaks [N] (Angeloni and Collar, 2009).

2.5.3. Water-holding capacity

Water holding capacity (WHC) is an indicator of the wheying-off defect (Lacey, 2002). It was evaluated by centrifugation, i.e. 222 g, 15 min, 20 °C (Lesme et al., 2019). The released water was weighed to calculate WHC as the percentage of residual curd to initial weight (Amatayakul et al., 2006).

2.6. Volatile compound analysis

Volatile compounds were extracted with a Turbomatrix HS-40 trap automatic headspace sampler and analyzed using a Clarus 680 gas chromatograph coupled to a Clarus 600T quadrupole mass spectrometer and identified and semi-quantified as described by Canon et al. (2021).

2.7. Organic acid quantification

Lactic, acetic, citric, and succinic acids were analyzed by High-Performance Liquid Chromatography (HPLC, Ultimate 3000, Thermo Fisher Scientific, Waltham, Massachusetts, USA). 1 mL of YA was ultra-filtered with vivaspin 2 centrifugal concentrator columns (10000 MWCO PES, Sartorius) at 8000 g for 1 h at 20 °C. The trials were prepared by a 20-fold dilution of filtrate in H₂SO₄ 0.005 M and stored at −20 °C until analysis. Analysis was run as previously described (Harlé et al., 2020).

2.8. Sensory evaluation

The sensory properties of the YAs, prepared as independent batches, were evaluated using a sorting task as previously described (Leyva Salas et al., 2018; Varela and Ares, 2012), by a panel of 30 untrained judges. YAs were prepared and stored at 4 °C for 7–8 days before sensory evaluation, in order to verify the absence of pathogens by a certified laboratory, Labocea (Combourg, France). Prior to tasting, samples were left for 30–60 min at 8 °C, then aliquots of 10 g of each sample were transferred in disposable cups coded with a 3-digit random number and served at room temperature. Eight products were presented to the judges in random order: the four recipes fermented by L and LP. Panellists were asked to group the samples perceived as the most similar and to give the characteristics they have mainly used to differentiate them.

2.9. Statistical analysis

Analyses of variance (ANOVA) were performed to determine whether acidification rates, physical properties (rheological, textural, and WHC), and the concentrations in volatile compounds and organic acids differed according to the mode of culture (five levels), the type of fat (two levels), and the protein ratio used (two levels) and their interaction, using the R function Anova (R version 3.5.1 (2018-07-02). RStudio. Inc.). The means of three replicates were compared using the Tukey post hoc test from the R package car (p-value < 0.05). Principal component analyses (PCA) of the volatile compounds was performed using the FactoExtra package of R. The sensory evaluation results were analyzed as recommended by Le and Worch (2015) in the R free software using the FactorMineR package by generating a contingency table (descfreq function) that calculated the number of occurrences of each
descriptor in the different samples \((p < 0.2)\). Then, a correspondence analysis (CA) was performed (functions plot.CA).

3. Results

3.1. Bacterial growth and acidification

At the end of the fermentation, \(i.e.\) when the pH reached 4.7, the bacterial counts in each YA reached \(10^9\) cfu/mL for all cultures. At inoculation time, the nonproteolytic strain P represented 75% of the bacterial population in cocultures with L and F, as expected, but its proportion decreased at the end of the fermentation, evidencing that the proteolytic strains F and L grew faster. However, P reached significantly higher proportions when cocultured with F \((56 \pm 10\%)\) compared to L \((33 \pm 9\%)\).

With all cultures except F, the YAs reached the targeted pH of \(4.7 \pm 0.1\), in less than 7 h or 12h, for L and P containing cultures, respectively. The pH of the YAs cultured with F was \(4.93 \pm 0.05\). Accordingly, L and LP acidified faster, followed by the three other cultures (Fig. 2). Protein ratio and fat type did not significantly impact bacterial growth and acidification (Fig. 2).

3.2. Milk and lupin proteolysis and release of free NH\(_2\) groups

Proteolysis and the concentrations in free NH\(_2\) groups varied according to the protein ratio and the LAB cultures used (Fig. 3), but not with the type of fat. There were significantly more free NH\(_2\) groups initially present in unfermented YA manufactured with a protein ratio of 50:50 compared to 67:33 (values indicated in the legend of the Fig. 3). Regarding the fermented YAs, those fermented by the strain F showed a strong hydrolysis of both milk and lupin proteins (Fig. 3A and B). In contrast, in YAs fermented with L, proteolysis was hardly observed, in agreement with a slight decrease in free NH\(_2\) groups compared to the unfermented YA (Fig. 3C). P significantly decreased the content in free NH\(_2\) groups compared to the unfermented preparation (Fig. 3C). YAs fermented by FP contained 27% less free NH\(_2\) groups and showed visually less protein hydrolysis on the SDS-PAGE electrophoregrams than YAs fermented by F. YAs fermented by LP contained slightly less free NH\(_2\) groups than YAs fermented by L and no visual differences in protein hydrolysis were observed on the electrophoregrams.

3.3. Physical properties of the yogurt alternatives

Flow curves were fitted with the power law model and showed the shear-thinning behavior of a non-Newtonian fluid, with a decline in the apparent viscosity as the shear rate increased (results not shown). The apparent viscosity at 50 s\(^{-1}\), which represents the approximate thickness felt in the mouth (Bourne, 2002), ranged from 0.73 to 1.76 Pa s (Fig. 4B). It significantly varied depending on fat type, protein ratio, and LAB culture. The apparent viscosity was significantly higher for YAs made with AMF, at a milk:lupin protein ratio of 67:33, and with L and LP, followed by P, FP, and F. Thixotropy, which represents the loss of structure of the YA during the measurement test, ranged from 1573 to 3349 Pa s\(^{-1}\) (Fig. 4D). It also significantly depended on fat type, protein ratio, and LAB culture. Thixotropy, \(i.e.\) structure degradation was higher in YAs containing AMF, with a protein ratio of 50:50, and fermented with L and LP.

Textural analysis simulates the breakdown of food when taking a spoon of YA, or as it occurs in the mouth, or during processing, and the results have often been correlated with the sensory textural attributes of the product (Bourne, 2002). Among the textural parameters studied, the firmness of the YAs depended on fat type, protein ratio, and LAB cultures (Fig. 4C). The YAs with the protein ratio of 67:33 exhibited higher firmness, and to a lesser extent, the ones fermented by L, P and LP. The water holding capacity (WHC) represents the ability of the gels to retain water. Water was significantly less retained in the presence of AMF and at a protein ratio of 67:33 (Fig. 4E). YAs fermented by F retained water the least, whereas no significant differences were observed between L, P, LP. FP led to an increase in the quantity of water retained in the gel compared to F. An interaction between protein ratio and culture was also observed: more differences were obtained between strains at a protein ratio of 50:50.

3.4. Organic acid produced and consumed in the yogurt alternatives

LAB cultures impacted the concentrations of all quantified organic acids (Fig. 5). All cultures produced lactate, with significantly lower concentrations observed in YAs fermented with F and P compared to the

![Fig. 2. Maximal acidification rates in the yogurt alternatives (dpH/h).](image)
a,b,c: Statistical differences \((p < 0.05)\) between cultures for one given milk:lupin protein ratio (50:50 on the left, 67:33 on the right) and fat type (AMF in pink and COCO in blue).
Fig. 3. SDS-PAGE and global net change in concentration of free NH$_2$ groups in the four YAs fermented by the proteolytic strains L and F and their cocultures with the non-proteolytic strain P. The unfermented YA preparations are presented as controls. Fermentation times ranged from 7 h for L and LP to 12 h for F and FP at 30 °C.

a,b,c,d: Statistical differences between the means observed for LAB cultures for one given protein ratio and fat type. The values of the free NH$_2$ free groups in the unfermented YAs (controls) were as follows: COCO50 = 135 ± 2 mg NH$_2$/mL; COCO67 = 111 ± 13 mg NH$_2$/mL; AMF50 = 131 ± 10 mg NH$_2$/mL and AMF67 = 103 ± 5 mg NH$_2$/mL.
three other LAB cultures. In contrast, acetate concentrations were significantly higher in F-fermented YAs followed by FP, then P, compared to the unfermented preparations, L and LP. F was the only strain to consume citrate and FP resulted in less citrate consumed compared to F. P was the only strain to produce succinate, but succinate was not detected in any of P cocultures. Fat type only impacted citrate concentration (higher with COCO), and protein ratio also influenced citrate concentration (higher with the ratio 67:33) as well as succinate concentrations (higher with the ratio 50:50).

3.5. Volatile compound profiles of the yogurt alternatives

A total of 26 volatile compounds were identified and exhibited significant changes between unfermented preparation and YAs (Table 2). Three compounds were specific to the fat type used and were not impacted by fermentation: 1,3-dimethylbenzene (DMBZ), associated with AMF, and ethyl octanoate (EOA), and 5-hydroxyoctanoic acid lactone (HOAL), associated with COCO. DMBZ, which is associated with a plastic aroma, most likely originated from the packaging in which AMF was stored. Eight volatile compounds, 2-pentanone (PTN), nonanal (NAN), 2-pentylfuran (PF), octanal (O), 1,3-di-tert-butylbenzene (BBN), heptanal (H), and 3-methylbutanal (MBT) were reduced from 3.6 to 44.7 times in YAs compared to the unfermented preparations (controls). They include aldehydes associated with a “green” flavour. Their concentrations were significantly higher in YAs with a protein ratio of 50:50. P decreased significantly more the concentrations in MBT, HP and H compared to F and L, while F decreased significantly less all these compounds compared to the four other cultures. Benzaldehyde (BZH), 2-butanone (BTN), acetoin (AC), 3-methyl-1-butanol (MB) and 1-hexanol (HX) concentrations were significantly higher in YAs with a 50:50 protein ratio and BTN and butanoic acid (BA) concentrations were significantly higher when AMF was used instead of COCO. Concentrations in 2-nitroethyl propionate (NP), 2-methylpropionic acid (MPA), hexanoic acid (HA), octanoic acid (OA) and nonanoic acid were significantly lower in YAs, regardless of the type of fat or protein ratio.
acid increased from 6 to 49-fold between the controls and YAs, with no distinction between cultures. The highest positive fold-changes between cultures and controls varied from ~2.4 for BTN to ~427 for AC. The greatest fold changes were observed with F, FP and/or LP for most (10 out of 14) volatiles (Table 2).

F was the only strain to produce cyclohexanecarboxylic acid (CHCA), associated with fruity, acidic, and metallic flavors, and produced significantly more BTN and A, associated with fruity and pungent flavors, respectively, compared to L and P. P produced significantly more diacetyl (D), associated with a buttery flavor, while L produced significantly more dimethyl sulfone (DS, sulfurous flavor) and less MB (fusel oil, alcoholic flavors), AC (buttery flavor) and HX (ethereal and fruity flavors) compared to F and P. No coculture contributed in a significant change in concentration of any volatile compounds, compared to the monocultures of the two strains composing it.

A principal component analysis (PCA) (Fig. 6) was performed to summarize the differences in volatile profiles between the YAs prepared. The first two PCs accounted for 56.7% of the total variability. The biological replicates of the cultures appeared closely localized, demonstrating the good global reproducibility of the experiments and analyses. The PC1-PC2 plan differentiated the three monocultures, on the basis of the concentration of AC, acetic acid (A), CHCA, MB, and D. The cocultures exhibited intermediary profiles. The plan also differentiated the YAs based on the concentration of three volatile compounds specific to AMF or to COCO as indicated above. The protein ratios were not differentiated on the first two dimensions of PCA.

3.6. Sensory evaluation of eight selected yoghurt alternatives by a sorting task

The correspondence analysis (CA) map built from sensory evaluation data of the eight YAs fermented with L and LP separates YAs on dimension 1 and 2 according to the protein ratios and the fat type, respectively (Fig. 7). The LAB cultures were, in contrast, not differentiated on this map. The YAs manufactured with the protein ratio of 67:33, negatively associated with dimension 1, were characterized as pleasant, textured (hard gel) and nonhomogenous, while the ones with the protein ratio of 50:50, positively associated with dimension 1, were described as unpleasant, bitter, and with a mellow texture. The YAs containing AMF, positively associated with dimension 2, were described as milky, lactic and “goaty”, and at the opposite, the YAs containing COCO, were described as fruity, fresh and nutty. The coconut flavor was also well identified.

4. Discussion

The aim of this study was to evaluate the impact of different formulations and LAB cultures on the physico-chemical and sensorial properties of YAs. Formulations included two fat types: AMF and COCO, two milk:lupin protein ratios: 50:50 and 67:33. Cultures were mono- and cocultures of strains known to interact positively in a chemically defined medium (CDM) (Canon et al., 2021).

The different LAB cultures used impacted the final YA...
characteristics, according to the strain properties. All strains grew in monocultures in the YAs prepared in the present study, while the non-proteolytic strain P was previously shown as unable to grow in a CDM with milk and lupin proteins as the sole nitrogen sources (Canon et al., 2021). This is due to the presence of peptides and free amino acids, coming from milk and lupin isolate, in sufficient amount in the YAs to sustain the growth of P, in contrast to CDM. The YAs fermented by the proteolytic *E. faecalis* strain, F, differed from the others, because of the slow and limited acidifying-capacity of this strain and its high proteolytic activity. Actually, the acidification rates of F were twice slower than those of L (Fig. 2), as previously observed in CDM (Canon et al., 2021), and the final pH reached higher value (Fig. 4). Consequently, the F-fermented YAs showed intermediary properties between that of F- and P-fermented YAs (Figs. 2 – 4). Sensory analysis by the trained panelists revealed that the sensory characteristics of the F-fermented YAs were closer to those of the P-fermented YAs than to those of L-fermented YAs.

Table 2

| Compound                     | m/z | Ab. | Identification | Associated aroma (theogoodscents.com) | CAS n° | LRI | Max fold change | YA with the max fold change |
|------------------------------|-----|-----|----------------|----------------------------------------|--------|-----|----------------|-----------------------------|
| 2-Butanone                   | 72  | BTN | DB, LRI        | Ethereal, fruity                        | 78-93-3 | 861 | 2.4            | F, AMF50                    |
| 3-Methylbutanal              | 58  | MBT | S, DB, LRI     | Ethereal, aldehydic                     | 590-86-3 | 886 | 44.7          | OCDC60                      |
| 2-Pentanoate                 | 71  | PTN | LRI            | Sweet, fruity                           | 107-87-9 | 961 | 3.6            | AMF, B50                   |
| Niacin                       | 41  | NNL | DB, LRI        | Sweet, creamy, buttery                  | 431-03-8 | 972 | 7.5            | P, AMF50                    |
| Hexanal                      | 44  | H   | DB, LRI        | Green                                   | 66-25-1 | 1068 | 40.2          | OCDC60                      |
| 1.3-Dimethylbenzene          | 106 | DMBZ| LRI            | Plastic                                 | 108-38-3 | 1113 | 30.2 *         | AMF67                      |
| Heptanal                     | 70  | HP  | LRI            | Green                                   | 111-71-7 | 1180 | 25.6          | AMF50                      |
| 3-Methyl-1-butanol           | 70  | MB  | LRI            | Fuel oil, alcoholic                      | 123-51-3 | 1213 | 24.9          | F, AMF50                    |
| 2-pentylfuran                | 138 | PF  | DB, LRI        | Earthy, beany                           | 5777-69-0 | 1224 | 14.9          | AMF50                      |
| Acetoin                      | 88  | AC  | DB, LRI        | Milky, butter                           | 513-86-0 | 1269 | 427.5         | P, AMF50                    |
| Octan-10-ol                  | 84  | O   | DB, LRI        | Milky, buttery                          | 124-13-0 | 1274 | 17.0          | OCDC60                      |
| 1-Hexanal                    | 56  |HX  | LRI            | Ethereal, fruity, alcoholic              | 111-27-3 | 1356 | 4.1           | F, AMF50                    |
| Nonanal                      | 41  | NNL | DB, LRI        | Waxy, aldehydic                         | 124-19-6 | 1376 | 11.7          | OCDC60                      |
| 1.3-Di-tert-butylbenzene     | 175 | BBN | DB, LRI        | ND                                      | 1014-60-0 | 1413 | 19.7          | AMF50                      |
| Ethyl octanoate              | 88  | EO  | DB, LRI        | Waxy, sweet, fruity                      | 106-32-1 | 1427 | 180.5 *        | OCDC60                      |
| Acetic acid                  | 60  | A   | D, LRI         | Fupgent, sour, overripe fruit            | 64-19-7 | 1453 | 23.8          | FP, AMF67                  |
| Benzaldehyde                 | 51  | BZI | S, LRI         | Nutty                                   | 100-52-7 | 1523 | 7.3           | P, AMF50                    |
| 2-Nitroethyl propionate      | 30  | NP  | DB             | ND                                      | 1545-5.7 | FP, AMF67 |
| 2-methylpropanoic acid       | 88  | MPA | DB, LRI        | Buttery, rancid                         | 79-31-2 | 1566 | 49.3          | LP, AMF50                   |
| Butanoic acid                | 73  | BA  | S, DB, LRI     | Cheesy, buttery                         | 107-92-6 | 1610 | 26.3          | FP, AMF67                   |
| Hexanoic acid                | 73  | HA  | S, DB, LRI     | Cheesy, fruity phenolic                  | 142-62-1 | 1815 | 15.5          | LP, AMF50                   |
| Dimethyl sulfone             | 94  | DS  | DB             | Sulfurous, burnt                        | 67-71-6 | 1850 | 5.5           | L, COCOS6                    |
| 5-Hydroxocyclohexanol acid   | 99  | HOAL| DB             | Sweet, coconut, creamy                   | 698-76-0 | 1870 | 47.9 *        | L, COCOS6                   |
| Octanoic acid                | 73  | OA  | S, DB, LRI     | Rancid, sticky                           | 124-07-2 | 2011 | 11.6          | L, COCOS6                   |
| Cyclohexanecarboxylic acid   | 55  | CHCA| DB             | Fruity, acidic, metallic                 | 98-49-5 | 2017 | 14.7          | FP, AMF50                   |
| Nonanoic acid                | 73  | NA  | DB             | Fatty, waxy and cheesy                   | 112-05-0 | 2038 | 13.1          | P, AMF50                    |

F = *E. faecalis* CIRM-BIA2412, L = *L. lactis* NCDO2125, P = *L. plantarum* CIRM-BIA1524.

Fermentation times ranged from 7 h for L and FP to 12 h for F and PP at 30 °C. Only the volatile compounds that showed significant difference in abundance between YAs and unfermented preparations (controls), and/or between YAs were selected. Compound were named according to the approximate retention index (R.I.) as determined in the mass spectral data library of the National Institute of Standards and Technology (NIST). Max fold change: maximal ratio of abundance between cultures and unfermented preparation, except for the values marked with * for which the ratio depends only on the ingredients used in the preparations.

For yogurt alternative (YA) codes see Table 1.
Figure 6. Principal component analysis of the volatile compounds identified in the yogurt alternatives. Variable map of the first two dimensions of the principal component analysis performed using the abundance of 26 selected volatile compounds, after log transformation and Pareto scaling, observed in 12 yogurt alternatives prepared according to the four formulations described in Table 1 and fermented at 30°C for 7-12 h by the five following cultures: L (L. lactis NCDO2125), F (E. faecalis CIRM-BIA2412), P (L. plantarum CIRM-BIA1524), and the cocultures LP and FP. Replicate experiments are represented using the same symbols. For the compound abbreviations see Table 2. The ellipses are drawn around the group mean point with a confidence level of 0.95 (package FactoMineR).

were very similar to the YAs fermented by L alone (Figs. 2-6), including on the sensory properties (Fig. 7). The different fermentation times, 7 h with L vs 12 h with F might also have influenced P growth, favoured in FP coculture because the medium stayed not too acid for a longer time. In addition, the high proteolytic activity expressed by F could result in the production of bioactive peptides and the decrease in protein allergenicity (Worsztynowicz et al., 2019) leading to FP-fermented YAs a potential added value with multifunctional properties compared to P and F taken individually. Finally, LAB strains also differed in the production and conversion of flavour compounds. F degraded less undesirable volatile compounds such as hexanal, associated with a “green” flavour. Both F and P produced diacetyl, acetoin which is in accordance with citrate utilization (Fig. 5) (Gänzle, 2015). FP-fermented YAs showed intermediary properties between that of F- and P-fermented YAs, thus leading to added functionalities, while LP-fermented YAs showed very similar properties in terms of final pH, texture, and sensory properties.

The type of fat did not influence bacterial growth, acidification rate, and proteolysis intensity. However it impacted the physical properties of the YAs: AMF gave firmer, more structured and viscous products, compared to COCO. These results are expected from the thermo-physical properties of AMF and COCO, since AMF is firmer and viscous at 4°C due to a higher melting point (Devi and Khatkar, 2017). They are also in accordance with the results observed by Barrantes et al. (1996), who compared the textural properties of set-type yogurts made with AMF or vegetable oils. Surprisingly, YAs made with COCO had a higher WHC. It
might result in a better capture of water in the gel protein network and filling of the gel pores with the fat droplets. The size of fat droplets was measured after heat treatment and did not differ between COCO and AMF (data not shown). We thus hypothesize that the interface formed by the proteins surrounding the fat droplets during the emulsification step could differ depending on the two types of fat. This could lead to different interactions between the protein network and the fat droplets, thus rendering the YAs made with COCO less subjected to gel contraction compared to AMF. It is well admitted that caseins are more willing to go to the interface, able to unfold for covering the fat droplets and in turn stabilize the fat droplet, in contrast to whey proteins (Chevallier et al., 2019). We do not have such information regarding lupin proteins, yet. We did not characterize the interface composition in the present study but it could help further understanding the effect of fat type notably on WHC. The volatile compounds profile also distinguished COCO and AMF with the presence of some compounds, markers of fat type (Table 2, Fig. 6). The type of fat was also sensorially distinguished, although no preference was expressed by the panellists between COCO and AMF (Fig. 7).

Protein ratios did not impact bacterial growth and acidification rates. However, proteins were more hydrolysed in YAs containing more milk protein (Fig. 3), likely because milk caseins, which are unstructured proteins, are more easily hydrolysed than globular proteins such as whey and lupin proteins. Protein ratios significantly impacted texture: ratio 67:33 led to firmer and more viscous YAs and higher WHC. The YAs prepared with the ratio 67:33 contained more whey proteins, which, when denatured after heat treatment, interact with caseins and participate in the formation of a firmer network (Donato and Guyomarc’h, 2009; Loveday et al., 2013). The lupin proteins are globular proteins with an isoelectric point near pH 4.6 (Duranti et al., 2008), close to that of the caseins. The latter can be used as molecular chaperones of whey and lupin proteins, to facilitate the solubilization of lupin protein and insure the thermal stability of both lupin and whey proteins (Yong and Foegeding, 2010). Lupin proteins as well as whey proteins are able to form aggregates upon heating (Berghout et al., 2015; Nicolai, 2019). Their capability to form thermal-induced aggregates in the case of the process of yogurt depends on the type of proteins. This can change the capability of gelation with firmer gels observed for whey proteins compared to soy and lupin proteins (Berghout et al., 2015; Nicolai, 2019; Roesch and Corredig, 2006). However, we cannot conclude from our experiments whether the whey and the lupin proteins can co-aggregate in YAs and interact with caseins as usually observed (Donato and Guyomarc’h, 2009) or just act as space filler into the gel. Thixotropy, which is an indicator of structure degradation, was higher in the YAs prepared with a protein ratio of 50:50. The initial shear stress was higher with the ratio 67:33, indicating that the YAs with the protein ratio 50:50 were initially less structured and more sensitive to shearing. Besides, regarding aroma formation, YAs with the 50:50 ratio also showed a higher concentrations in some volatile compounds associated with a “green” aroma, which could lead to an unpleasant flavour. The YAs prepared with the 67:33 ratio contained more citrate, a precursor of some desirable aroma compounds (Bintsis, 2018). The ratio 67:33 appears thus as globally preferable to obtain more desirable aroma profile and appropriate firmness.

Results from sensory analysis agreed well with those observed from instrumental analyses, notably regarding the “green” attributes, also associated with the 50:50 protein ratio (Table 2, Fig. 7). Sensory evaluation did not distinguish L and LP, which is in agreement with the fact that their volatile profiles did not significantly differ. Texture attributes such as “firm” and “hard” were also mentioned, to describe more specifically the YAs prepared with the 67:33 ratio, which is consistent with their higher firmness and viscosity. The YAs prepared with the 50:50 ratio were perceived as less acceptable by the panellists compared to the 67:33, which agrees with the fact that lupin isolate contains undesirable “green” flavour compounds. Similarly, ratios of milk:pea proteins of 70:30 and 60:40 were previously found optimal for acceptability (Youssef et al., 2016).

5. Conclusion

In LAB cocultures, both strains contributed to the final properties of the YAs when positive interactions occurred, i.e. when the proteolytic
strain stimulated the other, non-proteolytic, strain. The strains used in this study were chosen on the basis of their interactions previously shown in a CDM (Canon et al., 2021). It would be interesting to extend the screening to find strains that are able to interact and produce more sensory properties. It is possible to use flavorless coconut oil if its specific flavor or different aroma compounds and textural agents such as exopolysaccharides. Textural and sensory analyses showed that YAs manufactured with a milk-lupin protein ratio of 67:33 were more acceptable compared to the 50:50 ones. More knowledge on the effect of pre-treatment such as homogenization and preheating of the protein suspension and on the network formation between these proteins is needed to be able to improve the texture with a higher content in lupin. Using LAB strains producing more or different aroma compounds could also increase the acceptability of the YAs at a milk-lupin ratio of 50:50. Fat content is an important factor for the acceptability of yogurts. Coconut oil could substitute milk fat as it did not negatively impact the acceptability of lupin protein isolates. The strains used in this work reported in this paper.

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.

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