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Insect flour as milk protein substitute in fermented dairy products

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ABSTRACT

Fermented dairy products (FDP) are consumed worldwide, due to their nutritional attributes and sensory properties. In the last decade, edible insects have been the subject of research as a sustainable source of protein to be introduced into the food industry. The mealworm (*Tenebrio molitor*) and buffalo worm (*Alphitobius diaperinus*) have demonstrated the highest consumer potential. However, this study aims to demonstrate the effectiveness of other possible options such as black soldier fly (BSF) and drone brood (DB) flours in FDP, with or without probiotics, as a viable substitute of milk powder during refrigerated storage for 21 days. The nutritional composition of the products remained unchanged despite the addition of insect flour, with a fat content of ~0.95% and a protein content of ~4.7%. The products acidity increased from 0.78% to 1.06% lactic acid during storage. The colour of the FDP was typical yellowish-white, except for those containing BSF flour which had a darker colour. For all FDP, syneresis values were below 10% after the first day of storage, particularly for those with DB flour with syneresis as low as 5%. The probiotics had the highest values of *Lactobacillus* spp. (>6 log CFU/g) and *Streptococcus* spp. (>10 log CFU/g). The addition of the BSF flour was found to have a negative impact in the consumers' perception of the products, according to the sensory analysis.

1. Introduction

Fermented dairy products (FDP), particularly yoghurts, have been consumed worldwide by humans since their childhood (Chadha et al., 2022). In addition to being tasty, they also offer several health benefits and play a vital role in maintaining the balance of our gut bacterial flora (Silanikove et al., 2015). Particularly, the probiotics found in these products are crucial for enhancing the immune system and improve digestion, showcasing the benefits of these beneficial bacteria (Silanikove et al., 2015; Sun et al., 2022).

Food products are constantly evolving to meet consumer demands for taste, texture, and nutritional profiles. However, in order to meet these evolving dietary preferences, the need for alternative and sustainable sources of protein and fat is on the rise (Sogari et al., 2019). In recent years, the inclusion of insects in human diets, also known as entomophagy, has received significant attention (Ribeiro et al., 2018). Insects have been found to have physicochemical properties and digestive benefits, as well as a potentially lower environmental impact compared to traditional livestock. They emit fewer greenhouse gases and minimize ammonia release (Ordoñez-Araque & Egas-Montenegro, 2021; Sogari et al., 2019). Furthermore, insect farming is a cost-effective practice that promotes the use of food by-products, creating a sustainable cycle in which the by-products serve as food for the insects themselves (Ribeiro et al., 2022).

Insects are gaining increased interest in Western cuisine due to their nutritional value. They are rich in protein, essential amino acids, healthy fats, vitamins, and minerals that are all vital for human health (Ordoñez-Araque & Egas-Montenegro, 2021). Based on these factors, the incorporation of insect formulations into dairy products shows potential as a means of increasing their protein content and enhancing their nutritional value by providing essential nutrients such as dietary fibers and healthy fats (Finke, 2015).

The black soldier fly (*Hermetia illucens*) and drone bee (*Apis mellifera*) are two insect species that have gained significant attention in recent

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years as potential human food sources due to their relatively low environmental footprints and promise as sustainable protein sources. This makes them appealing options for nutrition and eco-conscious food choices (FAO, 2010; Ordoñez-Araque & Egas-Montenegro, 2021). BSF contributes to environmental sustainability by efficiently upcycling organic waste and reducing food waste (Franco et al., 2021; Ribeiro et al., 2022). However, there are current limitations and regulatory considerations that need to be addressed for the adoption of BSF flour in food industry (EPCEU, 2015). Similarly, DB is also not allowed, although there are already applications to the European Commission to include *Apis melifera* as a novel food.

Insect flour has been tested and incorporated into various food products, such as baked goods (bread, cookies, muffins, etc), protein bars, pasta and snacks, to enhance their nutritional profile, sustainability, and potentially offer new flavours and textures (Marinopoulou et al., 2023; Montevecchi et al., 2021; Osimani et al., 2018; Roncolini et al., 2019; Zielińska et al., 2021; Zielińska & Pankiewicz, 2020). However, its use in fermented dairy products has been vaguely explored with only one study found (Kim et al., 2017).

The aim of this study was to explore the potential of incorporating black soldier fly (BSF) and drone brood (DB) flour as sustainable protein sources in fermented dairy products (FDP). To achieve this, the impact of the flours on the physicochemical and microbiological properties of the products, as well as the benefits of probiotic enrichment, were evaluated. Additionally, consumers preferences were assessed through sensory analysis. This research aims to explore the potential of using insect-based ingredients in enhance the quality of FDP, promote sustainability, and meet the nutritional requirements of consumers.

2. Materials and methods

To investigate the potential use of insect flours as sustainable protein sources, two groups of fermented dairy products (FDP) were prepared: one with conventional yoghurt strains (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*), and the other with probiotics (*Bifidobacterium animalis* subsp. *lactis*, *Lactobacillus acidophilus* and *Streptococcus thermophilus*). Three variants were produced for each fermented dairy product type: the control (containing milk powder), one with black soldier fly (BSF) flour, and the other with drone brood (DB) flour. The nutritional composition of the BFS and DB flours and of the FDP were analysed. Subsequently, a physicochemical and microbiological evaluation of the products was performed over 21 days under refrigerated storage. On the 9th day, a sensory analysis was conducted to assess consumer preferences.

2.1. Preparation of insect flours

The BSF larvae used in the FDP formulations were supplied provided by the Coimbra Agriculture School rearing pilot unit. The larvae were fed with raw fruit and vegetable waste from the campus' canteen. To minimize microbiological contamination and darkening, the larvae were blanched in water at 85 °C for 2 min. Then, they were immediately immersed in ice to halt the cooking process and stored at −18 °C until use. After freeze-drying (Labconco, Kansas City, MO, USA) at −40 °C for 48 h, the larvae were ground (Classic 123, 700 W, Moulinex, Écully, Lyon, France) to produce BSF flour.

Drone pupae and larvae were collected from drone frames produced in the hives of the apiary at Viseu Agriculture School, Portugal. To ensure the preservation and quality of the drone brood (pupae and larvae), the drone frames were kept frozen at −20 °C until use (Jensen et al., 2019; Rutka et al., 2021). The larvae and pupae were manually extracted from the frames on a cold surface (1 °C) in a refrigerated box (11 °C) specifically designed for this operation. The combs placed on the cold surface were broken into small pieces and the drone larvae/pupae were separated by removing the wax and other materials. After extraction, the drone brood was frozen until freeze-dried (Christ - Alpha 1–4 LSC basic freeze-drier) at 1.54 mbar, −48.8 °C for 48 h. Dried drone brood (DB) was ground (La Moulinette 1, 2, 3 AD560120, Moulinex, Écully, Lyon, France) and sieved through a 0.5 mm mesh stainless steel sieve (Retsch, Haan, North Rhine-Westphalia, Germany) to produce BD flour.

2.2. Nutritional composition of insect flours

Moisture was determined according to AOAC 925.09–1925 method, and dry matter was calculated by subtracting from 100%. Ash content was determined using the AOAC 923.03–1923 method. For fat, protein and fiber determination, dried samples (105 °C) were used. The fat content of flour samples was determined by the AOAC 930.09–1930 method. Total nitrogen was evaluated by the Kjeldahl method AOAC 978.04–1978, with a conversion factor of 5.33 to determine the protein content (Boulos et al., 2020). The dietary fiber content was determined using the Total Dietary Fiber Assay Kit (Megaezyme, Bray, County Wicklow, Ireland) following AOAC 985.29-1986 (2003). Carbohydrate content was calculated by subtracting the sum of moisture, ash, crude fat, crude protein, and crude fiber from 100. The analyses were performed in triplicate ($n = 3$).

2.3. Preparation of fermented dairy products

A local milk producer supplied the bovine milk, which was skimmed using a Westfalia™ separator type ADB (GEA Group, Oelde, North Rhine-Westphalia, Germany) to reduce its fat content to 1.5% (w/w). The semi-skimmed milk (10 L) was divided into six portions to produce two variants of FDP supplemented with milk powder (MP) (36% protein, 0.9% fat, 53% carbohydrates and 1% salts), two with BSF flour and two more with DB flour (Table 1). Sucrose (6%) was added to all variants. The ingredients in each variant were mixed and heated to 70 ± 1 °C before being homogenized at 20 MPa. The mixtures (Table 1) were then pasteurized at 90 ± 1 °C for 25–30 s. Prior to filling and packaging, the mixtures were stirred for 10 min at 45 °C and inoculated. A conventional culture (0.005%, w/w) of *Lactobacillus bulgaricus* and *Streptococcus* thermophilus (FD-DVS YF-L903 - Yo-Flex, CHR HANSEN, Madrid, Spain) was used for the conventional variants CC, CB and CD. A probiotic culture (0.09 g/L) containing *Bifidobacterium animalis* subsp. *lactis* (BB12), *Lactobacillus acidophilus* and *Streptococcus thermophilus* (ABT-3, CHR HANSEN, Melbourne, Victoria, Australia) was used for probiotic variants PC, PB and PD. The fermentation was performed in 100 mL cups for compositional, physicochemical, and microbiological analysis, and in 30 mL cups for sensory analysis, at a constant temperature of 43 ± 1 °C until the product's pH reached 4.6 ± 0.1. The FDP were then stored at 4 ± 2 °C. Physicochemical and microbiological composition, as well as functional properties, of some FDP samples were evaluated after one day of cool storage, while the remaining samples were evaluated on the 7th, 14th and 21st day.

Table 1
Variants of FDP produced according to the starter culture and protein source (milk powder, BSF flour or DB flour).

Variant	Starter	Milk powder	BSF Flour	DB Flour
CC	Conventional	5%	–	–
CB	Conventional	3%	2%	–
CD	Conventional	3%	–	2%
PC	Probiotic	5%	–	–
PB	Probiotic	3%	2%	–
PD	Probiotic	3%	–	2%

CC - conventional starter with milk powder; CB - conventional starter with BSF flour; and CD - conventional starter with DB flour; PC - probiotic starter with milk powder; PB - probiotic starter with BSF flour; and PD - probiotic starter with DB flour.

2.3.1. Compositional analyses of the FDP

Dry matter was determined according to NP-703:1982, and ash content according to AOAC 945.46–1945. The fat content was analysed by the Gerber method according to NP-469:2002. The total nitrogen was evaluated by the Kjeldahl method according to AOAC 991.20-1994 (1996), and a conversion factor of 6.38 was used to determine the protein content. Compositional analyses were performed on all FDP variants on 7th day. Each analysis was performed as triplicates of independent samples ($n = 3$).

2.3.2. Physicochemical stability of the FDP

Texture analysis was performed using a TA.XT Express Enhanced model texturometer (Stable Micro Systems, Godalming, Surrey, UK) with a TA.24 cylindrical probe. A TPA test was performed with a pre-test speed of 5 mm/s, a test speed of 2 mm/s, a post-test speed of 5 mm/s, and a distance of 20 mm. The TA.XTExpress software (version 1,1,9,0 (Unicode), OCX version 2,0,0,11, from Stable Micro Systems, Godalming, Surrey, UK) was used to calculate the results of the TPA test. Hardness values were recorded. After conducting texture and microbiological analyses, the FDP samples were carefully homogenized to ensure accurate and representative sampling for subsequent analyses. The pH was determined directly with a HI 9025 pH meter (Hanna Instruments, Leighton Buzzard, UK). The total acidity of FDP was assessed according to NP-701:1982 and expressed as a percentage of lactic acid. The products colour was measured using the colorimeter Minolta Chroma Meter, model CR-200B (Konica Minolta, Shibuya, Tokyo, Japan) with the CIEL*a*b* coordinate system. To determine the syneresis index, 20 g of each FDP were centrifuged at 5 °C (Hettich Rotanta 460R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Baden-Württemberg, Germany) for 10 min at 350 *g*. The syneresis index was calculated by determining the weight percentage of the supernatant in relation to the total mass of the sample (Campos et al., 2024). Rheology analyses were conducted on the 1st and 21st day of storage using the methodology described by Campos et al. (2024). Elastic modulus (G'), viscous modulus (G"), complex viscosity (η*) and damping factor (tan δ) were measured in the range of 0.05–1.00 Hz at 3 Pa at 5 °C, and recorded at 1 Hz for comparison. Texture, pH, total acidity, colour and syneresis were conducted on the 1st, 7th, 14th and 21st day of storage. Each analysis was performed in triplicate using independent samples ($n = 3$).

2.3.3. Microbiological stability of FDP

The microbiological profile of the FDP was evaluated through refrigeration storage, analysing total aerobic bacteria, lactic acid bacteria (*Lactobacillus* spp. and *Streptococcus* spp.), yeasts and moulds and *Escherichia coli*. All samples were diluted with Ringer's solution (Merck, Darmstadt, Hesse, Germany). Total aerobics were analysed according to ISO 4833–1:2013 using the pour-plate technique, incorporating 1 mL of each sample dilution with PCA (Liofilchem, Roseto degli Abruzzi, Teramo, Italy). After 72 h of incubation at 30 °C, visible colonies were counted. *Lactobacillus* spp. and *Streptococcus* spp. were determined according to ISO 7889:2003. For *Lactobacillus* spp., 1 mL of each sample dilution was incubated with MRS (Biokar Diagnostics, Allonne, France) at 37 °C in an anaerobic environment and visible colonies were enumerated after 72 h. For *Streptococcus* spp., 1 mL of each sample dilution was incubated with M17 (OXOID, Basingstoke, Hampshire, UK) at 37 °C for 48 h until visible colonies were counted. Yeasts and moulds were determined according to ISO 21527–1:2008. Each sample dilution (0.1 mL) was placed on DRBC Agar Petri dishes (VWR, Geldenaaksebaan, Leuven, Belgium). After an incubation period of 5 days at 25 °C, typical colonies were counted. Screening for *E. coli* bacteria was performed according to ISO 16649–2:2001. From each initial dilution of samples, 1 mL was incubated in TBX (Alliance Bio Expertise, Bruz, Brittany, France) for 24 h at 44 °C until blue colonies were enumerated. All analysis were performed as duplicates of three independent samples ($n = 6$).

2.3.4. Sensorial analysis

The sensory analysis was conducted on the 9th day of refrigerated storage with the participation of 27 untrained individuals ranging in age from 17 to 60. Panellists were asked to rate colour, aroma, texture and taste using a 9-point hedonic scale (ranging from 1 "very unsatisfied" to 9 "very satisfied"). The samples were presented in individual cups, each coded with a unique random three-digit identifier. The ethical guidelines were rigorously followed to protect the rights and privacy of the participants, including voluntary participation, informed consent, and strict data confidentiality (ethical approval nº 36 CEIPC/2024). The participants were fully informed of the study's requirements and risks and assured of their right to withdraw at any time.

2.4. Statistical analysis

All data are expressed as mean values ± standard deviation. Statistical analysis was performed using GraphPad Prims software version 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). An unpaired *t*-test was used to compare the BSF and DB flours. For the compositional analysis of the samples a one-way ANOVA with Tukey's test was performed to evaluate the statistical significance between FDP. A two-way ANOVA using Tukey's test (or Sidak's test for rheology) was performed to determine the differences between the means of the physicochemical and microbiological results, with type of FDP and storage time as factors. A 5% significance level was used for all analyses. The data from the sensorial analysis were subjected to a Kruskal-Wallis test at a significance level of 5%, following Dunn's multiple comparison test.

3. Results and discussion

The samples produced in this work allowed the evaluation of the use of insect flour (black soldier fly (BSF) and drone brood (DB)) as a replacement for milk protein in the production of fermented dairy products (FDP). The effect of a probiotic starter (*Bifidobacterium* spp., *L. acidophilus* and *S. thermophilus*) instead of a conventional yoghurt starter (*L. bulgaricus* and *S. thermophilus*) used in dairy products was also evaluated.

3.1. Nutritional composition of insect flours and FDP

The nutritional composition of the insect flours is shown in Table 2. The total solids content is very similar for both flours (*ca.* 95%), with significant differences only at $p < 0.0001$. The ash content of BSF flour was significantly higher (8%) compared to that of DB flour (3%). Both flours have high fat and protein content, with BSF containing 23% fat and 40% protein, and DB containing 26% fat and 33% protein. The total dietary fiber content is approximately 3% for BSF flour and 5% for DB flour, which may be attributed to the insect's exoskeleton that is rich in chitin (Bergeron et al., 1988). The carbohydrate content was higher in DB flour (around 28%), and significant differences were found between the insect flours ($p = 0.0002$).

Liland et al. (2017) and Shumo et al. (2019) have also investigated the nutritional composition of BSF flour. Liland et al. (2017) reported values of approximately 5% for ash content, 34% for fat content and for protein content. In comparison to the present results, the fat content is much higher (40%–23%, respectively), while the ash and protein content are lower (8%–5%, and 34%–40%, respectively). Shumo et al. (2019) reported values of 84% for total solids, 10% for ash content and 39% for protein content (recalculated with a 5.33 conversion factor). These results are similar those presented in Table 2. Regarding DB flour, Ghosh et al. (2016) reported values of 4% ash, 35% protein (recalculated with a 5.33 conversion factor), 15% fat and 40% carbohydrates for larvae and pupae (mean values of both). The ash and protein content of the insects are comparable to the values presented in Table 2. However, the fat content is significantly lower, and the carbohydrate content is higher. It is worth noting that the nutritional composition of insects may

Table 2
Nutritional composition of the black soldier fly flour (BSF) and drone brood flour (DB), and the fermented dairy products (FDP).

	Total Solids (%, DM)	Ash (%)	Fat (%, DM)	Protein (%, DM)	Total Dietary Fiber (%, DM)	Carbohydrates (%, DM)
Flours						
BSF	95.9 ± 0.1[a]	7.9 ± 0.0[a]	22.8 ± 0.7[b]	40.4 ± 0.0[a]	2.9 ± 0.1[b]	21.5 ± 0.7[b]
DB	94.2 ± 0.0[b]	3.2 ± 0.0[b]	25.7 ± 0.0[a]	33.0 ± 0.0[b]	4.5 ± 0.2[a]	27.8 ± 0.1[a]
P-value	*<0.0001*	*<0.0001*	*0.0022*	*<0.0001*	*0.0003*	*0.0002*
Fermented Dairy Products						
CC	19.9 ± 0.0[B]	0.94 ± 0.09[A]	0.90 ± 0.00[BC]	4.5 ± 0.7[A]	–	13.6 ± 0.8[A]
CB	19.8 ± 0.1[BC]	0.95 ± 0.11[A]	1.00 ± 0.00[AB]	4.1 ± 1.1[A]	–	13.8 ± 1.0[A]
CD	20.3 ± 0.0[A]	0.95 ± 0.03[A]	1.00 ± 0.00[AB]	4.8 ± 0.7[A]	–	13.5 ± 0.7[A]
PC	19.9 ± 0.1[BC]	0.92 ± 0.08[A]	0.87 ± 0.06[C]	5.1 ± 0.5[A]	–	13.0 ± 0.6[A]
PB	19.7 ± 0.1[C]	0.99 ± 0.01[A]	1.07 ± 0.06[A]	4.9 ± 0.7[A]	–	12.8 ± 0.7[A]
PD	20.3 ± 0.0[A]	0.87 ± 0.05[A]	1.03 ± 0.06[A]	4.8 ± 0.8[A]	–	13.5 ± 0.8[A]
P-value	*<0.0001*	0.4863	*0.0004*	0.7114	–	0.6120

BSF, black soldier fly; DB, drone brood; CC, FDP with conventional starter and milk powder; CB, FDP with conventional starter and BSF flour; CD, FDP with conventional starter and DB flour; PC, FDP with probiotic starter and milk powder; PB, FDP with probiotic starter and BSF flour; PD, FDP with probiotic starter and DB flour. Different small letters within the same column represent statistical differences between flours ($p < 0.05$). Different capital letters within the same column represent statistical differences between FDP ($p < 0.05$).

vary depending on the substrate and environmental conditions in which they grow. According to Devi et al. (2023), the protein and fat content of edible insects varies between 35-60% and 10%–50%, respectively, depending on the stage of development and the death process.

The nutritional properties of the FDP were evaluated on the 7th day of refrigerated storage and are presented in Table 2. Total solids ranged from 19.7% (PB) to 20.3% (CD and PD), with the variants containing DB flour significantly differing from the others ($p < 0.05$). The ash content did not differ significantly ($p = 0.4863$) among the variants, with values ranging from 0.87% (PD) to 0.99% (PB). The FDP variants with insect flour (CB, CD, PB and PD) had higher fat values (ranging from 1.00% to 1.07%) compared to the control variants (CC and PC) with fat values of 0.90% and 0.87%, respectively. As previous mentioned, the insects' flour has a fat content of 23–26%, while the milk powder has a fat content of 0.9%. This difference in fat content may have contributed to the higher fat values in the FDP variants with insects' flour. No significant differences ($p > 0.05$) were found for the protein and carbohydrate contents. The mean values for protein and carbohydrate were 4.7% and 13.4%, respectively.

Zahid et al. (2022) investigated the impact of probiotic bacteria on low-fat (0.015%) yoghurts. The authors used *Bifidobacterium lactis*, *Lactobacillus casei* and *Lactobacillus rhamnosus*, along with the conventional starter cultures *L. bulgaricus* and *S. thermophilus*. The results for total solids (*ca.* 31%), ash (*ca.* 2.7%) and protein (*ca.* 1.37%) were similar for both types of FDP, as in the present study. However, the values are higher than those presented in Table 2, particularly in total solids and ash content. This may be due to the authors' use of a higher concentration of milk powder in their formulations (14% compared to 3%–5%), which increases the mentioned parameters.

Few studies have evaluated the impact of using BSF or DB flour in other food products on their nutritional properties. For instance, Montevecchi et al. (2021) studied the addition of different concentrations of BSF flour (2% or 4%) to baked products similar to bread, while the control only used wheat flour. The authors observed differences in moisture, ash and protein content between the baked products. The addition of BSF flour resulted in a decrease in moisture content, while ash and protein content increased significantly. In the present study, the ash and protein content in FDP with BSF flour was found to be similar to that of the control FDPs. The variation in study results may be attributed to the distinct characteristics of the different food product. Marinopoulou et al. (2023) conducted research on the use of drone flour in human foods and found that the progressive incorporation of drone brood flour led to an increase in total dietary fibre and insoluble dietary fibre in bakery products. However, no information was provided on the levels of protein, fat or carbohydrates. In the present study, neither BSF nor DB flour significantly altered the nutritional values of the FDP. These results are promising as both insects have a smaller environmental impact than milk powder.

3.2. Physicochemical and microbiological stability of FDP

The fermentation step was stopped when the pH reached 4.6 and the products were stored in refrigeration for 21 days and analysed weekly. During this period, the pH value stabilized, but prolonged refrigeration of FDP can lead to the proliferation of microorganisms, particularly lactic acid bacteria, which can cause acidification of the products. Although no decrease in pH was observed, the acidity of FDP increased as expected (Fig. 1). Overall, the samples exhibited a slight decrease in pH from day 1 to day 7. However, by day 14, the pH values had risen again and stabilized at levels similar those observed on day 1 (Fig. 1A). Despite of minor pH fluctuations observed during storage time and between samples, significant differences ($p < 0.0001$) were detected for

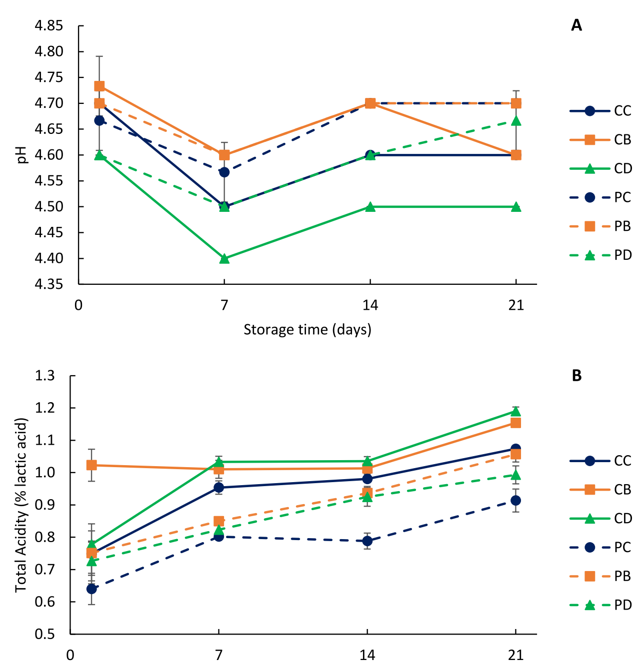

Fig. 1. pH (A) and total acidity (B, % lactic acid) of the fermented dairy products (FDP) made with milk powder (CC and PC), milk powder and BSF flour (CB and PB), and milk powder and DB flour (CD and PD). Full lines correspond to FDP produced with conventional starter (CC, CB and CD). Dotted lines correspond to FDP produced with probiotic starter (PC, PB and PD).

both factors analysed (type of FDP and storage time) and their interaction. The total acidity of all products increased over time (Fig. 1B). The initial acidity was *ca.* 0.73% lactic acid, except for CB which was 1.02% lactic acid. The final acidity was approximately 1.14% lactic acid for FDP with conventional starter (CC, CB and CD) and 0.99% lactic acid for FDP with probiotic starter (PC, PB and PD). Significant differences ($p < 0.0001$) were found in both factors and their interaction. Despite these differences, the pH and total acidity of the control FDP are very similar to those of the FDP with insects' flours, suggesting that the insect flours have no significant impact on the FDP acidity.

Only one study was found that investigated the incorporation of insects powder in FDP. Kim et al. (2017) evaluated the addition of *Oxya chinensis sinuosa* (grasshopper) powder to yoghurts, concluding that the products with insect powder showed similar results in pH and total acidity to the control yoghurts (without grasshopper powder). The study indicates that the total acidity of the yoghurts with insect powder ranged from 0.93% to 1.10%. These results are in line with those found in the present study (Fig. 1), despite the different type of FDP and insect used.

The colour of the homogenized FDP was determined weekly for 21 days using the CIEL*a*b* colour system and the results are shown in Fig. 2. This characteristic is one of the most relevant for consumer acceptance, as the visual stimulus has a great influence on the decision to buy a product. The lightness (L*, Fig. 2A) of the samples is high (between 78 and 98) due to the white colour of the milk. The FDP with the lowest values were those containing BSF flour (between 78 and 95), which had a more brownish colour. The a* coordinate indicates the variation between green (−a) and red (+a), and the values ranged from −1.3 and −4.6, indicating that the samples tend to have a greener colour spectrum (Fig. 2B). The b* coordinate varies between blue (−b) and yellow (+b). All samples have positive values, indicating a tendency towards yellowish tones ranging from 5.7 to 11.5 (Fig. 2C). In general, all the products tended towards a yellowish-white colour during refrigeration. Over time, all FDP showed a decrease in the values of the L* and b* coordinates, and an increase in the a* coordinate, which may indicate a degradation of the proteins responsible for the white colour. This led to significant differences ($p < 0.0001$) over the storage time. The probiotic starter had no effect on the colour of the products. The addition of the insect flour, particularly the BSF flour, caused a darker FDP (lower L*) and higher a* and b*, which represent a closer approximation to the red and yellow hues respectively. This is due not only to the fact that BSF flour is darker than DB flour or milk powder, but also to the fact that the particles of BSF flour did not dissolve well in the milk, resulting in a deposit at the bottom of the packages (hence the homogenization prior to analysis). These are significant differences between the products ($p < 0.0001$).

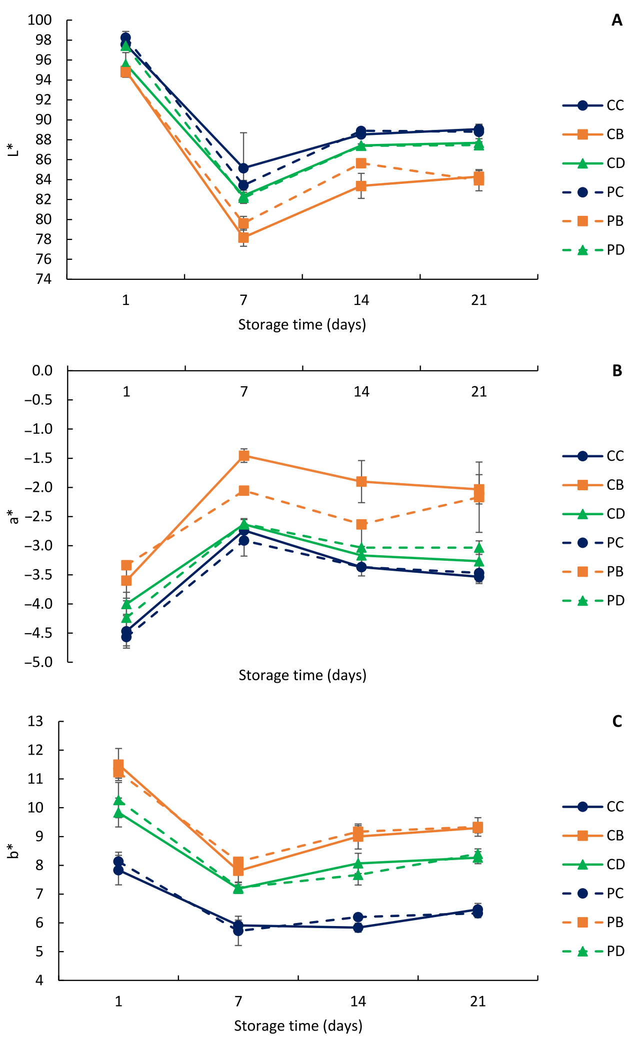

Fig. 2. Colour parameters L* (A), a* (B) and b* (C) of the fermented dairy products (FDP) made with milk powder (CC and PC), milk powder and BSF flour (CB and PB), and milk powder and DB flour (CD and PD). Full lines correspond to FDP produced with conventional starter (CC, CB and CD). Dotted lines correspond to FDP produced with probiotic starter (PC, PB and PD). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

With regard to yoghurts, the study by Kim et al. (2017) only evaluated the colour by sensory analysis. However, other studies explore the addition of insect flour to other foods, namely *Tenebrio molitor* was used in cookies (Sriprablom et al., 2022a), muffins (Zielińska et al., 2021) and shortcake biscuits (Zielińska & Pankiewicz, 2020); *Zophobas atratus* was used in cookies; *Gryllodes sigillatus* was used in muffins; and bread (Marinopoulou et al., 2023) was produced with *Apis melliffera*. In all studies, the addition of insect flour made food darker than the control (without insect flour). Increasing the concentration of insect flour also resulted in darker products. These characteristics were attributed to the innate brownish colour of the flours. However, as noted by (Zielińska et al., 2021), changing the colour of the product may not result in a worse consumer rating.

Syneresis is the process of separating the liquid part of the solid matrix, resulting in the formation of a layer of whey on top of the gel (Papaioannou et al., 2022). In FDP such as yoghurts, it is normal for the syneresis values to decrease over time during refrigeration time (Guénard-Lampron et al., 2020). This is due to the fact that the whey proteins in these FDP tend to gradually become less soluble and more prone to aggregating, forming a gel network that traps the whey proteins and prevents them from leaking out of the matrix. As a result, the syneresis index decreases, resulting in a firmer product. Fig. 3A shows the results of the FDP syneresis, which follow a similar trend. The syneresis index was higher in the probiotic products, particularly on the 1st day of refrigerated storage, with values of 18.7%, 17.3%, and 10.5% for PC, PB, and PD, respectively. These values decreased to 4.1%, 7.0%, and 2.3% on the 21st day. For the conventional starter, the syneresis index started at *ca.* 5% and slightly decreased to *ca.* 3.5% for CC and CB, and 1.8% for CD, on the 21st day. These variations in storage time and between FDP resulted in significant differences at $p < 0.0001$. In terms of the impact of the insect flour, FDP with BSF flour (CB and PB) showed more similar results to the control (CC and PC) than those with DB flour (CD and PD). The latter exhibited the lowest syneresis values of all FDP within their categories (conventional or probiotic starter), suggesting that DB flour has a positive effect on reducing syneresis and maintaining the FDP structure. BSF flour did not have a beneficial impact on syneresis due to its poor solubility in milk. This resulted in particle deposition, which may have caused discontinuities in the gel and made the whey separate more easily.

As previously stated, protein content directly affects syneresis. A

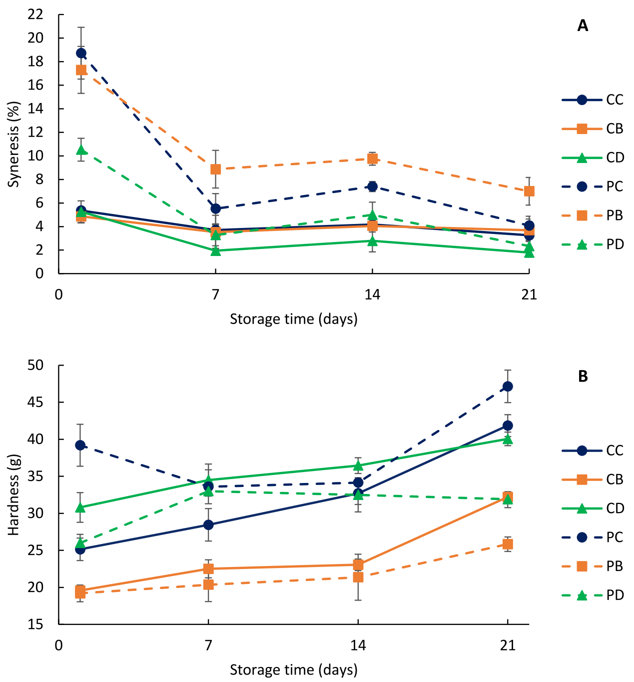

Fig. 3. Syneresis index (A, %) and hardness (B, g) of the fermented dairy products (FDP) made with milk powder (CC and PC), milk powder and BSF flour (CB and PB), and milk powder and DB flour (CD and PD). Full lines correspond to FDP produced with conventional starter (CC, CB and CD). Dotted lines correspond to FDP produced with probiotic starter (PC, PB and PD).

lower protein concentration can result in a weaker structure, causing more whey to be expelled from the solid matrix and leading to higher syneresis. The FDP protein content showed no significant differences (Table 2), so the higher syneresis in the probiotic FDP (PC, PB and PD) may be attributed to the use of the probiotic cultures. These results are consistent with those found in the study by Papaioannou et al. (2022), which also compared conventional starters with probiotic-added starters. The probiotic yoghurts in their study also exhibited a higher syneresis index than the control. However, the syneresis in all their products was much higher (ranging from 23.6% to 39.9%) than the syneresis found in the present study (ranging from 1.8% to 18.7%, as shown in Fig. 3A).

Hardness is a parameter that refers to the analysis of the product's texture, characterized by a firmer or denser consistency, which can occur due to the higher amount of solids present in the FDP. The results (Fig. 3B) show that hardness increased over time in all FDP. This increase may be due to the continuous loss of whey (already described above in syneresis), which makes the products more solid over time. The use of BSF flour in the FDP resulted in lower hardness, with values of 19.6 g and 19.2 g on the 1st day and 32.2 g and 25.8 g on the 21st day, for CB and PB, respectively. In contrast, the hardness of control products (CC and PC) and those made with DB flour (CD and PD) increased from 25.1 g on day 1 of refrigerated storage (the minimum value for CC) to 47.2 g on day 21 of refrigerated storage (the maximum value for PC). The hardness of the FDP was not influenced by the starter culture, which is consistent with the findings of Cui et al. (2021) who studied conventional and probiotic yoghurts and found no impact of the starter culture on hardness after 14 and 21 days of storage. However, significant differences were observed between FDP and over time at $p < 0.0001$.

The rheological profile (Table 3) was evaluated only on the 1st and 21st days of refrigerated storage. In all products, the elastic modulus (G′) was higher than the viscous modulus (G″), suggesting that the FDP exhibits stronger elastic than viscous behaviour (Walstra & Jenness, 1984). This behaviour has also been reported by other authors who evaluated plain yoghurts produced with conventional and probiotic starters (Papaioannou et al., 2022). During the 21-day storage period, both G′ and G″ increased over time, with G′ showing a more significant increase. On the first day, the control FDP (CC and PC) exhibited higher G′ and G″ values compared to DB (CD and PD) and BSF (CB and PB) FDPs. However, on the 21st day, the probiotic FDP had higher values for G′ and G″ compared to conventional ones. Additionally, FDP with DB flour demonstrated the higher elastic modulus. Papaioannou et al. (2022) reported that the effect of the probiotic on the rheological properties of yoghurts was not clear. However, for G' moduli, probiotic plain yoghurts showed higher values than conventional ones, which is consistent with the results presented in Table 3 for the FDP.

The complex viscosity (η*) of the samples increases over time. This effect is more pronounced in samples containing insect flour. The damping factor (tan δ), which is the ratio of the viscous and elastic moduli, decreases over time. This effect is more noticeable in CB, PC and PB. The tan δ values indicate that the products have a more elastic behaviour, as the values are lower than 1. These results were also observed by Papaioannou et al. (2022), although the tan δ was between 0.52 and 0.75, which is higher than the values reported in Table 3 (between 0.29 and 0.37). Significant differences were found for all attributes in terms of storage time and type of FDP ($p < 0.05$).

This study investigated the microbiological stability of all FDP during refrigerated storage between the 7th and 21st days (Fig. 4). The total aerobic bacteria count ranged from 3.1 to 6.5 log CFU/g (Fig. 4A), with lower counts generally observed on the 7th day of storage and an increase over time. During storage, the products made with DB flour (CD and PD) had the highest counts, with ~3.65 log CFU/g on the 7th day and between 5.7 and 6.5 log CFU/g on the 21st day. The FDP containing probiotic cultures (PC, PB and PD) showed the greatest increase in flora over the refrigeration period. Significant differences were observed between the FDP and the other products over refrigeration ($p < 0.0001$).

Regarding yeasts and moulds (Fig. 4B), the values obtained varied between 2.5 and 3.8 log CFU/g on the 7th day of storage, with an

Table 3
Rheological profile of the FDP: elastic modulus (G′), viscous modulus (G″), complex viscosity (η*) and damping factor (tan δ).

Parameter	Storage time (days)	CC	CB	CD	PC	PB	PD
G' (Pa)	1	127.9 ± 4.9^{Aa}	30.8 ± 2.1^{Bc}	77.7 ± 9.3^{Babc}	148.8 ± 9.4^{Ba}	49.5 ± 10.6^{Bbc}	109.1 ± 3.7^{Bab}
	21	169.1 ± 53.5^{Ac}	199.3 ± 24.3^{Ac}	234.4 ± 23.5^{Ac}	331.6 ± 45.5^{Ab}	232.7 ± 53.0^{Ac}	488.1 ± 30.5^{Aa}
G'' (Pa)	1	39.6 ± 2.0^{Aab}	11.1 ± 0.6^{Bc}	23.9 ± 1.9^{Babc}	48.7 ± 3.4^{Ba}	18.3 ± 3.4^{Bbc}	35.4 ± 1.1^{Bab}
	21	50.9 ± 15.6^{Ad}	58.2 ± 6.6^{Acd}	67.2 ± 6.8^{Acd}	95.3 ± 12.7^{Ab}	76.3 ± 13.1^{Abc}	148.3 ± 8.8^{Aa}
η* (Pas)	1	21.3 ± 0.8^{Aa}	5.2 ± 0.3^{Bc}	12.9 ± 1.5^{Babc}	24.9 ± 1.6^{Ba}	8.4 ± 1.8^{Bbc}	18.3 ± 0.6^{Bab}
	21	28.1 ± 8.9^{Ac}	33.0 ± 4.0^{Ac}	38.8 ± 3.9^{Ac}	54.9 ± 7.5^{Ab}	39.0 ± 8.6^{Ac}	81.2 ± 5.0^{Aa}
tan δ	1	0.31 ± 0.01^{Ab}	0.36 ± 0.01^{Aa}	0.31 ± 0.02^{Ab}	0.33 ± 0.01^{Ab}	0.37 ± 0.01^{Aa}	0.32 ± 0.00^{Ab}
	21	0.30 ± 0.00^{Ab}	0.29 ± 0.00^{Bb}	0.29 ± 0.00^{Ab}	0.29 ± 0.00^{Bb}	0.33 ± 0.02^{Ba}	0.30 ± 0.00^{Ab}

CC, FDP with conventional starter and milk powder; CB, FDP with conventional starter and BSF flour; CD, FDP with conventional starter and DB flour; PC, FDP with probiotic starter and milk powder; PB, FDP with probiotic starter and BSF flour; PD, FDP with probiotic starter and DB flour. Different capital letters for each parameter within the column represent statistical differences through storage time ($p < 0.05$). Different small letters within the same row represent statistical differences between FDP ($p < 0.05$).

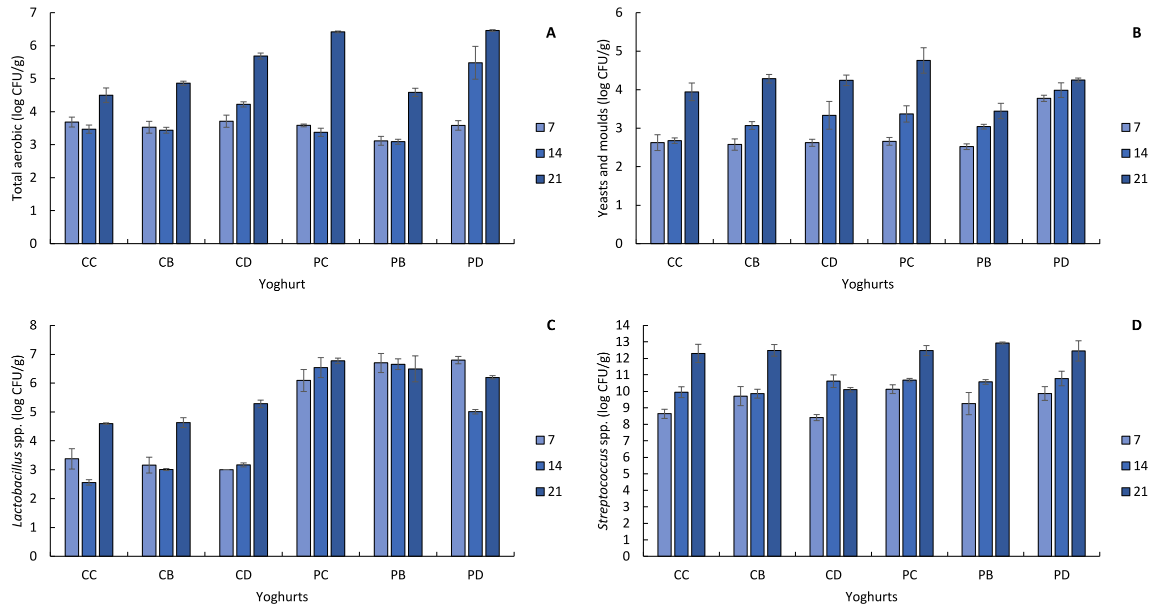

Fig. 4. Microbiological profile (log CFU/g) through storage time (7, 14 and 21 days) of the fermented dairy product (FDP) made with milk powder (CC and PC), milk powder and BSF flour (CB and PB), and milk powder and DB flour (CD and PD).

increase over the refrigeration period reaching between 3.4 and 4.8 log CFU/g at the 21st day. These microorganisms grew more from day 7th to 21st in the products with conventional starter (CC, CB and CD) than with probiotic starter (PC, PB and PD). As will be described later, the FDP with probiotics had a higher amount of lactic acid bacteria, which may have competed with the yeasts and moulds, causing them to be lower in these FDP variants. The study byOsimani et al. (2018)) evaluated yeasts and moulds in bread made with cricket powder and the study by Roncolini et al. (2019) with mealworm powder. The control dough had 8.0 log CFU/g of yeasts, while the dough with different concentrations of the cricket powder had 8.3–8.4 log CFU/g. In the study with mealworm powder, the control dough had 7.8 log CFU/g of yeasts, while the dough with different concentrations of mealworm had 7.7–7.9 log CFU/g. In both studies, the yeasts' colonies were similar between the control dough and insect added dough. These results agree with those observed in Fig. 4B for the conventional starter, as the insect FDP also had similar values to the control ones. In the probiotic FDP, the control had higher counts of yeasts and moulds than the insect FDP, which may suggest that insect's powder could contribute to better product stability.

The lactic acid bacteria, *Lactobacillus* spp. and *Streptococcus* spp., evaluated in this study play a crucial role in dairy products. To be considered probiotic, some authors suggest obtaining values greater than 10^6 log CFU/g (Cui et al., 2021; Silva et al., 2023), while others suggest values higher than 10^9 log CFU/g to modify the microbiota and have healthy effects on the host (Meybodi et al., 2020). For both microorganisms assessed, there was an increase in the counts during the refrigeration period ($p < 0.0001$). The increase in lactic acid bacteria was expected due to the corresponding increase in total acidy, expressed in lactic acid, over time (Fig. 1B). On the 21st day, *Lactobacillus* spp. counts ranged from 4.6 to 5.3 log CFU/g for FDP with conventional starters (CC, CB and CD) and 6.2 to 6.8 log CFU/g for products with probiotics (PC, PB and PD) (Fig. 4C). The impact of the probiotic starter on the products was significant in this analysis, as higher counts for these microorganisms were consistently recorded in the probiotic FDP throughout the storage time ($p < 0.0001$). Other authors have described that probiotic cultures may positively influence the bioavailability of simple sugars for *Lactobacilli* strains (Cui et al., 2021). These higher counts of *Lactobacillus* spp. in the probiotic FDP may be explained by the presence of *L. acidophilus* alongside *Bifidobacterium lactis*, a well-known probiotic strain.

In relation to *Streptococcus* spp. (Fig. 4D), after 21 days of refrigeration, the log CFU/g ranged from 10.1 to 12.5 for CC, CB and CD, and from 12.4 to 12.9 for PC, PB and PD. Although significant differences were found between FDP in both parameters ($p < 0.0001$), the counts for the control FDP (CC and PC) were similar to those with insect flour (CB, CD, PB and PD). The use of the insect flour did not have a negative impact on the growth of the specific microflora in these food products. Additionally, the products containing probiotic strains met the requirements to be considered probiotic products (>10^6 log CFU/g for *Streptococcus* spp. and >10^9 log CFU/g for *Lactobacillus* spp.). The study by Cui et al. (2021) evaluated yoghurt made from cow's milk and conventional starter with probiotic cultures (*Lactobacillus rhamnosus*, *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus*) during refrigerated storage. Similar to the findings of this study, the log CFU/g of *Streptococcus* spp. was higher in probiotic yoghurts (close to 9 log CFU/g) than in the conventional yoghurts (close to 8 log CFU/g).

Microbial communities are commonly found in insects, originating from their intestinal tract, including some pathogenic microorganisms (Grabowski & Klein, 2017). One such microorganism is *E. coli,* which was evaluated in the FDP through during refrigeration. CC, PC and PD showed less than 1 CFU/g on the 7th day of storage. At the 14th and 21st day of storage, colonies were not observed which may suggest competition with lactic acid bacteria that reached their highest values on the last day of refrigeration and may have prevented the growth of these pathogenic microorganisms.

3.3. Sensory analysis of FDP

On the 9th day of refrigerated storage, 27 untrained individuals drawn within the Coimbra Agriculture School community evaluated the aroma, colour, texture, and taste parameters of the FDP. The results are presented in Fig. 5. For the colour parameter, all the products scored similarly (between 7.7 and 8.3), except for FDP with BSF flour that had the lowest score (6.6 for CB and 5.7 for PD). This can be explained by the dark deposit created by the BSF flour in the products, which was visible to the consumer. With regard to aroma, the results were similar for all the samples. However, the FDP with milk powder scored higher (7.6 for CC and 7.0 for PC) than the products with insect flour (between 6.1 and

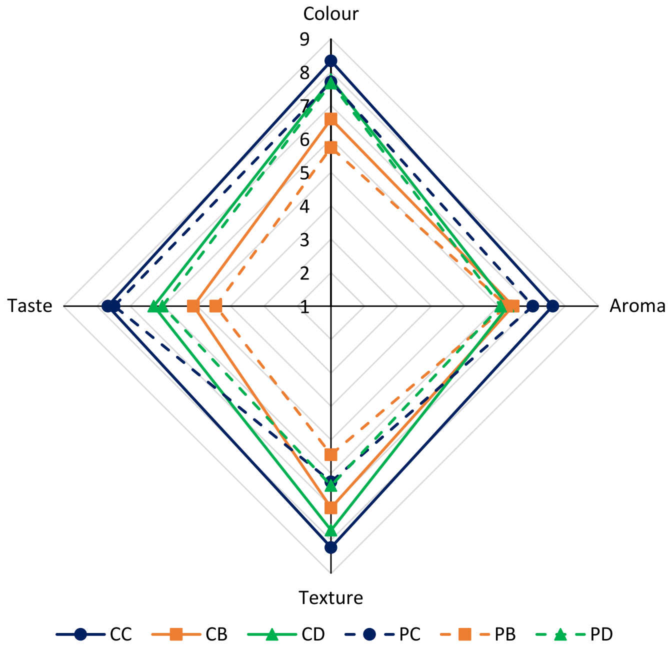

Fig. 5. Sensory evaluation of the fermented dairy product (FDP) made with milk powder (CC and PC), milk powder and BSF flour (CB and PB), and milk powder and DB flour (CD and PD). Full lines correspond to FDP produced with conventional starter (CC, CB and CD). Dotted lines correspond to FDP produced with probiotic starter (PC, PB and PD).

6.4). These results may indicate that the insect flour has transferred characteristic aromatic compounds to the FDP, revealing their different composition. In addition, at the end of the tasting, some tasters indicated that they smelled a honey aroma on the FDP with DB flour. The texture score for FDP with conventional starter cultures was higher (between 7.0 and 8.2) than with probiotics (between 5.4 and 6.4). These results were to be expected, as syneresis was higher in the probiotic products, indicating greater dissatisfaction on the part of the consumers. The taste evaluation also showed that, despite of the probiotics, the control FDP scored higher (7.7 and 7.5 for CC and PC, respectively), followed by the FDP with DB flour (6.3 and 6.0 for CD and PD, respectively) and finally the FDP with BSF flour (5.1 and 4.4 for CB and PB, respectively). It was noticeable that the products with probiotics scored lower than those with the conventional starter. Significant differences ($p < 0.05$) were found between FDP for all parameters. Finally, the products with BSF flour scored lower for most of the parameters, which could also be a consequence of the visible deposit that could have influenced the consumer's perception of the parameters.

Kim et al. (2017), investigated the addition of grasshopper powder to yoghurts and evaluated taste, aroma, colour and texture attributes. The authors tested four concentrations of the insect powder in the yoghurts (0%, as control, 0.5%, 1.0% and 2.0%). All yoghurts scored very similarly on all attributes, with the exception of the yoghurts with 2% grasshopper powder, which scored slightly lower. Sriprablom et al. (2022) evaluated the acceptance of cookies with different concentrations of *Tenebrio molitor* and *Zophobas atratus* flour. The attributes of colour, aroma, texture, and taste of the control cookies (without insects' flour) received higher scores than the other cookies. Additionally, as the concentration of insect flour increased, the scores of all parameters decreased. These results are in accordance with those presented in the study (Fig. 5). This indicates that further research is needed to enhance consumer acceptance of insect flour in other food products.

In a sensory analysis conducted by Mazurek et al. (2023) 60 participants aged 19–23 evaluated wheat pancakes containing mealworm (*Tenebrio molitor*), buffalo worm (*Alphitobius diaperinus*), and cricket (*Acheta domesticus*) at various concentrations (10%, 20% and 30%). Similarly to our study, the control samples (without insects) were found to be more acceptable. Furthermore, the acceptability of the samples decreased with increasing insect concentration. Within the insect-incorporated options, mealworm received the most favourable scores, followed by cricket and lastly buffalo worm. The study by Skotnicka et al. (2023)) evaluated the acceptability of cream soups (tomato and vegetable) fortified with mealworm, cricket, buffalo worm and grasshopper (*Ruspolia differens*). The results demonstrated lower acceptability as the control group, in a panel of 104 participants (49 with ages between 18 and 29 and 55 with ages above 65). Notably, consumer scores for taste and texture were the primary reasons for the lower ratings in the insect-containing soups. Çabuk and Yılmaz (2020) explored consumer perception of egg pasta enriched with grasshopper and mealworm flour using a sensory panel of 20 semi-trained participants (aged 18–45). The control samples scored higher here as well, and the odour characteristic emerged as the most significant factor influencing lower acceptability for insect-fortified pasta. The darker colour of these products also played a crucial role in the products lower score. However, the authors highlighted the nutritional value boost associated with insect flour addition. Finally, Ribeiro et al. (2021) conducted a questionnaire study with 282 participants, investigating consumer attitudes towards insect inclusion in bread. Their results suggest a potential link between pre-existing consumption of specialty breads (low-salt, high-fiber, etc.) and a greater acceptance of insect-fortified foods. This could be attributed to an openness towards novel ingredients and a focus on health benefits. These studies align with our research, which indicates that consumer acceptance of insect-based food products remains a significant challenge. They also highlight the necessity for further investigation into strategies to enhance consumer perception.

The present study employed black soldier fly larvae and drone brood as insect sources. While these options hold promise for sustainable protein, they may be less familiar to consumers compared to reported higher-acceptance insects like mealworm or buffalo worm. Furthermore, the processing techniques for incorporating these specific insects in fermented dairy products may require further optimization. Despite the observed sensory evaluation scores, this research paves the way for exploring alternative insect sources in dairy applications. Future studies comparing a broader range of insect species, coupled with continued development of processing methods for black soldier fly larvae and drone brood, can provide valuable insights for improving consumer acceptance of insect-based dairy products.

4. Conclusions

Due to legal restrictions, there have been limited studies on the use of insect flour in food products. Only one study has been found that incorporated insects in fermented dairy products (FDP). Therefore, this work significantly contributes to deepening the knowledge gap that still exists regarding the incorporation of black soldier fly (BSF) and drone brood (DB) as a substitute for milk powder in FDP. The study also evaluated the effects of probiotic cultures compared to conventional cultures in the FDP with insect flours. The addition of insect flour did not negatively impact the nutritional, physicochemical and microbiological composition of the FDP. However, the sensory analysis revealed noticeable differences in taste and aroma between the control products and those containing insect flour. Especially in products made with BSF flour, which has a darker colour, a more distinctive taste, aroma, and incomplete dissolution of the flour was observed. The use of probiotic cultures resulted in a significant difference in texture, with higher syneresis indexes and less firmness, as noted by the panellists in the sensory analysis. In general, further studies are needed to improve the final products and increase consumer acceptability. Heat treatments can be applied to insect flours to remove the fatty acids present and improve flavour. Homogenization can also be conducted to prevent deposits caused by insect flours in the fermented dairy products.

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Ethics approval

PARECER N.° 36 CEIPC/2024.

Consent to participate

Ethical guidelines were rigorously followed to protect participant rights and privacy, including voluntary participation, informed consent, and strict data confidentiality. Participants were fully informed of the study's requirements and risks and assured of their right to withdraw at any time.

Availability of data and material

Data will be made available on reasonable request.

Code availability

Not applicable.

CRediT authorship contribution statement

Vítor Neves: Writing – original draft, Methodology, Investigation, Formal analysis. **Lara Campos:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Nuno Ribeiro:** Writing – review & editing, Resources, Methodology, Conceptualization. **Rui Costa:** Writing – review & editing, Resources, Methodology. **Paula Correia:** Writing – review & editing, Resources. **João Gonçalves:** Writing – review & editing, Resources. **Marta Henriques:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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