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Can a functional cheese spread incorporating Akkermansia muciniphila deliver beneficial physicochemical and biological properties while enhancing probiotic stability and viability during aerobic storage and in vitro digestion?

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ABSTRACT

In the present study, next generation probiotic *Akkermansia muciniphila* was incorporated into a dairy matrix containing Portuguese whey cheese and Greek-style yoghurt in a proportion of 3.5:1, respectively. Subsequently, this innovative food was characterized in terms of microbiological and physicochemical parameters, total phenolic content and antioxidant, antidiabetic, and antihypertensive activities, as well as its protective effect on *A. muciniphila* viability during 21 d of refrigerated aerobic storage and when subjected to simulated gastrointestinal passage. The probiotic cheese spread displayed high microbiological quality, low total phenolic content (0.36 mg gallic acid equivalents/g of dried cheese) and interesting biological activities, including antidiabetic (98.10% α-glucosidase inhibition) and antihypertensive (49.18% angiotensin converting enzyme inhibition). Simultaneously, this food ensured a high *A. muciniphila* viability (>10^8 CFU/g) during 21 d of refrigerated aerobic storage with subsequent in vitro digestion. Additionally, this probiotic cheese presented a similar profile in terms of texture, color, water activity and pH when compared to the cheese control (without *A. muciniphila*), suggesting a potentially high acceptance among consumers. In conclusion, the developed cheese spread seems to be a promising and suitable food vector to safeguard *A. muciniphila* viability during refrigerated aerobic storage for at least 21 d with subsequent gastrointestinal passage.

1. Introduction

Increasing evidence-based information about diet and its impact on human health is driving consumer demand for functional foods that combine high nutrient value with health benefits (Rolim et al., 2020). The functional food market is rapidly expanding, with carotenoids, dietary fibers, fatty acids, minerals, prebiotics, probiotics, synbiotics, vitamins, and minerals dominating as bioactive ingredients (Turkmen et al., 2019). Probiotics, which are living microorganisms that have positive health effects when consumed in appropriate amounts (Hill et al., 2014), are among them. In addition, probiotics may be used as technological agents to improve the properties of food matrices (Rolim et al., 2020). The addition of probiotics to food matrices may indeed enhance certain properties, such as sensory, antiproliferative, anti-diabetic, antimicrobial and immunomodulatory properties (Madureira, Pintado, et al., 2011; Mushtaq et al., 2019).

Recently, the intestinal commensal bacterium *Akkermansia muciniphila* has attracted the attention of the scientific community as a novel probiotic candidate, also referred to as a next-generation probiotic, due to its relevant biological effects in a variety of metabolic and inflammatory disorders (Almeida et al., 2020; J. C. Barbosa et al., 2022). *Akkermansiamuciniphila* is a bacterium that belongs to the Verrucomycrobia phylum and represents approximately 0.5–5% of the bacteria present in the human gastrointestinal tract (GIT) (de Vos, 2017). This bacterium is a crucial symbiont of the intestinal microbiota as it can influence the host's immune responses and immune tolerance to commensal microorganisms (Derrien et al., 2004). Although *A. muciniphila* has been reported to be strictly anaerobic, recent studies

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have shown that it exhibits some tolerance to aerobic environments, maintaining a high level of culturability (Machado et al., 2020). Additionally, *A. muciniphila* has demonstrated high survival rates when exposed to in vitro GIT conditions (Machado et al., 2020). Therefore, the combination of aerotolerance and GIT resistance makes *A. muciniphila* a promising candidate from a technological standpoint (Almeida et al., 2022). The study conducted by Depommier et al. (2019) demonstrated the safety profile of *A. muciniphila* and its potential to improve metabolic parameters in overweight/obese insulin-resistant volunteers. This highlights *A. muciniphila's* potential as a candidate for novel food and pharmaceutical formulation development, as noted by Andrade et al. (2020).

Specifically, *Akkermansia muciniphila* DSM 22959 is a type strain and the most extensively described strain in the literature (Cozzolino et al., 2020; Machado et al., 2020, 2022). This strain is frequently used in biotechnology, particularly in the development of novel foods due to the extensive knowledge available on this specific strain (Barbosa et al., 2022a; Machado et al., 2023; Marcial-Coba et al., 2019; Vedor et al., 2023).

Dairy products are often used as carriers for probiotics due to their broad consumer acceptance and excellent nutritional profile (Rolim et al., 2020). Greek yoghurt, also known as strained yoghurt in Europe, is becoming increasingly popular due to its attractive sensory attributes and nutritional properties (Gyawali et al., 2022). Probiotic bacteria have been added to strained yoghurt, along with starter cultures, to create innovative probiotic yoghurts with enhanced functional, physicochemical, sensory, and microbiological safety properties (Yang & Yoon, 2022). Another excellent protective dairy matrix for probiotic cells is cheese. The probiotic microorganisms are protected during storage and gastrointestinal passage due to the unique characteristics of the matrix structure, which includes nutrient availability, fat and protein content, high buffering capacity, low oxygen levels, and high pH values (Rolim et al., 2020).

From a circular economy perspective, whey cheese is considered a sustainable solution for the use of whey, the main by-product of rennet and acid coagulation cheesemaking (Bintsis & Papademas, 2023; Garcia et al., 2022). These cheeses are produced by denaturing whey proteins through heating at 88–92 °C and have different names according to the country and region of origin (Bintsis & Papademas, 2023; Pintado et al., 2001). Portuguese whey cheese, also known as 'Requeijão', has been identified as a valuable food matrix for incorporating probiotics. This is due to its ability to support high survival rates of these beneficial microorganisms during storage and simulated gastrointestinal conditions (Garcia et al., 2022; Madureira et al., 2006, 2008, 2011). In this context, Faustino and colleagues developed a cheese spread containing *Osmundea pinnatifida* red algae extract, using Portuguese whey cheese and Greek-style yoghurt as the dairy matrix. This innovative dairy product has high microbiological quality and interesting bioactivities, mainly prebiotic and antihypertensive properties (Faustino et al., 2023).

Considering this rationale, the present work aimed to develop a cheese spread incorporating the next generation probiotic *A. muciniphila* using a dairy matrix based on Portuguese whey cheese and Greek-style yoghurt in a proportion of 3.5:1, respectively. This innovative food was then characterized in terms of microbiological, physicochemical parameters (namely texture, color, water activity, and pH), total phenolic compounds content and antioxidant, antidiabetic, and antihypertensive activities. Furthermore, the protective effect of this dairy matrix on viability and stability of *A. muciniphila* during refrigerated aerobic storage and when exposed to in vitro gastrointestinal passage was evaluated. In this respect, this study is the first to provide evidence of the ability of a cheese spread composed of Portuguese whey cheese and Greek-style yoghurt to efficiently protect the next-generation probiotic *A. muciniphila* during aerobic storage and in vitro passage through the GIT.

2. Material and methods

2.1. Bacterial strain and culture conditions

In the present work, the freeze-dried *Akkermansia muciniphila* DSM 22959 strain from the DSMZ collection (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was used. The strain was stored frozen at −80 °C in PYG broth supplemented with 1 g/L mucin [PYGM; media composition as specified in DSMZ (2023) except for the absence of resazurin], with 20 mL/100 mL glycerol (Fisher Scientific, Loughborough, UK) for long-term storage. For each experiment, *A. muciniphila* DSM 22959 glycerol stock was thawed and grown in PYGM broth at 37 °C for 20–24 h under anaerobic conditions (85% N_2, 5% H_2, and 10% CO_2) in an anaerobic incubator (Whitley A35 HEPA anaerobic workstation, Bingley, UK). The bacterial cultures were then propagated through at least two subsequent culture steps by inoculating in fresh PYGM broth at proportion of 1:10. The cultures were incubated under the same growth conditions. The resulting cultures underwent centrifugation at 12000×*g* for 30 min at 4 °C using a Sorvall LYNX 4000 Superspeed Centrifuge (Thermo Scientific, MA, USA). Subsequently, they were washed once with the same volume of 8.5 g/L NaCl (Sigma-Aldrich, St. Louis, MO, USA). The bacterial pellet was then resuspended in 8.5 g/L NaCl to achieve a cell concentration of approximately 10^9 colony-forming units (CFU) per milliliter (CFU/mL). The resulting bacterial suspension was used either directly as a control for free cells or to produce cheese spread.

2.2. Cheese spread production

Commercial whey cheese made from cow's milk and Greek-style yoghurt without added sugar were purchased from a local supermarket (Lidl, Porto, Portugal). Table 1 presents the nutritional information for both products. The recipe for the cheese spread was based on the previous work of Faustino et al. (2023) with some modifications. Briefly, the cheese spreads were prepared by mixing 21 g of whey cheese with 6 g of Greek-style yoghurt per batch in sterile containers. The mixture was then subjected to thermal treatment in a water bath (GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany) for 10 min at 90 °C. Afterwards, the pasteurized cheese spreads were cooled down to room temperature. The probiotic cheese spread was prepared by mixing the *A. muciniphila* pellet (approximately 3×10^{10} CFU) with a sterile spatula. It is important to note that the *A. muciniphila* pellet used in each batch of cheese spread was obtained by centrifuging 30 mL of *A. muciniphila* saline suspension (with a concentration of about 10^9 CFU/mL) at 12000×*g* for 30 min and subsequently removing the supernatant. Additionally, control batches of processed cheese spread without *A. muciniphila* were included in the experiments.

2.3. Microbiological evaluation of cheese spread

To assess the viability and stability of *A. muciniphila* in the cheese spread, as well as to identify potential microbial contaminations, microbiological analyses were conducted on three randomly selected cheese spreads incorporating *A. muciniphila*. These analyses were

Table 1
Nutritional declaration of Portuguese whey cheese and Greek-style yogurt per 100g.

Nutrition declaration per 100g		Whey cheese	Greek-style yogurt
Energy		585 kJ	442 kJ
Fat		9.4 g	8.5 g
	of which saturated	6.7 g	5.4 g
Carbohydrates		5.1 g	4.3 g
	of which sugars	4.0 g	3.6 g
Proteins		8.9 g	3.2 g
Salt		0.63 g	1.11

performed on the day of production (day 0) and after 7, 14, and 21 d of refrigerated (at 4 °C) aerobic storage. For the analysis, a sample weighing 1 g from each batch was diluted in 9 mL of sterile phosphate buffer saline (PBS; VWR, Radnor, PA, USA) and homogenized using a vortex. Serial decimal dilutions were then prepared in PBS and plated in triplicate on various solid media: PYGM agar for enumerating *A. muciniphila*, de Man, Rogosa and Sharpe agar (MRS, BIOKAR Diagnostics, Beauvais, France) for counting lactic acid bacteria, plate count agar (PCA, Merck, Darmstadt, Germany) for detecting mesophilic aerobic bacteria, potato dextrose agar (PDA, BIOKAR Diagnostics, Beauvais, France) for counting yeasts and molds, and violet red bile glucose agar (VRBGA, BIOKAR Diagnostics, Beauvais, France) for detecting and enumerating Enterobacteriaceae. The inoculated agar plates were then incubated under specific conditions: PYGM agar at 37 °C anaerobically for 3–7 d, MRS agar at 37 °C under both aerobic and anaerobic conditions for 2 d, PCA at 30 °C aerobically for 2–5 d, PDA at 20 °C aerobically for 5 d, and VRBGA at 37 °C aerobically for 2 d. After the respective incubation periods, colony numbers were counted, and the results were expressed as CFU per gram (CFU/g) of cheese spread.

2.4. Physicochemical characterization of the cheese spread

One day after production, the physicochemical parameters including texture, color, water activity, and pH were analyzed in triplicate for both the cheese spread incorporating *A. muciniphila* and the control cheese (i.e. without *A. muciniphila*). Texture properties were evaluated by measuring the force-time curve using a TA. XT equipment (Stable Micro Systems, Surrey, UK). An extrusion test was performed, as suggested by the manufacturer for this type of dairy product. Briefly, a back extrusion rig (A/BE) composed by a sample container and a disc plunger with 40 mm were used. The container was filled up to 75% of its capacity, to exclude base interferences. The disc plunger performs a compression test which extrudes the product up and around the edge of the disc. This test measures the consistency of viscous products, such as yoghurt, creams and sauces, and the results relate to measurements of viscosity. The disc plunger was attached to a 5 kg load cell, which was calibrated using a 2 kg weight. The extrusion was performed in triplicate at room temperature (22 ± 2 °C). Distance was set at 30 mm with a trigger force of 0.5 g and a test speed of 1 mm/s. This test allowed the measurement of various attributes such as firmness, consistency, and cohesiveness.

The color evaluation was performed using a colorimeter (Chroma meter CR 400, Konica Minolta, Osaka, Japan). The device provided values for lightness (L*), green-red chromaticity coordinate (a*), and blue-yellow chromaticity coordinate (b*). The color differences (ΔE*) between the cheese spread with *A. muciniphila* and cheese spread control were calculated using the equation:

$$\Delta E^* = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \right]^{\left(1/2\right)} \quad (1)$$

where ΔL*, Δa*, and Δb* are the difference between the two samples (cheese spread with *A. muciniphila* and cheese control) in L*, a*, and b*, respectively. The perception of the color difference ΔE* varies according to the observed color and the sensitivity of the human eye. The human eye only distinguishes color difference if ΔE* is larger than 1–3 (Bodart et al., 2008).

The water activity (aw) of the cheese spreads was analyzed using a water activity meter (LabMaster-aw neo, Lachen, Switzerland), while pH measurements were performed by immersing the electrode probe of a pH meter (Basic 20 Crison Instruments, Barcelona, Spain) into the cheese spreads.

2.5. In vitro simulation of the gastrointestinal tract

The standardized digestion method (Brodkorb et al., 2019), with slight modifications, was used to perform the in vitro gastrointestinal tract simulation 21 d after cheese spread production. Independent tubes were used for either 0.5 mL of free cells or 0.5 g of cheese spread incorporating *A. muciniphila*, with two replicates per timepoint and per condition. To replicate the temperature and peristaltic movements of the human gastrointestinal tract, an orbital shaker incubator (Wiggen Hauser, Berlin, Germany) set at 37 °C and 200 rpm was used. During the gastric phase, the samples were exposed to 2 mL of simulated gastric fluid (pH 3) containing pepsin (3.33×10^{-5} kat/mL – sourced from porcine gastric mucosa; Sigma Aldrich, St. Louis, MO, USA) for 120 min. Subsequently, intestinal conditions were simulated by adding 4 mL of simulated intestinal fluid containing pancreatin (based on the trypsin activity at 1.66×10^{-6} kat/mL in the final mixture; Sigma Aldrich, St. Louis, MO, USA) and bile salts (Sigma Aldrich, St. Louis, MO, USA). The pH was adjusted to 7, and the exposure time was 180 min. To evaluate the impact of gastric and intestinal conditions on the viability of *A. muciniphila* (both free and incorporated in cheese spread), samples were collected after the gastric and intestinal phases. Enumeration of *A. muciniphila* cells was carried out according to the previously described procedure. The in vitro digestion protocol was conducted under aerobic conditions, while the PYGM plates were incubated under anaerobic conditions.

2.6. Extract preparation for total phenolic content, antioxidant, antidiabetic, and antihypertensive activities determination

Cheese spread extracts from cheese spread with *A. muciniphila* and cheese spread control were prepared according to the procedure described by Ribeiro et al. (2021) with minor modifications. Briefly, previously lyophilized cheese spread with *A. muciniphila* and cheese spread control (each in triplicate) were homogenized with 30 mL of methanol acidified with formic acid (9:1 v/v), using an orbital shaker (Wiggen Hauser, Berlin, Germany) at 250 rpm, for 1 h. The homogenized samples were centrifuged at 3850×*g*, at 4 °C for 10 min (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany), and the supernatant was stored at −20 °C overnight, to allow for protein precipitation. The resulting slurry was centrifuged again under the previous conditions to remove soluble proteins. The extract was evaporated using a rotavapor (Buchi, Flawil, Switzerland) under the following conditions: bath temperature of 45 °C, pressure of 100 atm, for approximately 30 min. Finally, the final volume of each extract was adjusted to 5 mL, by adding deionized water.

2.7. Total phenolic compounds content evaluation

Following the protocol described by Singleton and Rossi (1965) and Coscueta et al. (2018) with minor modifications, the total phenolic content of cheese spread with *A. muciniphila* and cheese spread control was determined. A calibration curve for gallic acid, ranging from 0.025 to 0.200 mg/mL, was established to express the results in milligrams of gallic acid equivalents per milliliter of sample (mg GAE/mL). The assay consists of adding 30 μL of each sample (or its required dilution), 100 μL Folin-Ciocalteu solution (20 mL/100 mL) and 100 μL anhydrous sodium carbonate solution (74 g/L) to each designated well. The microplate was wrapped in aluminium paper and incubated for 30 min at 25 °C in the dark. A multi-detection plate reader (Synergy H1, VT, USA) operated by Gen5 software was used to read the resulting blue mixtures at 765 nm. Results were expressed as milligrams of gallic acid equivalent per gram of dried cheese spread (mg GAE/g). All assays were performed in triplicate.

2.8. 2,2-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging assay

The antioxidant activity was evaluated using the ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) scavenging assay described by Gonçalves et al. (2009) with slight modifications. First, an initial

absorbance of 0.70 (±0.02) at 734 nm was obtained by adjusting the concentration of the ABTS working solution. Simultaneously, a Trolox solution was prepared by dissolving 0.0125 g Trolox (Sigma-Aldrich, MO, USA) in 1 mL methanol (Fischer Chemical, MA, USA) and making up to 50 mL with ultrapure water. A calibration curve of Trolox (0.0063–0.044 g/L) was then plotted to allow results to be expressed as mg of Trolox equivalents per gram of sample. For the assay, each well of a 96-well microplate was filled with 20 μL Trolox, sample or solvent and 180 μL ABTS working solution. The microplate was incubated at 30 °C for 5 min and the absorbance was measured at 734 nm using a multi-detection plate reader (Synergy H1, VT, USA). All assays were performed in triplicate.

2.9. α-Glucosidase inhibition assay

Antidiabetic activity was evaluated using the alpha-glucosidase inhibitory activity assay described by Kwon et al. (2008) with minor modifications. Briefly, 50 μL of samples were mixed with 100 μL of phosphate buffer (prepared according described in Kwon et al. (2008)) containing α-glucosidase solution (1.67×10^{-8} kat/mL) in each well of a 96-well microplate. The mixture was incubated for 10 min at a temperature of 25 °C for later addition of 50 μL of 1.51 g/L *p*-nitrophenyl-α-D-glucopyranoside solution in phosphate buffer to each well. Absorbance was read and reaction mixtures were incubated at 25 °C for 5 min for further absorbance readings using a multi-detection plate reader (Synergy H1, VT, USA) at 405 nm. The assay included a negative control containing 50 μL buffer instead of the sample and a positive control containing 50 μL acarbose at a concentration of 10 mg/mL. All assays were performed in triplicate. The α-glucosidase inhibition was calculated using the following formula:

$$\alpha - Glucosidase\ inhibition\ (\%) = \left(\frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}}\right) x\,100 \quad (2)$$

where $\Delta Abs_{control}$ is the variation of absorbance of the control and ΔAbs_{sample} is the variation of absorbance of the samples.

2.10. Angiotensin-I converting enzyme (ACE)-inhibitory activity assay

The antihypertensive activity was determined using the angiotensin converting enzyme (ACE) inhibitory activity assay, following the protocol described by SENTANDREU and TOLDRA (2006), with slight modifications. Briefly, 40 μL of ultrapure water or ACE working solution (7.0×10^{-6} mkat/mL) was added to each well of the microtiter plate. By adding ultrapure water to the blanks and the respective samples to the color control or sample wells, the final volume of 80 μL was adjusted. The enzymatic reaction was then initiated by adding 160 μL of substrate solution and the mixture was incubated at 37 °C. After 30 min, the excitation and emission wavelengths were set at 350 nm and 420 nm, respectively, and the fluorescence generated was measured using a multi-detection plate reader (Synergy H1, VT, USA). All assays were performed in triplicate.

For the calculation of ACE inhibitory activity (iACE), the following formula was used:

$$iACE\ (\%) = ((F_{CTL} - F_{BLK}) - (F_{SPL} - F_{SPLB})) * \frac{100}{F_{CTL} - F_{BLK}} \quad (3)$$

where F_CTL represents the fluorescence intensity of the control, F_BLK represents the fluorescence intensity of the blank, F_SPL represents the fluorescence intensity of the sample, and F_SPLB represents the fluorescence intensity of the color sample control.

2.11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of replicates and analyzed using IBM SPSS Statistics 28.0 software (Chicago, IL, USA). Parametric tests were performed if the data followed a normal distribution according to the Shapiro-Wilk test (normality test). Thus, for the statistical analysis of the comparison between cheese spread with *A. muciniphila* and control in terms of texture, water activity, pH, total phenolic content and antidiabetic and antihypertensive activities, the t-student test for independent samples was used. Statistical differences were considered significant with P values < 0.05.

3. Results and discussion

3.1. Microbiological parameters of cheese spread

A paramount concern for the food industry and the general community pertains to the potential contamination of food by deleterious microorganisms and environmental pathogens. Consequently, the assurance of microbiological quality assumes a critical aspect, particularly in field of milk and its related products (Fusco et al., 2020). According to Madureira et al. (2011), whey cheeses have been described in the literature as readily available nutrient-rich matrices that coupled with their high pH, high water content, low salt concentration and water activity close to 1, may promote the growth of spoilage microorganisms. To counteract this issue, thermal treatments have been recommended as effective strategies to prevent microbial contamination and maintain the microbiological quality and safety of final products (Soni et al., 2021). Taking this into consideration, a thermal treatment (90 °C for 10 min in water bath) was applied to the cheese spread before incorporating *A. muciniphila* to prevent microbial contamination during 21 d of refrigerated aerobic storage. The microbiological analysis showed that microbial counts of lactic acid bacteria, aerobic mesophilic microorganisms, fungi and Enterobacteriaceae in cheese spread incorporating *A. muciniphila* were below the limit of detection of the CFU plating technique, i.e., lower than 1000 CFU/g, in all sampling timepoints (0, 7, 14, and 21 d after refrigerated storage). Thus, these results indicate that this probiotic cheese spread is safe for consumption in regards of contamination and spoilage for at least 21 d under refrigerated storage conditions, in accordance with the previous findings of Faustino and colleagues (Faustino et al., 2023). The viability of probiotics in food products is another crucial factor. Factors such as pH, temperature, and processing techniques may impact the viability of the probiotic during manufacturing and subsequent storage (Terpou et al., 2019). Taking this into consideration, the viability of *A. muciniphila* was evaluated throughout the refrigerated aerobic storage for 21 d. As it can be observed in Fig. 1, *A. muciniphila* DSM 22959 was successfully

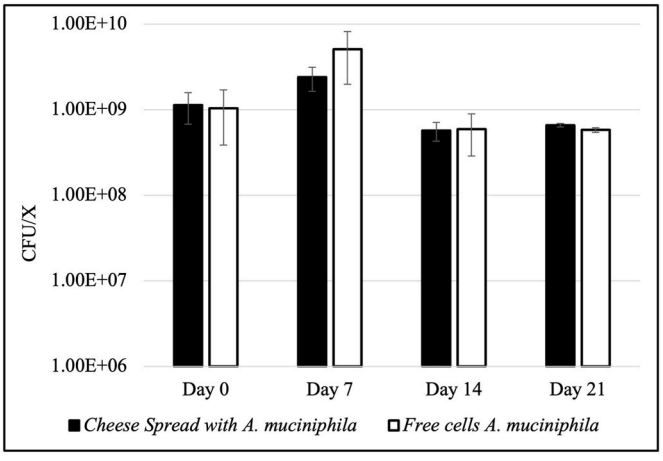

Fig. 1. Viable cell numbers of *A. muciniphila* DSM 22959 incorporated in cheese spread (expressed in CFU/g – black bars) and free cells (expressed in CFU/mL – white bars) and their evolution throughout refrigerated (4 °C) aerobic storage for 21 d. CFU has been expressed per x, as the free cells concentration is in liquid form (mL), while the results obtained from a solid food (cheese spread) are expressed per g.

incorporated into the dairy matrix, as the viable cell numbers in the cheese spread and free forms were similar at the day of cheesemaking, i.e., $1.13 \times 10^9 \pm 4.54 \times 10^8$ CFU/g and $1.04 \times 10^9 \pm 6.54 \times 10^8$ CFU/mL, respectively. After an aerobic storage period of 7 d at 4 °C, both the free cells and the cheese incorporated with *A. muciniphila* exhibited an increase in viability. The observed increase in the survival rate may be attributed to the recovery of the microbial cells, which may have suffered damages during the production and/or incorporation phases, in particular during the centrifugation steps. Specifically, in the case of *A. muciniphila*, which possesses relatively small cells (0.6–1.0 μm according to Derrien et al. (2004)), higher centrifugation speeds (12.000×*g* as reported by Barbosa et al. (2022)) were necessary to obtain the maximum cell yield. After 14 d of storage, a slight decrease in *A. muciniphila* cell viability was observed independently of its form – incorporated in the cheese spread or as free cells. At the end of storage (21 d), the viability of *A. muciniphila* incorporated in cheese spread and in free form was $6.57 \times 10^8 \pm 3.06 \times 10^7$ CFU/g and $5.78 \times 10^8 \pm 3.54 \times 10^7$ CFU/mL, respectively (Fig. 1). In fact, the *A. muciniphila* viability loss either in cheese or free form was lower than 0.5 log cycle in relation to day 0. Furthermore, these results suggest that cheese spread could be an adequate and suitable food matrix to safeguard viability and stability of *A. muciniphila* during refrigerated aerobic storage. In fact, *A. muciniphila* viability was maintained at acceptable levels during storage, since it is generally accepted that probiotic products should have a minimum concentration of 10^6 CFU/mL or gram (Kechagia et al., 2013). In literature, it has been described that the incorporation of probiotic bacteria has been successfully performed in whey cheese matrices. Several strains of *Lactobacillus acidophilus*, *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) and *Bifidobacterium animalis* were able to maintain viability levels above 10^7 CFU/g during at least 21 d of refrigerated storage in whey cheeses with or without additives (Garcia et al., 2022; Madureira et al., 2006, 2008, 2015).

3.2. Physicochemical characteristics of cheese spread

The physicochemical characteristics of cheese spread with and without the incorporation of *A. muciniphila*, are detailed in Table 2. In general, the addition of *A. muciniphila* did not lead to significant changes in texture, water activity, and pH values compared to the control cheese spread without *A. muciniphila* ($p > 0.05$). Additionally, the color difference (ΔE) between the cheese spread with *A. muciniphila* and the control was below the minimum detection limit ($\Delta E = 0.771 < 1$), indicating that the two types of cheese spread were indistinguishable to the human eye.

Thus, it is possible to conclude that, at day 1 of storage, there are no significant physicochemical alterations in the cheese spread incorporating *A. muciniphila* when compared with the control counterpart. These observations are desirable as they may indicate a potentially high acceptability of this probiotic cheese spread by the consumers (Madureira et al., 2015). A study involving the incorporation of *A. muciniphila* in a chocolate matrix, demonstrated similar results, highlighting how the incorporation of *A. muciniphila* does not significantly impact the physicochemical properties (Vedor, 2023). However, these analyses were performed only at day 1 of storage, so to better understand if the *A. muciniphila* throughout the storage period changed the physicochemical characteristics of the cheese spread, it would be of great importance to perform the same analyses along the storage period.

Table 2
Physicochemical characteristics of cheese spread with and without *A. muciniphila*.

	Cheese spread with *A. muciniphila*	Cheese spread without *A. muciniphila* (control)
Texture		
Firmness (kg)	0.692 ± 0.011	0.919 ± 0.139
Consistency (kg/s)	12.329 ± 0.877	14.718 ± 2.517
Cohesiveness (kg)	−0.470 ± 0.065	−0.616 ± 0.078
Work of cohesion (kg/s)	−0.647 ± 0.163	−0.787 ± 0.180
Color		
L	77.68 ± 0.67	76.97 ± 1.52
a	1.95 ± 0.06	1.77 ± 0.03
b	3.41 ± 0.31	3.62 ± 0.03
Water activity	0.966 ± 0.005	0.968 ± 0.003
pH	6.00 ± 0.04	5.98 ± 0.01

The symbol * marks the statistically significant differences between cheese spread with *A. muciniphila* and control cheese spread regarding each specific physicochemical parameter.

3.3. Total phenolic content and antioxidant activity of cheese spread

The most important dietary sources of polyphenols are wine, beer, coffee, tea and foods of plant origin (Nardini, 2022). Phenolic compounds are chemical substances produced during plant metabolism and consist essentially of several phenolic groups. These compounds are highly effective in neutralizing free radicals, have antioxidant properties and have anti-tumour, anti-microbial and anti-mutagenic activities (Khan et al., 2018; Shui & Leong, 2002). The total phenolic content depends on several factors, including the food matrix, the nature of the process, and the duration of the treatment (Arfaoui, 2021).

As it can be observed in Table 3, the total phenolic content present in cheese spread with *A. muciniphila* and in control was relatively low, with concentrations ranging from 0.36 to 0.38 mg gallic acid equivalents/g for dried cheese spread with *A. muciniphila* and cheese spread control, respectively. In literature, the phenolic compounds content and antioxidant activity have been correlated in direct proportionality, i.e., high levels of phenolic compounds have been linked to high antioxidant activity (Khan et al., 2018). Thus, given the low concentration of phenolic compounds in both cheese spreads, the antioxidant activity was below the limit of detection of the ABTS assay (lower than 0.0156 mg of Trolox equivalents/g of dried cheese spread). Even so, it would be useful to carry out a qualitative analysis to understand exactly which phenolic compounds are present in the matrix, even if in minor quantities (Khoddami et al., 2013). This analysis could be performed by spectrophotometric or chromatographic techniques (Khoddami et al., 2013).

As phenolic compounds are a type of secondary metabolite mainly synthesized by plants (Lin et al., 2016), it would be expected that this cheese spread would contain a low concentration of these compounds as it is a product of animal origin. It is known that the phenolic compounds present in cow's milk, such as phenols (thiophenol, cresols, ethyl phenols), hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and anthocyanidins, are derived from the diet, although they may also result from the conversion of amino acids (O'Connell & Fox, 2001). Furthermore, there are no statistically significant differences in total phenolic

Table 3
Total phenolic content and antioxidant activity measured using ABTS assay, of cheese spread incorporating *A. muciniphila* and control.

	Cheese spread with *A. muciniphila*	Cheese spread without *A. muciniphila* (control)
Total phenolic content (in mg gallic acid equivalents/g of dried cheese spread)	0.36 ± 0.04	0.38 ± 0.02
ABTS scavenging activity (in mg of Trolox equivalents/g of dried cheese spread)	<0.0156	<0.0156

The symbol * indicates the statistically significant differences ($p < 0.05$) between cheese spread incorporating *A. muciniphila* and cheese control in each parameter: total phenolic content and ABTS scavenging activity.

content and ABTS scavenging activity between the control cheese and the cheese spread incorporating *A. muciniphila* ($p > 0.05$), which suggests that *A. muciniphila* does not influence the phenolic compounds amount present in the cheese spread. Since this activity was determined on the day of production (day 0), this result was expected. To evaluate if *A. muciniphila* presents some kind of metabolic activity, the determination of the total phenolic content and antioxidant activity should have been performed throughout or at the end of the storage. Moreover, it is important to note that these results are in accordance with Faustino et al. (2023) study that reported a low ABTS scavenging activity (ABTS radical scavenging percentage around 10%) in a cheese spread with similar composition to that produced in this work.

3.4. Antidiabetic activity of cheese spread

According to the World Health Organization, diabetes "is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys and nerves" (World and Health Organization, 2023a). Previously, a systematic review and dose-response meta-analysis verified that a modest increase in daily intake of dairy products such as low-fat dairy, cheese and yoghurt may contribute to the prevention of type-2 diabetes (Gao et al., 2013). Taking this into consideration, the antidiabetic potential of cheese spread with and without *A. muciniphila* (control) was assayed, by determining the α-glucosidase inhibitory activity.

In the present study, cheese spread with *A. muciniphila* and control displayed percentages of α-glucosidase inhibition of 98.10% (±3.68%) and 101.73% (±1.67%), respectively. Furthermore, no statistically significant differences in α-glucosidase inhibitory activity were found between these cheeses ($p > 0.05$). This finding suggests that the addition of a probiotic strain such as *A. muciniphila* does not impact the α-glucosidase inhibitory activity either positively or negatively. It is important to note that *in vivo* studies highlight the potential of *A. muciniphila* in reducing the accumulation of body mass and the levels of plasma TNF-α. Furthermore, it has the potential to enhance the number of goblet cells, promote mucin secretion, and restore the integrity of the gut barrier (Deng Si-si et al., 2022). Collectively, these effects contribute to ameliorating symptoms associated with diabetes. The assay performed in the current work was focused on antidiabetic activity does not specifically address insulin sensitivity; rather, it evaluates the inhibition of α-glucosidase. Consequently, establishing a robust correlation between the incorporation of *A. muciniphila* into the cheese spread matrix and an enhanced antidiabetic potential proves challenging, as distinct parameters are under evaluation. In their work, Vedor et al. reached similar conclusions with the incorporation of *A. muciniphila* having no impact on the α-glucosidase inhibitory activity and therefore requiring further *in vivo* studies to evaluate the antidiabetic activity of *A. muciniphila* (Vedor, 2023). Despite the absence of an influence on α-glucosidase inhibitory activity in the cheese matrices with the addition of *A. muciniphila*, the inclusion of this probiotic in the cheese spread may still be valuable, given its potential to exert antidiabetic effects in the human body post-consumption. To validate this hypothesis, additional *in vivo* studies targeting the antidiabetic potential of cheese spread incorporating *A. muciniphila* should be undertaken. Furthermore, the notably high antidiabetic activity of cheese spread (98.09 ± 3.68%) observed in this study diverges from the results reported by Faustino et al. (2023). In fact, these researchers recorded an α-glucosidase inhibitory activity below 2% in a cheese spread with similar composition (Faustino et al., 2023). Possible explanation for these discrepancies may be attributed to different protocols applied for preparation of extract that were used in the antidiabetic activity assay.

3.5. Antihypertensive activity of cheese spread

Hypertension, commonly referred to as high blood pressure, is a major cause of premature death worldwide (World and Health Organization, 2023b). Notably, the intake of dairy products has been associated with reduced risk of hypertension (Heidari et al., 2021). Taking this into consideration, the antihypertensive activity of cheese spread with and without *A. muciniphila* (control) was determined using the ACE inhibition assay.

In the present work, the ACE inhibition percentage was 49.18% (±25.54) and 41.35% (±11.18) for cheese spread incorporating *A. muciniphila* and control, respectively. Furthermore, there are no statistically significant differences in the antihypertensive activity between the cheese spread with *A. muciniphila* and the control ($p > 0.05$). Hence, these findings suggest that the *A. muciniphila* incorporation does not influence the antihypertensive effects of the cheese spread (measured in terms of ACE inhibition percentage). It is noteworthy that this assessment was conducted solely on the day of production, limiting the inference to the inherent properties of the matrix. To substantiate this notion, an *in vivo* investigation focusing on the antihypertensive activity of the cheese spread incorporating *A. muciniphila* is essential. Furthermore, the current work recorded higher ACE inhibition percentages for the cheese spread compared to the values documented in the Faustino and coworkers' study, i.e., reporting undetectable ACE inhibition levels for a cheese spread with similar composition (Faustino et al., 2023). Possible explanation for these disagreements may be attributed to the same reason appointed for the antidiabetic activity namely different protocols applied for extract preparation.

3.6. Survival of Akkermansia muciniphila incorporated in cheese spread and free cells when exposed to simulated gastrointestinal passage

As previously mentioned, the probiotic cells must survive the harsh gastrointestinal conditions, reaching the intestine in adequate viability levels, to exert beneficial effects (Hill et al., 2014). Taking this into consideration, cheese spread incorporating *A. muciniphila* and its free counterpart after 21 d of refrigerated aerobic storage were exposed to simulated gastrointestinal passage, with the aim to investigate the potential protective effect of the dairy matrix (cheese spread) on *A. muciniphila* viability throughout gastrointestinal transit.

As can be observed in Table 4, *A. muciniphila* cells incorporated in the cheese spread maintained their viability throughout gastrointestinal passage. In opposite, free cells suffered viability reductions of at least 2 log cycles when exposed to in vitro digestion protocol, at 21 d of refrigerated aerobic storage. These results suggest that the cheese spread matrix offers protection towards *A. muciniphila* viability during gastrointestinal passage, even after prolonged refrigerated aerobic storage for 21 d. Furthermore, these results are in agreement with previous studies demonstrating that cheese matrices offer a high protection to probiotic microorganisms throughout gastrointestinal transit (Pitino et al., 2012). This protective effect may be attributed to the cheese's unique traits such as availability of nutrients, fat and proteins that form a solid matrix with high buffering capacity and low oxygen content that safeguard probiotic microbial cells during the passage through stomach to the intestine. (Rolim et al., 2020).

Table 4
Evolution of viable cell numbers of *A. muciniphila* DSM 22959 in free form (CFU/mL) or when incorporated in cheese spread (CFU/g) during in vitro gastrointestinal passage at 21 days.

	CFU/X ± S.D.	
	Free cells *A. muciniphila* DSM 22959	*A. muciniphila* DSM 22959 incorporated in cheese spread
Initial phase	$5.78 \pm 0.35 \times 10^8$	$6.57 \pm 0.31 \times 10^8$
Gastric phase	$9.20 \pm 3.96 \times 10^6$	$1.86 \pm 0.71 \times 10^9$
Intestinal phase	$1.95 \pm 1.62 \times 10^6$	$6.47 \pm 3.56 \times 10^8$

4. Conclusion

The incorporation of *A muciniphila* in a dairy matrix containing 77 g of Portuguese whey cheese/100$g_{final\ product}$ and 23 g of Greek-style yoghurt/100$g_{final\ product}$ led to the development of an innovative food, in cheese spread form, with high microbiological quality and interesting antidiabetic and antihypertensive activities. Concurrently, this novel food ensured a high *A. muciniphila* viability level (higher than 10^8 CFU/g) after refrigerated aerobic storage for 21 d and when subsequently exposed to simulated gastrointestinal conditions. Advantageously, the cheese spread incorporating *A. muciniphila* exhibited a similar profile in terms of texture, color, water activity and pH, when compared with control cheese, suggesting a potentially high acceptance among consumers. In future perspective, the physicochemical parameters, and biological activities throughout storage at least 21 d should be evaluated. Such monitoring would provide insights into whether *A. muciniphila* is exerting any metabolic actions within the food matrix. Furthermore, additional studies aiming the determination of nutritional composition of this probiotic cheese spread as well as consumer acceptance testing focused on specific sensory attributes should be conducted. Additionally, *in vivo* studies concerning both antidiabetic and antihypertensive properties should be performed to understand the impact of adding *A. muciniphila* to the food matrix.

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CRediT authorship contribution statement

Mariana Fonseca: Writing – original draft, Methodology, Investigation, Formal analysis. **Rita Vedor:** Writing – original draft, Methodology, Investigation, Formal analysis. **Joana C. Barbosa:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Ana Maria Gomes:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Daniela Machado:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, 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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