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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

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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 were able to interact positively in a chemically defined medium containing milk and lupin proteins as sole nitrogen source. Positive interactions were favoured by the proteolytic activity of LAB strains, which furnished peptides and amino acids to nonproteolytic strains. The aim of this study was to evaluate the impact of different formulations, including two fat types: anhydrous milk fat (AMF) and coconut oil (COCO) and two milk:lupin protein ratios: 50:50 and 67:33, as well as 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/stlo_eng), and *Lactococcus lactis* NCDO2125 (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 galacto-oligosaccharides (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 (MRS) for lactiplantibacilli (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, Eurial, Nantes, France) was used to reconstitute milk at 3.3% (w/w) of proteins and a whey protein isolate (Ingrédia, 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 TM5 bowl (Vorwerk, Wuppertal, Germany). The mix was prehomogenized using an ultra-turrax at 24,000 rpm for 10 min in a water-bath at 65 °C, then homogenized at 250/50 bar (× 10^5 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 TM5 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

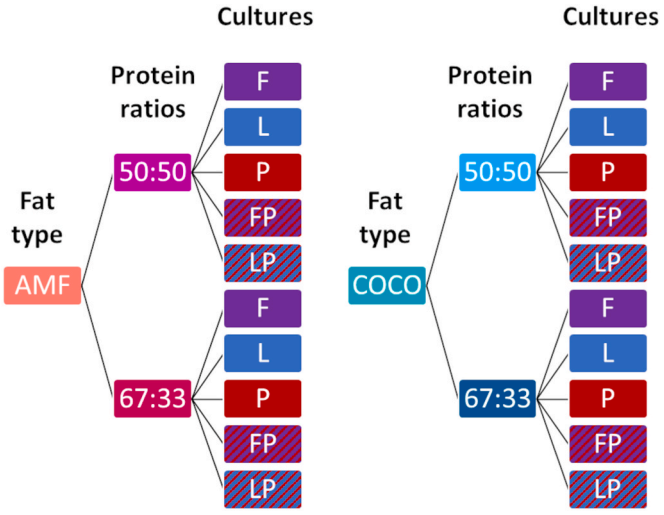

Fig. 1. Experimental plan containing three factors: 1) fat type with two levels: anhydrous milk fat (AMF) or coconut oil (COCO), 2) milk:lupin protein ratios with two levels: 50:50 or 67:33 and 3) Cultures with 5 levels: *Lactococcus lactis* NCDO2125 (L), *Enterococcus faecalis* CIRM-BIA2412 (F), and *Lactiplantibacillus plantarum* CIRM-BIA1524 (P) in monocultures and the cocultures FP and LP. The 20 combinations were prepared and analyzed in triplicates.

Table 1
Composition of the four set-type yoghurt alternatives (YAs, in g/kg).

	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[a]	32.6	55.4	0.5	101	101	101	101
Whey protein isolate[b]	85.1	5.5	1.0	0	13	0	13
Lupin protein isolate[c]	88.7	0.5	3.0	37	25	37	25
Coconut oil[d]	0	0	100	0	0	15	15
Anhydrous milk fat[a]	0.1	0	99.8	15	15	0	0
Sucrose[d]	0	100	0	10	10	10	10
Distilled water	-	-	-	*qs* 1 kg	*qs* 1 kg	*qs* 1 kg	*qs* 1 kg

[a] Eurial, Nantes, France.
[b] Ingrédia, Arras, France.
[c] Prolupin GmbH., Grimmen, Germany.
[d] E. Leclerc, Ivry-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, 10^8 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 CO_2 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-phtaldialdehyde (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 NH_2 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 12% acrylamide gel (Bio-Rad, Marnes-la-Coquette, France) under reducing conditions. Each YA was mixed with the sample buffer (2:1) (1.2 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS (Bio-Rad, Hercules, CA, USA), 1% (v/v) DTT (Sigma-Aldrich, St. Louis, MO, USA). 20% (v/v) glycerol (Prolabo, VWR International, West Chester, PA, USA) and 0.02% (w/v) bromophenol blue. 15 µL were loaded per lane. A standard molecular weight marker (PrecisionPlus Protein Standards, 10,000–250,000, Bio-Rad) was used.

2.5. Physical characterization 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 Guyancourt France) with a gap of 1 mm. A stirring step was performed before 6 mL of YA were 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 (Llyod 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 Nexigen*Plus* software (version 3.0, Lloyd Instruments) as the maximum force that occurs when the gel initially breaks [N] (Angioloni and Collar, 2009).

2.5.3. Water-holding capacity

Water holding capacity (WHC) is an indicator of the wheying-off defect (Lucey, 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 ultrafiltered 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 H_2SO_4 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 sensorial 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 ± 10%) compared to L (33 ± 9%).

With all cultures except F, the YAs reached the targeted pH of 4.7 ± 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 ± 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

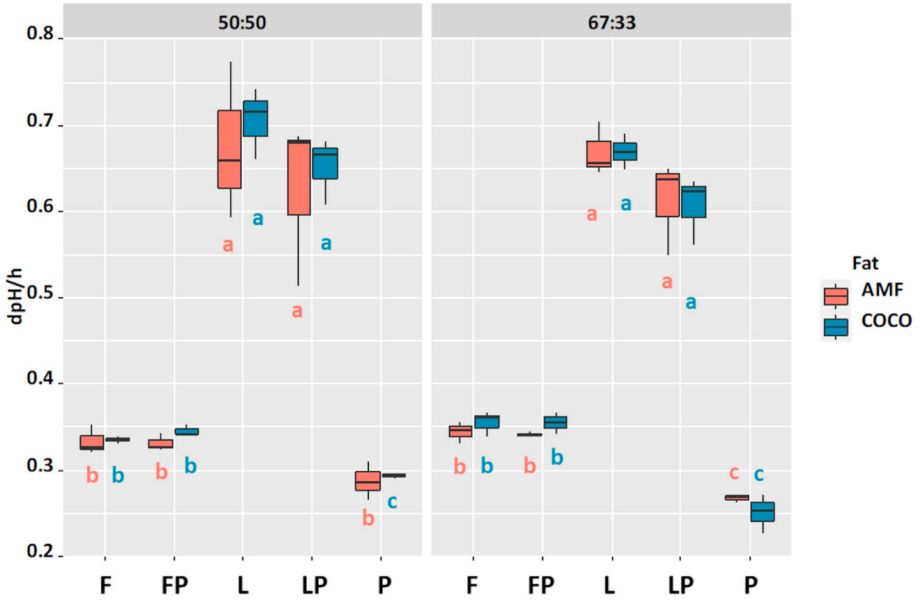

Fig. 2. Maximal acidification rates in the yogurt alternatives (dpH/h).
a,b,c: Statistical differences ($p < 0.05$) between cultures for one given milk:lupin protein ratio (50:50 on the left, 67:33 on the right) and fat type (AMF in pink and COCO in blue).

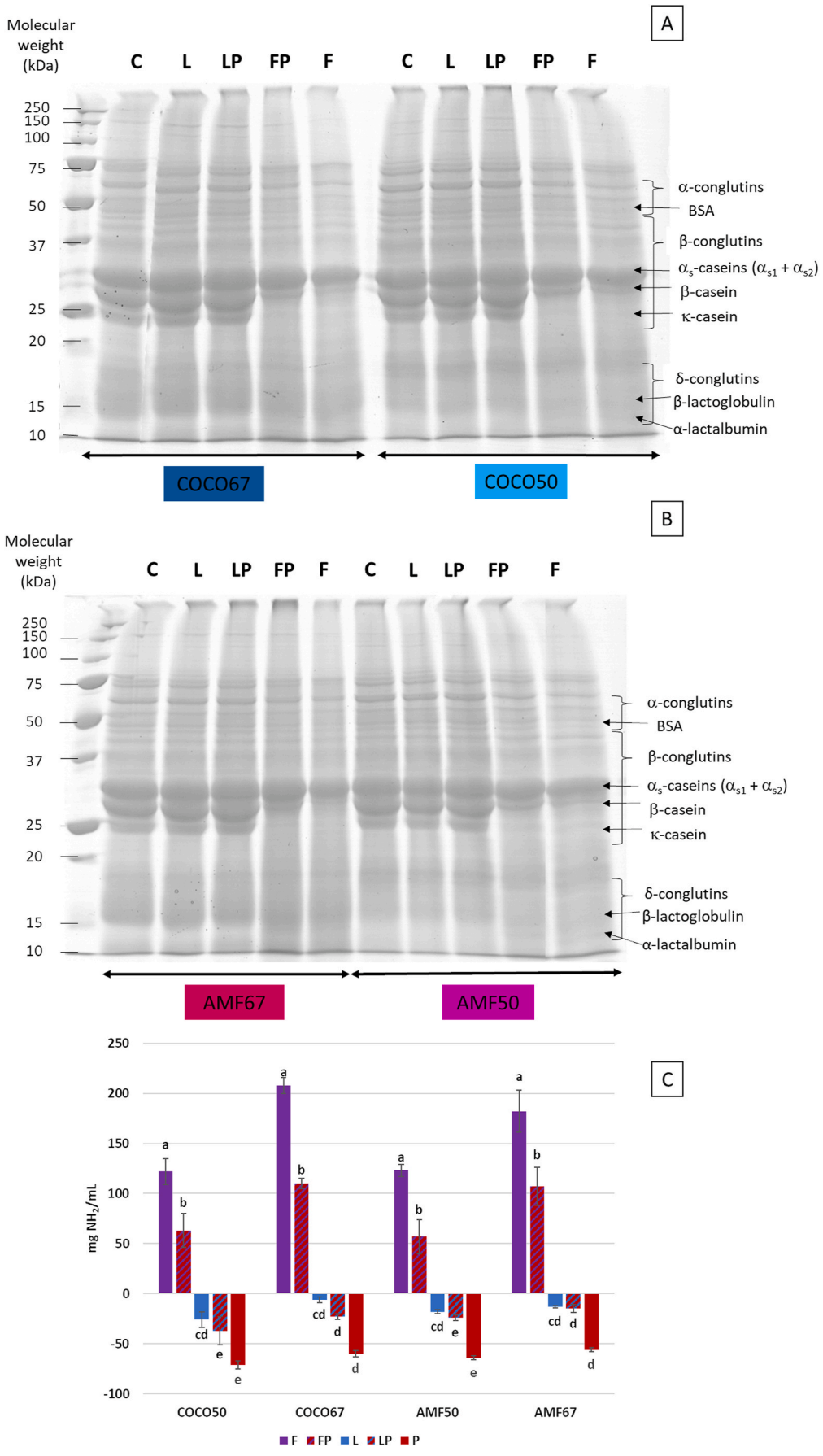

Fig. 3. SDS-PAGE and global net change in concentration of free NH_2 groups in the four YAs fermented by the proteolytic strains L and F and their cocultures with the non-proteolytic strain P. The unfermented YA preparations are presented as controls. Fermentation times ranged from 7 h for L and LP to 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.

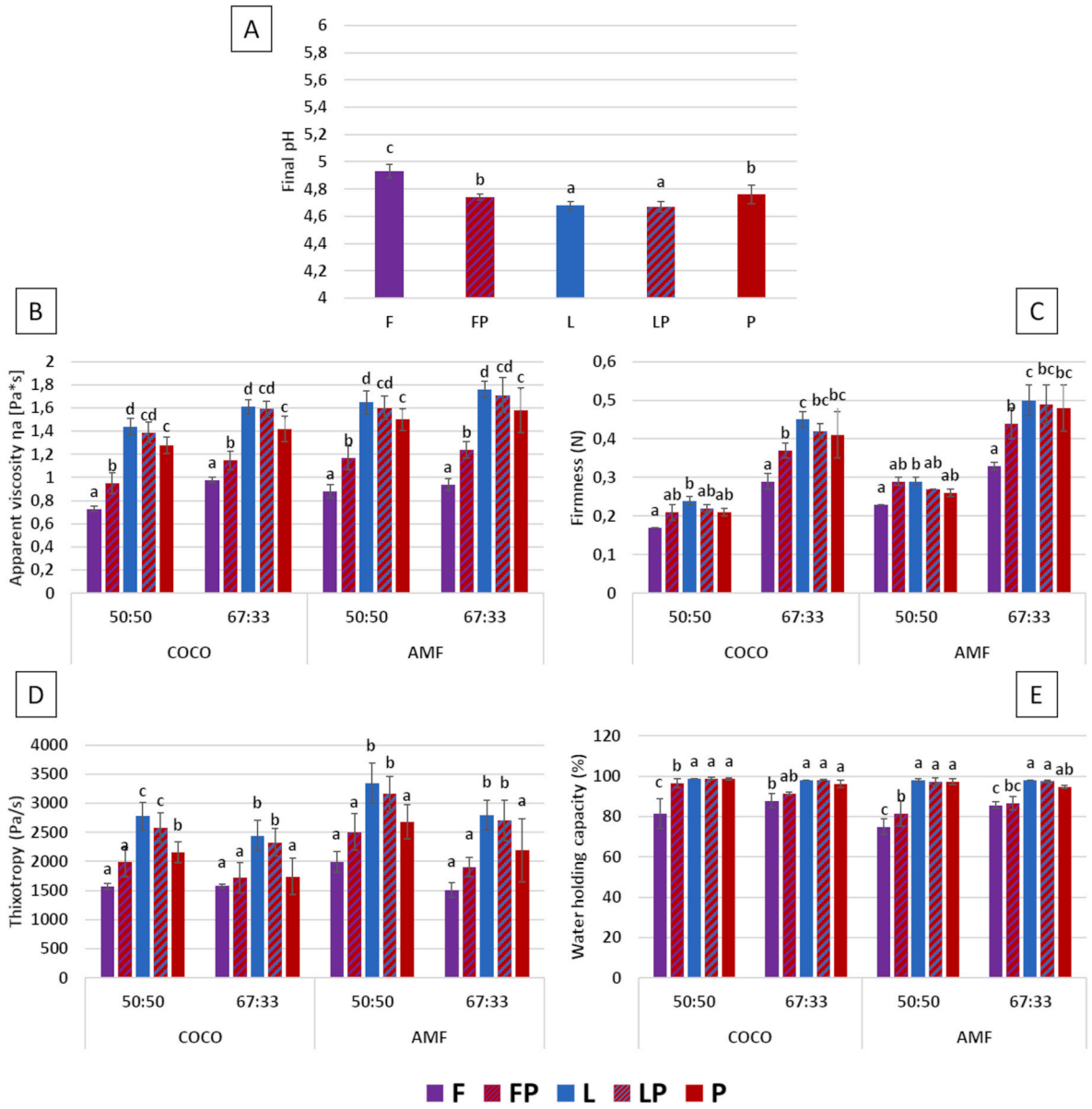

Fig. 4. pH and textural properties of the yogurt alternatives (YA). A) pH observed at the end of fermentation time for the YA inoculated with *Lactococcus lactis* NCDO2125 (L), *Enterococcus faecalis* CIRM-BIA2412 (F), and *Lactiplantibacillus plantarum* CIRM-BIA1524 (P) in monocultures and the co-cultures FP and LP, a,b,c: Statistical differences ($p < 0.05$) between cultures; textural properties with B) apparent viscosity, C) firmness, D) thixotropy and E) water holding capacity. a,b,c,d: Statistical differences between the means observed for LAB cultures for one given protein ratio and fat type.

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 ethly 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 (NNL), 2-pentylfuran (PF), octanal (O), 1,3-di-tert-butylbenzene (BBN), heptanal (HP), hexanal (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-methylpropanoic acid (MPA), hexanoic acid (HA), octanoic acid (OA) and nonanoic

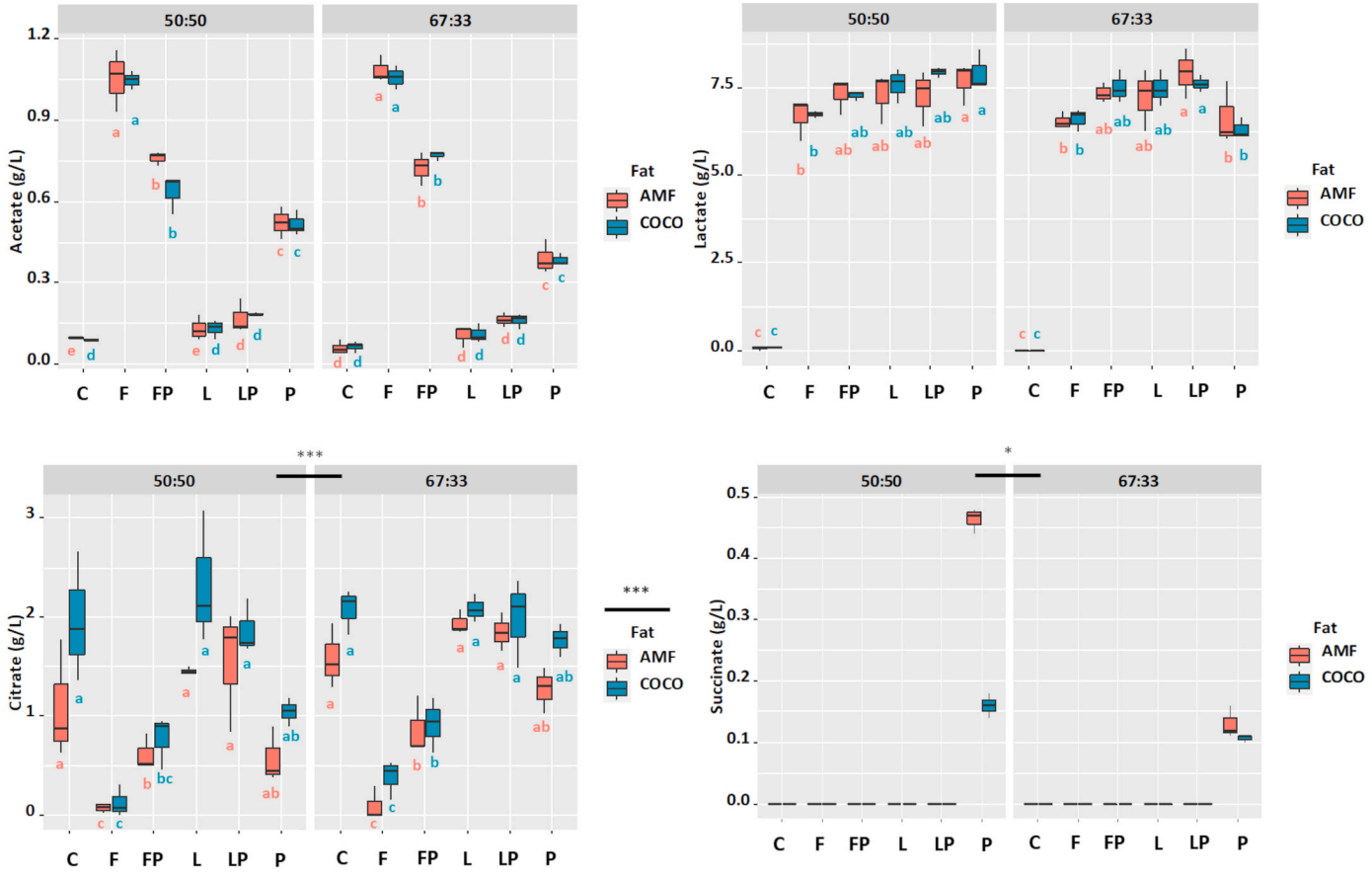

Fig. 5. Concentrations in acetate, lactate, citrate and succinate (g/L) in all the YAs and controls (unfermented milk and lupin mixes). Fermentation times ranged from 7 h for L and LP to 12 h for F and FP at 30 °C.
Statistical differences ($p < 0.05$) between cultures for one given milk:lupin protein ratio (50:50 on the left, 67:33 on the right) and fat type (AMF in pink and COCO in blue).
* represents a significant effect of fat type or protein ratio.
The names of the strains referred to *Lactococcus lactis* NCDO2125 (L), *Enterococcus faecalis* CIRM-BIA2412 (F), and *Lactiplantibacillus plantarum* CIRM-BIA1524 (P) in monocultures and the co-cultures FP and LP.

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 co-cultures 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 flavour 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

Table 2
Selected volatile compounds identified in yogurt alternatives after 7–12 h of fermentation at 30 °C with the strains *E. faecalis* CIRM-BIA2412, *L. lactis* NCDO2125, *L. plantarum* CIRM-BIA1524, in monocultures and the cocultures of *L. plantarum* with *E. faecalis* or *L. lactis*.

Compound	m/z	Ab.	Identification	Associated aroma (thegoodscentscompany.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	COCO50
2-Pentanone	71	PTN	LRI	Sweet, fruity	107-87-9	961	3.6	AMF_R50
Diacetyl	86	D	LRI	Sweet, creamy, buttery	431-03-8	972	7.5	P_AMF50
Hexanal	44	H	DB. LRI	Green	66-25-1	1068	40.2	COCO50
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	Fusel oil, alcoholic	123-51-3	1213	24.9	F_AMF50
2-pentylfuran	138	PF	DB, LRI	Earthy, beany	3777-69-3	1224	14.9	AMF50
Acetoin	88	AC	DB, LRI	Milky, buttery	513-86-0	1269	427.5	P_AMF50
Octanal	84	O	DB, LRI	Green	124-13-0	1274	17.0	COCO50
1-Hexanol	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	COCO50
1.3-Di-tert-butylbenzene	175	BBN	DB, LRI	ND	1014-60-4	1413	19.7	AMF50
Ethyl octanoate	88	EOA	DB, LRI	Waxy, sweet, musty, pineapple	106-32-1	1427	180.5 *	COCO67
Acetic acid	60	A	D, LRI	Pungent, sour, overripe fruit	64-19-7	1453	23.8	FP_AMF67
Benzaldehyde	51	BZH	S, LRI	Nutty	100-52-7	1523	7.3	F_AMF50
2-Nitroethyl propionate	30	NP	DB	ND	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, fruity	107-92-6	1610	26.3	FP_AM67
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	67-71-0	1840	5.5	L_COCO67
5-Hydroxyoctanoic acid lactone	99	HOAL	DB	Sweet, coconut, creamy	698-76-0	1870	47.9 *	L_COCO50
Octanoic acid	73	OA	S, DB, LRI	Rancid, soapy, cheesy	124-07-2	2011	11.6	P_COCO50
Cyclohexanecarboxylic acid	55	CHCA	DB	Fruity, acidic, metallic	98-89-5	2017	14.7	FP_AMF67
Nonanoic acid	73	NA	DB	Fatty, waxy and cheesy	112-05-0	2038	13.1	FP_AMF50

F = *E. faecalis* CIRM-BIA2412, L = *L. lactis* NCDO2125, P = *L. plantarum* CIRM-BIA1524.
Fermentation times ranged from 7 h for L and LP to 12 h for F and FP 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 IUPAC (International Union of Pure and Applied Chemistry) nomenclature, Ab = abbreviation of volatile compound name used in PCA (Fig. 6). CAS=Chemical Abstract Service registry number; Identification: compounds identified on the basis of: S = Retention time and mass spectrum from standard, LRI = Linear retention index, DB = 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.

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 had lower apparent viscosity at 50 s^{-1}, thixotropy, firmness, and WHC. These results agree well with the results of Körzendörfer and Hinrichs (2019), who demonstrated that a higher value of pH, 5 or 4.8, leads to less firm and viscous gels compared to gels at pH 4.6. Interactions between caseins are known to depend on the pH. The acid functional groups of certain amino acids, such as glutamic and aspartic acids and phosphoserine, fix the protons formed, leading to a progressive cancellation of the negative charge of the micelles, the formation of hydrophobic and electrostatic interactions between micelles, and thus the formation of a so-called "lactic" gel. The high final pH value of the F-fermented YAs can also represent a safety risk, as the low pH derived from the production of organic acids is crucial in pathogen inhibition, especially in a YA containing lupin protein isolate, in which *Bacillus cereus* can be present (Fritsch et al., 2015). In the case of yogurts, pH values range from 4.5 to 4.8, which is the usual target considered for having safe products (Corrieu and Béal, 2016).

We also hypothesize that the high proteolysis observed in the F-fermented YAs (Fig. 3A and B) leads to more peptides and free amino acids, as shown by a higher level of free NH_2 released (Fig. 3C) and therefore could have modified the textural properties. This result is in line with the lower firmness, viscosity, and WHC observed in these YAs, which can be attributed to lower peptide-water and peptide-peptide interactions between the fat droplets and/or the protein network than the protein-protein interactions (Lacou et al., 2016). In contrast, the YAs fermented by the proteolytic *L. lactis* strain, L, had the lowest pH and were characterised by the highest apparent viscosity, thixotropy, firmness, and WHC, as expected. The strain L, despite its potential proteolytic properties, did not express this capacity in YA, as shown by SDS PAGE protein profiles and global net decrease in concentration of free NH_2 groups (Fig. 3). This result can be explained by two related factors: YAs initially contained enough peptides and/or free amino acids to sustain L growth, and L in turn likely repressed its protease expression, as previously observed for another *L. lactis* strain, for which the expression of both the *prtP* and *prtM* genes encoding for the cell-envelop protease is controlled at the transcriptional level by the peptide concentration in the medium (Guédon et al., 2001; Meijer et al., 1996).

As a consequence of the difference in expression of their proteolytic activity, F and L also differently stimulated the growth of the non-proteolytic strain, P. In FP coculture, P growth was stimulated by the peptides and free amino acids provided (Fig. 3), and the resulting YAs showed intermediary properties between that of F- and P-fermented YAs (Figs. 2–6). In contrast, in LP coculture, P grew less and the resulting YAs

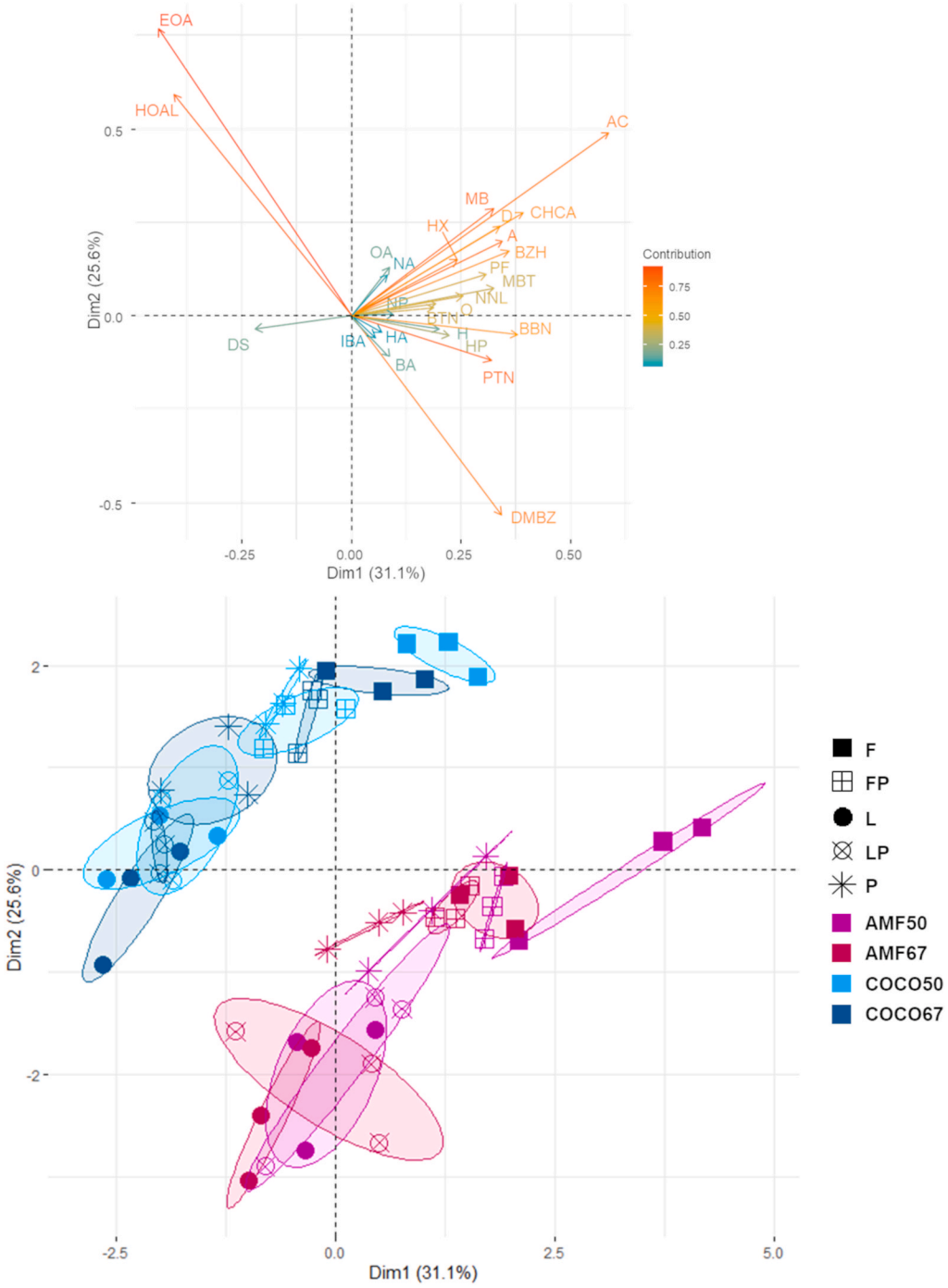

Fig. 6. Principal component analysis of the volatile compounds identified in the yogurt alternatives.
Variable map of the first two dimensions of the principal component analysis performed using the abundance of 26 selected volatile compounds, after log transformation and Pareto scaling, observed in 12 yogurt alternatives prepared according to the four formulations described in Table 1 and fermented at 30 °C for 7–12h 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 vegetal oils. Surprisingly, YAs made with COCO had a higher WHC. It

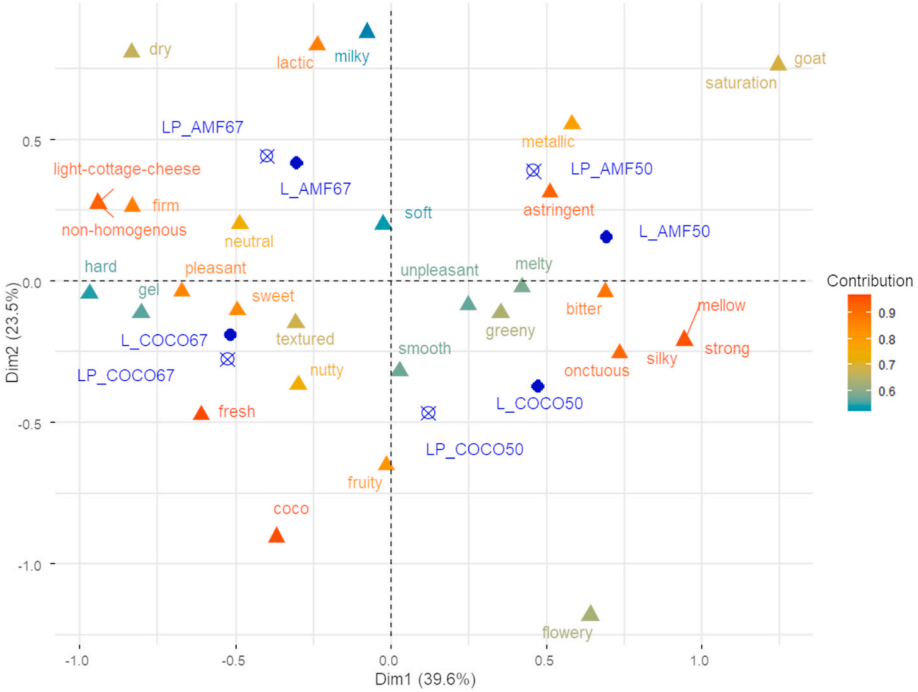

Fig. 7. Correspondence analysis map of sensory evaluation data obtained for eight yogurt alternatives submitted to a free sorting task. Only the contributions of the best attributes are represented (cos2 > 0.5). The eight yoghurt alternatives were prepared according to the four formulations described in Table 1 and fermented at 30 °C by L (*L. lactis* NCDO2125) and LP (*L. lactis* NCDO2125. in coculture with *L. plantarum* CIRM-BIA1524) until the pH reached 4.7 ± 0.2.

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 firmess.

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 (Yousseef 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 and/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 sensory properties. It is possible to use flavorless coconut oil if its specific flavor is an issue. This study was done in the context of food transition that requires incorporating more plant-based ingredients in our diet. The mixed dairy and plant-based YAs are interesting for the consumers starting the transition as it softly gets them acquainted with the unfamiliar properties of plant-based YAs.

CRediT authorship contribution statement

Fanny Canon: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Marie-Bernadette Maillard:** Investigation. **Marie-Hélène Famelart:** Validation, Writing – review & editing. **Anne Thierry:** 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.

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