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Incorporation of *Bifidobacterium animalis* subspecies *lactis* BB-12® and *Akkermansia muciniphila* in chocolate matrices

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ABSTRACT

Chocolate is a food product highly popular worldwide, and it has been proposed as a carrier for probiotic delivery. This work evaluated the viability of probiotic strains, *Bifidobacterium animalis* subsp. *lactis* BB-12® and *Akkermansia muciniphila* DSM 22959, in chocolate matrices with different cocoa percentages (33.6; 54.5 and 70.5%) throughout 28-days of aerobic storage. The pH, total phenolic content and antioxidant, antidiabetic, and antihypertensive activities were also determined at timepoints of 0 and 28-days. During storage, all chocolates showed high pH stability (variations lower than 1 unit) and it was observed a growing trend in total phenolic content and in bioactivities with the increase of cocoa content in the matrix. Regarding probiotic viability, a higher level for *B. animalis* subsp. *lactis* BB-12® was achieved in chocolate containing 70.5% cocoa (estimated in 10^8 CFU/g after storage). In opposite, *A muciniphila* DSM 22959 exhibited greater viability in chocolate with 33.6% and 54.5% cocoa (around 10^6 CFU/g after storage, meeting the minimum required amounts for probiotic products). Conjugating the data from physicochemical properties, bioactivities and probiotic viability, dark chocolate matrices with 54.5% and 70.5% cocoa, may be considered a promising food vector for *A. muciniphila* DSM 22959 and *B. animalis* subsp. *lactis* BB-12®, respectively.

1. Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Most commercial probiotics belong to genus *Bifidobacterium* and the genus formerly known as *Lactobacillus* (Zheng et al., 2020), being available to consumers in various food and nutraceutical products (Gomes et al., 2017). Among *Bifidobacterium* species, *Bifidobacterium animalis* subsp. *lactis* BB-12® is one of the most well-documented probiotics (Jungersen et al., 2014). This strain, originated from Chr. Hansen's dairy cultures collection, has been incorporated in infant formula (Holscher et al., 2012), dietary supplements, and fermented milks (Chr Hanson, 2014). Technologically, BB-12® is highly attractive due to its fermentative activity, high aerotolerance, suitable stability, resistance to acid and bile. When added to food products, it generally does not lead to dramatic changes in sensory properties, such as taste, appearance, and mouthfeel (Cuffia et al., 2018).

In addition to classical probiotics, intestinal commensals, such as *Akkermansia muciniphila*, have been proposed as novel probiotic candidates, also termed as next-generation probiotics (Almeida et al., 2020). *Akkermansia muciniphila* belongs to the Verrucomycrobia phylum, representing around 0.5–5% of the bacteria in the human gastrointestinal tract (GIT) (de Vos, 2017). This bacterium is a crucial symbiont of the intestinal microbiota since it can shape the host's immune responses and the immune tolerance to commensal microorganisms (Derrien et al., 2011). Despite its reported strict anaerobic nature, recently, it was demonstrated that *A. muciniphila* exhibits a certain tolerance to aerobic environments, maintaining a high level of culturability (Machado et al., 2020). Furthermore, *A. muciniphila* presented a high survival when exposed to *in vitro* GIT conditions (Machado et al., 2020). Thus, the aerotolerance, conjugated with the GIT resistance, makes *A. muciniphila* very promising from a technological point of view (Almeida et al., 2022).

Worldwide, chocolate is one of the most appealing food products, due to its potential to sensorially arouse pleasure and trigger positive emotions on a psychological level (Konar et al., 2016). Chocolate is constituted by a fatty matrix, dubbed cocoa butter, in which the cocoa and sugar are incorporated. Its variable composition segregates and classifies this food product into defined categories with specific properties (Montagna et al., 2019). The primary categories are dark, milk,

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and white chocolate, which differ in the formulation's cocoa solids, milk fat, and cocoa butter contents (Petyaev & Bashmakov, 2017). Although chocolate is, sometimes, seen as harmful for human health, several studies suggest that chocolate, particularly dark chocolate, may confer health benefits due to the presence of numerous bioactive compounds (De Araujo et al., 2016). The cocoa bean itself contains more than 300 chemicals compounds present in chocolate. The bioactive compounds can be classified as follows: polyphenols, including flavonoids and non-flavonoids; methylxanthines, comprising theobromine and caffeine; and minerals, such as magnesium, iron, and zinc, among others (Petyaev & Bashmakov, 2017). All the above-mentioned bioactive molecules benefit the human body; however, studies suggest flavonoids are essential in the prevention of cardiovascular diseases (Petyaev & Bashmakov, 2017). Furthermore, these compounds are in higher quantities in dark chocolate, as this type of chocolate is richer in cocoa solids (flavonoids account for 12–18% of dry weight cocoa beans (Lik Hii et al., 2009)) compared to white and milk chocolate.

Chocolate incorporating probiotics may be a valuable candidate for novel functional foods due to the combined health benefits of probiotics and chocolates' bioactive compounds (Silva et al., 2017). Indeed, chocolate has been proposed as a suitable carrier to deliver probiotics, either in their free or encapsulated forms, as a viable alternative to fermented dairy products (Hossain et al., 2021). However, the incorporation of certain probiotic bacteria, such as *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* into chocolate matrices remains limited (Hossain et al., 2021; Marcial-Coba et al., 2019). Given their anaerobic nature, these two probiotics have not yet been incorporated as free cells in chocolate. Together with the increasing demands for functional chocolates, this creates an opportunity to innovate the probiotic market as well as the confectionery and food industries. Thus, this study aimed to evaluate the viability and stability of. *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* DSM 22959 incorporated in chocolate matrices with different cocoa percentages (33. 6%; 54.5% and 70.5%) throughout 28-days of aerobic storage at room temperature. Simultaneously, chocolates were characterized in terms of physicochemical properties (namely pH and total phenolic content) and bioactivities, including antioxidant, antidiabetic, and antihypertensive activities.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Akkermansia muciniphila DSM 22959 obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and *B. animalis* subspecies *lactis* BB-12® from Chr. Hansen collection (Hoersholm, Denmark) were used in this study. For long-term storage, bacterial strains were kept at −80 °C in appropriate media with 20% (v/v) glycerol (Fisher Chemical, Loughborough, United Kingdom), namely: PYG broth supplemented with 0.05% (w/v) mucin (Sigma-Aldrich, St. Louis, MO, USA) for *A. muciniphila* DSM 22959 [PYGM media composition following DSMZ recommendations (DSMZ, 2020) except that no resazurin was added] and de Man Rogosa and Sharpe (MRS) supplemented with 0.05% (w/v) of L-cysteine·HCl (Alfa Aesar, Kandel, Germany) for *B. animalis* subsp. *lactis* BB-12®. For each experiment, a glycerol stock of each bacterial strain was thawed and grown in appropriate broth for 24 h at 37 °C under anaerobic conditions (85% N_2, 5% H_2, and 10% CO_2) achieved using an anaerobic incubator (Whitley A35 HEPA anaerobic workstation, Bingley, United Kingdom). For *A. muciniphila* DSM 22959, at least two subsequent culturing steps using the same growth conditions were performed, with a final incubation volume of 1 L of PYGM broth with 10% (v/v) of cell inoculation, to obtain a higher biomass yield. Then, *A. muciniphila* cells were harvested by centrifugation (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo Scientific, MA, USA) at 12000×*g*, 30 min, at 4 °C. The resulting bacterial pellet was washed once with the same volume of sterile phosphate buffer saline (PBS; VWR, Radnor, PA, USA) and resuspended in 200 mL physiological saline solution (NaCl; 0.9% w/v). For *B. animalis* subsp. *lactis* BB-12®, one sub-culturing step at the same growth conditions was performed, with a final incubation volume of 200 mL of MRS with 0.05% cysteine with 1% (v/v) of cell inoculation. *B. animalis* BB-12® cells were collected by centrifugation (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany) at 3850×*g*, 10 min, at 4 °C. The resulting bacterial pellet was washed once with the same volume of sterile PBS and resuspended in 200 mL of NaCl 0.9% (w/v) solution. Both saline bacterial suspensions were stored at 4 °C for a maximum period of 24 h until chocolate preparation.

2.2. Chocolate production and storage

For chocolate production, 70 mL of bacterial suspensions were centrifuged at 3850×*g*, 10 min, at 4 °C, (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany) and the supernatants were discarded, while the bacterial pellets were maintained for incorporation. After this, 70 g of chocolate nuggets [containing 33.6% (Recipe Nº 823), 54.5% (Recipe Nº 811) or 70.5% (Recipe Nº 70-30-38) cocoa content] from Callebaut (Lebbeke-Wieze, Belgium) were melted in a chocolate melting pot (Meilleur du Chef, Bassussary, France) until a temperature of 50 °C was reached. Next, the temperature was lowered by mixing and dripping the chocolate preparation until 37 °C (optimum temperature to guarantee cell viability). At this moment, bacterial pellets were incorporated into the chocolate by mixing thoroughly. This chocolate mixture was spread onto a polycarbonate mold with half-spherical wells. After filling the wells, the mold was tossed a few times to remove air bubbles eventually present. Chocolates were left to solidify at room temperature (20 ± 2 °C) for 4 h. Finally, chocolates were removed from the mold, obtaining around 15 single-serving chocolates, and stored in aluminum-wrapped Petri dishes at room temperature (20 ± 2 °C) under aerobic conditions. Also, plain/control (without probiotics) chocolates (for each cocoa content tested) were prepared following the previous steps except for the incorporation of bacterial pellets. Two batches of each chocolate (with probiotics and control) were prepared and analyzed.

2.3. Viability assessment in bacterial suspensions and probiotic chocolates throughout aerobic storage

The number of colony-forming units (CFU) of *A. muciniphila* DSM 22959 and *B. animalis* subsp. *lactis* BB-12® were determined at different time points: 1) saline bacterial suspensions immediately before their centrifugation to obtain pellets for incorporation into chocolate; 2) chocolate samples immediately after production (i.e., at day 0 of storage); and 3) chocolate samples after 7, 14, 21 and 28 days of aerobic storage at room temperature (20 ± 2 °C). For condition 1 bacterial suspensions were 10-fold diluted in PBS, and 10 μL of each dilution were plated, in triplicate, onto PYGM agar (1.5% (w/v) bacteriological agar, Biokar diagnostics, Beauvais Cedex, France) for *A. muciniphila* DSM 22959, and MRS with 0.05% (w/v) cysteine and 1.5% (w/v) agar for *B. animalis* subsp. *lactis* BB-12®. For conditions 2 and 3, two single-serving chocolate samples (technical duplicates) were weighed and dissolved in pre-warmed (at 37 °C) PBS (in a proportion of 1:9). From this suspension, decimal dilutions were performed in PBS, and 10 μL of each dilution were plated, in triplicate, on the appropriate media. Agar plates were incubated at 37 °C under anaerobic conditions during 5–7 days for *A. muciniphila* DSM 22959 and 48 h for *B. animalis* subsp. *lactis* BB-12®. After incubation, CFU enumeration was performed, and the results were expressed as mean ± standard deviation CFU/mL for bacterial suspensions (condition 1) and mean ± standard deviation CFU/g in probiotic chocolate samples (conditions 2 and 3).

2.4. Aspect evaluation

To monitor the aspect of the different chocolates throughout the 28-day period of aerobic storage pictures were taken of the single-serve doses, in duplicate at day 0 and day 28.

2.5. Physicochemical characterization of probiotic chocolate

2.5.1. pH measurement

The chocolate pH values were assessed at day of production/manufacture and after 28 days of aerobic storage by dissolving single-serving chocolates in pre-warmed (at 37 °C) PBS in a proportion of 1:9 and analyzing the resulting suspension using a pH meter (Crison Instruments, Barcelona, Spain).

2.5.2. Chocolate extracts preparation

Chocolate extracts were prepared according to the method described by Silva et al. (2017) with minor modifications. Initially, 5 mL of the previously diluted chocolate samples (section 2.3) were defatted using 10 mL n-hexane 97% (Ibis Scientific, NV, USA). This solution was homogenized by agitation, followed by sonication (Bath sonicator, Bandelin, Berlin, Germany) for 5 min and centrifugation at 2470×*g* for 5 min at 20 °C (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany). The supernatant was removed with a Pasteur pipette. This procedure was performed twice, and the samples were left to air-dry the residual n-hexane for approximately 1 h.

Posteriorly, the phenolic compounds extraction was performed by adding 80% (v/v) ethanol. Thus, 20 mL of absolute ethanol (VWR, PA, USA) were added to the remaining pellet and homogenized by a sonication step of 10 min, followed by a centrifugation step at 3850×*g* for 5 min at 20 °C (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany).The supernatant was collected in a new 50 mL-tube, and the procedure was repeated by adding 8 mL of absolute ethanol and 2 mL of deionized water. A rotavapor (Buchi, Flawil, Switzerland) was used to concentrate the phenolic content of the samples. Each sample was exposed to the rotavapor for approximately 30 min with a bath temperature of 45 °C and a pressure of 100 atm. A final volume of 5 mL of sample was obtained. These extracts were obtained from chocolates at day of production/manufacture and after 28 days of aerobic storage and were used for determination of total phenolic content and antioxidant, antihypertensive and antidiabetic activities.

2.5.3. Total phenolic content (TPC) determination

The TPC was determined using the Folin–Ciocalteu colorimetric method following the protocol described by Singleton and Rossi (1965) and Coscueta et al. (2018), with slight modifications. Firstly, a calibration curve of gallic acid (0.025–0.200 mg/mL) was prepared to express the results as milligrams of gallic acid equivalents per milliliter of sample (mg GAE/mL). The assay consists of adding 30 μL of each sample (or its necessary dilution), 100 μL of Folin-Ciocalteu solution (20% v/v) (Merck KGaA, Darmstadt, Germany), and 100 μL of anhydrous sodium carbonate solution (7.4% w/v) to each assigned well. The microplate, wrapped in aluminum paper, was incubated for 30 min at 25 °C in the dark. The resulting blue mixtures were read at 765 nm on the Multi-detection plate reader (Synergy H1, VT, USA) operated using the Gen5 software. The results were expressed as mg GAE per g of chocolate (mg GAE/g). All assays were performed in triplicate.

2.6. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging assay

The antioxidant activity was measured through ABTS scavenging assay following the protocol described by Gonçalves et al. (2009), with slight modifications. Firstly, the concentration of the ABTS working solution was adjusted to an initial absorbance of 0.70 (±0.02) at 734 nm. Then, the Trolox solution was prepared by weighing 0.0125 g of Trolox (Sigma-Aldrich, MO, USA) and dissolving in 1 mL of methanol (Fischer Chemical, MA, USA), completing the 50 mL volume with deionized water. To express the results as Trolox equivalents a standard curve (25μM–175μM) was calculated. For the assay, 20 μL of Trolox or sample or solvent and 180 μL of ABTS working solution were added to each well. The 96-well microplate was incubated for 5 min at 30 °C, and the absorbance was measured at 734 nm with a Multi-detection plate reader (Synergy H1, VT, USA). All assays were performed in triplicate.

2.7. α-Glucosidase inhibition assay

The antidiabetic potential was measured through the assessment of α-glucosidase inhibitory activity following the procedure described by Kwon et al. (2008), with some modifications. Firstly, 50 μL of samples were mixed with 100 μL of 0.1 M phosphate buffer (pH = 6.9) containing a-glucosidase solution (1.0 U/mL) per each well, and the mixture was incubated at 25 °C for 10 min. Afterward, to each well was added 50 μL of 5 mM p-nitrophenyl-a-D-glucopyranoside solution in 0.1 M phosphate buffer (pH = 6.9). Then, the absorbance was read, and the 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.

For this assay, a negative control containing 50 μL of buffer solution in the place of the sample and positive control containing 50 μL of acarbose at a concentration of 10 mg/mL were used. All assays were performed in triplicate. α-Glucosidase inhibition was calculated as following:

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

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

The antihypertensive activity was determined via ACE-inhibitory activity assay following the protocol described by Sentandreu and Toldra (2006), with minor modifications. Firstly, 40 μL of ultrapure water or ACE working solution (42 mU/mL) were added to each correspondent well. Next, the final 80 μL volume was adjusted by adding ultrapure water to blank, control, or samples. Finally, the enzyme reaction was started by adding 160 μL of substrate solution (0.45 mM), and the mixture was incubated at 37 °C. The generated fluorescence was measured for 30 min using a Multi-detection plate reader (Synergy H1, VT, USA), with excitation and emission wavelengths at 350 and 420 nm, respectively. All assays were performed in triplicate. The ACE inhibitory activity (iACE) percentage was calculated as following:

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

2.9. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of replicates and analyzed using a statistical package for the social science 17.0 software (SPSS; Chicago, IL, USA). Parametric tests were carried out if the data followed a normal distribution according to Shapiro-Wilk test (Normality test). With this, for statistical analysis of bioactivities, One-Way analysis of variance (ANOVA) test was used to evaluate differences between different conditions and cocoa contents, while for comparison between two timepoints t-Student test for paired samples was applied. Additionally, for statistical comparison between control and probiotic chocolate, t-Student for independent samples test was performed. Statistical differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Viability of probiotics before, during chocolate preparation and throughout aerobic storage

One important factor to consider when incorporating probiotics into food, such as chocolate matrices, is their viability throughout food production and subsequent storage (Silva et al., 2017; Succi et al., 2017). Although there is no unanimity regarding the minimum number of probiotic microorganisms needed to confer health-beneficial effects, it is widely accepted that a minimum level of 10^6–10^7 CFU/mL or CFU/g of viable probiotic cells must be in the final product at the consumption time (Kechagia et al., 2013; D. M. Rasika et al., 2021).

As it can be observed in Fig. 1(a) and (b), the initial bacterial suspensions of *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* DSM 22959, used for the incorporation in chocolate, presented viable cell numbers between 10^9 and 10^{10} CFU/mL. After incorporation in chocolate, a viability decrease was observed for both bacteria in all three different cocoa percentages. Less than 1 log-cycle reduction was reported for *B. animalis* subsp. *lactis* BB-12® whereas for *A. muciniphila* DSM 22959 the viability reduction was higher than 1 log-cycle, particularly in the 70.5% cocoa percentage chocolate, where a reduction greater than 2 log CFU was observed. Concerning the aerobic storage of *B. animalis* subsp. *lactis* BB-12®, a gradual decrease was observed throughout the 28-days in all chocolate matrices. However, the highest survival was verified for the chocolate with the highest cocoa content (mean value of 0.55 log CFU/g reduction, when comparing day 0 versus day 28), followed by the chocolate matrix with 33.6% of cocoa (mean value of 1.30 log CFU/g reduction, day 0 versus day 28). Contrarily, the chocolate matrix containing 54.5% cocoa content presented the lowest protective power (mean value around 2.95 log CFU/g loss, day 0 versus day 28). Despite the viability reduction, all chocolate matrices allowed the survival of *B. animalis* subsp. *lactis* BB-12® at levels acceptable for a probiotic product (Kechagia et al., 2013; D. M. D. Rasika et al., 2020, pp. 339–384). In the literature, it has been reported that certain bioactive compounds present in chocolate, such as flavonoids, may act either as a prebiotic (supporting/stimulating the viability of the probiotic bacteria) or as antimicrobial agents (Foong et al., 2013; Nazzaro et al., 2020, pp. 35–89). Recent studies concluded that total dietary polyphenols are responsible for shaping the microbiota by 1) exerting prebiotic effects on beneficial bacteria and 2) having selective antimicrobial action against gastrointestinal pathogens (Kumar Singh et al., 2019; Tzounis et al., 2011). Thus, present results suggest that the chocolate with 70.5% cocoa content could act as a prebiotic substrate for *B. animalis* subsp. *lactis* BB-12®.

Regarding *B. animalis* BB-12®, our results suggest that polyphenols may exert beneficial prebiotic properties, when at higher quantities, in a more accessible form, which is the case of the chocolate matrix with 70.5% cocoa. Furthermore, the chocolate matrix with 54.5% cocoa has the highest sugar content; a higher sugar content may increase osmotic pressure, which may lead to a viability reduction. Hereby, a possible explanation for the growth inhibition in the matrix with 54.5% cocoa could be the presence of unidentified trace elements, which could have antibacterial properties. Additionally, it could be hypothesized that these trace elements may not have inhibitory activity by themselves but could promote interactions with the phenolic compounds or other elements, which could cause the inhibition of bacterial growth. Secondly, it is important to note that chocolate matrices 33.6% and 54.5% are produced with cocoa butter, while the chocolate matrix with 70.5% cocoa content is manufactured with fat-reduced cocoa powder. Therefore, it is speculated that the different chocolates contain diverse phenolic compounds, which should be further characterized using spectrophotometric or chromatographic techniques (Khoddami et al., 2013).

During the aerobic storage of chocolates incorporated with *A. muciniphila* DSM 22959, the largest viability reduction was observed after 14 days, with a viability reduction ranging between 1 and 2 logs CFU/g depending on the chocolate matrix (Fig. 1b). After this period, viability decreased but in lesser magnitude. The chocolate matrices with 33.6% and 54.5% cocoa exhibited a similar protective effect on *A. muciniphila*, allowing a survival around the minimum number required of 10^6 CFU/g after 28-days of aerobic storage. Only the matrix with 70.5% cocoa did not achieve the minimum required levels for probiotic products.

In the literature, it has been described that cocoa polyphenols have antimicrobial activity against Gram-positive and Gram-negative bacteria; however, a 2017 study has demonstrated that after "Dutching", an alkalization process used in the manufacture of several chocolates, the antibacterial power is greater against Gram-negative bacteria (Todorovic et al., 2017). Considering that *A. muciniphila* is a Gram-negative bacterium, the observed decrease in viability with increased cocoa polyphenols content was expected. In the literature, the incorporation of NGP, namely *A. muciniphila*, into chocolate matrices remains poorly

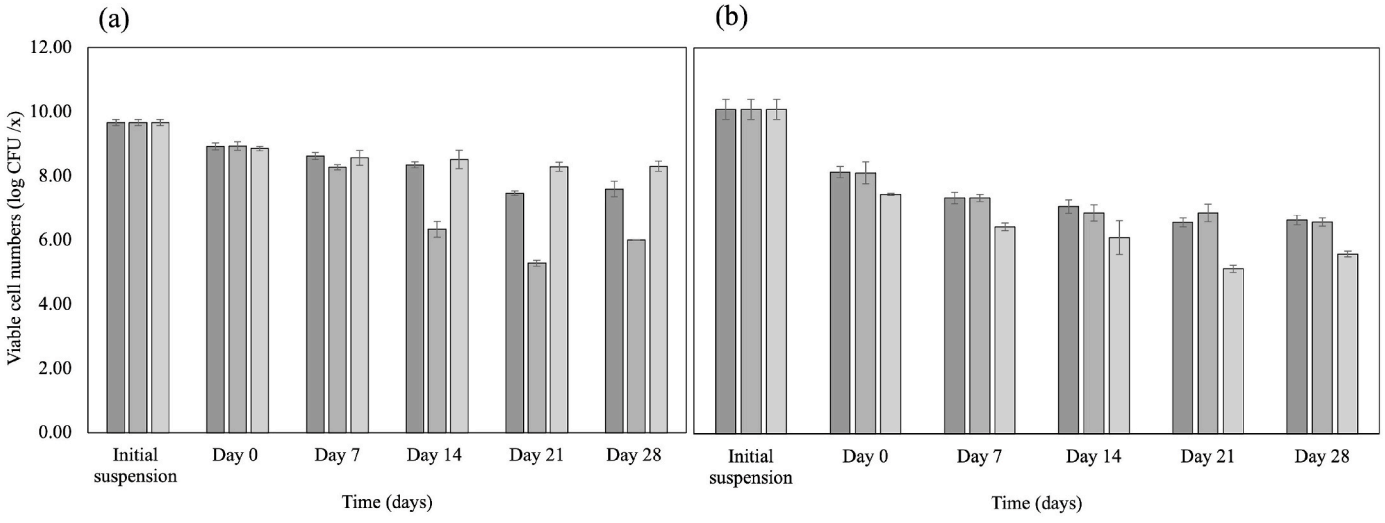


Fig. 1. Viable cell number of *Bifidobacterium animalis* subspecies *lactis* BB-12® (a) and *Akkermansia muciniphila* DSM 22959 (b) in initial suspension (log CFU/ml) and their evaluation in different cocoa content chocolates (log CFU/g) during aerobic storage at room temperature for 28 days. 33.6% cocoa content (dark grey bars); 54.5% cocoa content (medium grey bars) and 70.5% cocoa content (light grey bars). Log CFU has been expressed per x, as the initial concentration is in liquid form (mL), while the results obtained during storage originate from a solid food (chocolate) and are therefore expressed per g.

explored. In fact, Marcial-Coba et al. (2019) were pioneers when they used 70% dark chocolate as a carrier for freeze-dried microencapsulated *A. muciniphila* in a xanthan/gellan gum matrix and evaluated bacterial survival during anaerobic storage and *in vitro* gastric transit. Embedding into dark chocolate, conferred increased protection to the encapsulated *A. muciniphila*, resulting in viability higher than 10^6 CFU/g after 60 days of anaerobic storage (at 4 °C and 15 °C) (Marcial-Coba et al., 2019). However, the anaerobic storage conditions applied by Marcial-Coba et al. do not correspond to a feasible storage modality (more expensive and unsuitable for a household context). Moreover, using an encapsulation procedure before the incorporation in chocolate increases the production costs of this functional chocolate, which becomes less attractive to the industry.

3.2. Aspect evaluation of the chocolates

One property, which is important to monitor during storage is the overall quality aspect of the chocolate (Table 1). When comparing the three different tested conditions, it is possible to conclude that the color varies according to the cocoa content of the chocolate. The chocolate with the lowest cocoa content has the lightest color among the three chocolate types, while the chocolate with the highest cocoa content has the darkest shade.

When assessing the chocolate overall quality aspect throughout a storage period, two important phenomena might occur, namely sugar and/or fat bloom. Chocolate's physical behavior is mainly determined by the cocoa butter, thus the requirement of strict tempering and storage temperatures, to assure the best physical properties. When chocolate is handled unproperly, undesirable properties might resurface, namely a bloom, which might be classified either as sugar or fat bloom (Kinta & Hatta, 2012).

By analyzing the figures in Table 1 and it is possible to observe that the three chocolate matrices at time point 0 have a shining surface, which is gradually lost throughout the 28-day storage period. The phenomenon of fat bloom is particularly evident in the chocolate matrices of 33.6% and 70.5% cocoa content. Although fat bloom might alter organoleptic properties such as aspect and flavor, it does not affect cell viability, as both affected matrices had the highest cell survival rate (Fig. 1a)).

Also, in the case of chocolate incorporated with *A. muciniphila*, the overall quality aspect of the chocolate was monitored during the 28-day aerobic storage period. Based on Table 2, the same conclusions as before can be withdrawn. As mentioned earlier, the chocolates' color becomes darker with the increase in cocoa content.

By evaluating the photographs in Table 2 and it is possible to observe that only the dark chocolate matrices at time point day 0, upon production, have a shining surface, which is progressively lost throughout the storage period. The phenomenon of fat bloom is particularly evident in all three chocolates, starting as early as upon production (at timepoint day 0) for the 33.6% cocoa content chocolate matrix. This phenomenon is mainly due to unmanageable temperature oscillations, which leads to alteration in the fine crystal structure. Once again, it is important to note that, although fat bloom might alter organoleptic properties such as overall quality aspect and flavor, it does not affect cell viability, as both affected matrices had the highest cell survival rate (Fig. 1b)).

3.3. pH evaluation of chocolates

Natural cocoa powder is characterized as a low acidic product with pH values ranging from 5.3 to 5.8, due to organic acids presence, such as acetic and lactic acids, which result from sugars fermentation. However, cocoa powder undergoes an alkalization process which results in a darker color of the cocoa mass and consequently raises the pH values to 6.8–8.1. In chocolate, the pH is responsible for the reduction of microbial activity, therefore avoiding the possible growth of food pathogens, such as *Salmonella* spp. Lower pH values of chocolate matrices lead to increased sour and astringent taste. Contrarily, higher pH values result in an intensified dark color and consequently affect flavor and taste of the cocoa mass (Aprotosoaie et al., 2016; Valverde García et al., 2020). As shown in Fig. 2, pH values ranged between 6.09 and 6.80. Furthermore, a decrease in pH with an increase in cocoa content can be observed in both control and probiotic chocolates.

Importantly, adding probiotics to chocolate did not significantly change the pH compared to those of the plain chocolate control ($p > 0.05$). Also, pH values in all chocolates (with/without probiotics), remained highly stable throughout the 28-days of aerobic storage at room temperature, only with slight oscillations, i.e., lower than 1.0 in the pH scale. These findings are in accordance with previous studies involving functional chocolates incorporating probiotic lactobacilli and bifidobacteria strains and indicate that the added probiotic strains either had a slowed down metabolism or were metabolically inactive throughout storage. This characteristic is of great importance for preserving food products throughout their storage period (Silva et al., 2017; Succi et al., 2017).

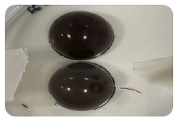
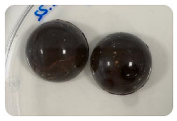
3.4. Total phenolic compounds and antioxidant activity determination

Currently, it is well established that the functional properties of chocolate are attributed to phenolic compounds principally to flavonoids and phenolic acids (Deus et al., 2021). Therefore, the TPC in different chocolates (probiotic and control) at day of production and after 28 days of aerobic storage was evaluated. As shown in Fig. 3, a clear pattern concerning chocolates' TPC can be identified; specifically, with the increase of cocoa content in a chocolate matrix, an increment in TPC was observed. Indeed, TPC ranged between 5.65 and 7.27, 24.23–25.04, and 43.27–80.17 mg GAE/g for chocolates with cocoa

Table 1
Evolution of the overall quality aspect of chocolates incorporated with *Bifidobacterium animalis* subspecies. *lactis* BB-12® throughout 28 days of aerobic storage.

	Cocoa Percentage [% (w/w)]		
	33.6%	54.5%	70.5%
Day 0			
Day 28			

Table 2
Evaluation of the overall quality aspect of chocolates incorporated with *Akkermansia muciniphila* throughout 28 days of aerobic storage.

	Cocoa Percentage [% (w/w)]		
	33.6%	54.5%	70.5%
Day 0			
Day 28			

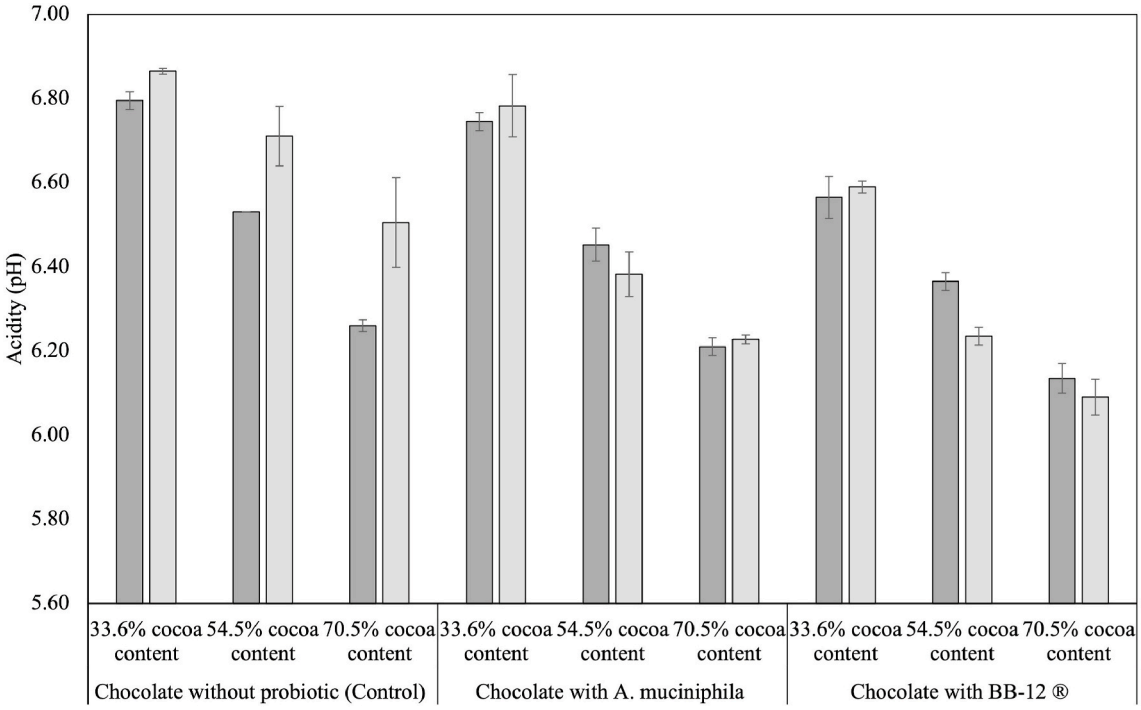


Fig. 2. Evaluation of pH values between 0 (dark grey bars) and 28 (light grey bars) days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *Akkermansia muciniphila* DSM 22959 and chocolate incorporating *Bifidobacterium animalis* subspecies *lactis* BB-12®.

percentages of 33.6%, 54.5%, and 70.5%, respectively. Regarding this, Mikolajczak and Tanska verified that cocoa mass content in chocolate bars was strongly correlated with phenolic compounds and antioxidant capacity (Mikołajczak & Tańska, 2021). Also, Kemsawasd et al. demonstrated that white, milk, and dark chocolates (containing 0%, 10%, and 50% of cocoa, respectively) presented values of total polyphenols in ascending magnitude (Kemsawasd et al., 2016).

Moreover, within the same cocoa content, the plain chocolate (control) and both probiotic-incorporated chocolates showed similar values for the TPC ($p > 0.05$) and mostly did not present statistical differences throughout 28-day aerobic storage at room temperature ($p > 0.05$, Fig. 3). Similarly, Silva et al. (2017) demonstrated semisweet chocolate incorporated with *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 did not present statistical differences in the TPC when compared with the control chocolate (Silva et al., 2017).

As chocolate is both a source of polyphenols and polyamines, it has a high antioxidant potential (Ellam & Williamson, 2013). A majority of studies on chocolate's impact on human health demonstrated positive outcomes regarding cardioprotective effects of cocoa and chocolate flavonoids. This specific effect was correlated with the antioxidant potential of cocoa flavonoids (Jalil & Ismail, 2008). In this scope, the antioxidant activity of chocolates (probiotic and control) at the day of production and after 28-days of aerobic storage was assessed. As depicted in Fig. 4 and analogously to observed in TPC, an increasing pattern was detected, i.e., the increase of cocoa content in a chocolate matrix leads to an increment in antioxidant activity. Indeed, antioxidant activity varied between 52146.43 and 81702.45, 205909.00–232146.81, and 349916.87–416219.14 μmol of Trolox equivalent per g of sample for each chocolate 33.6%, 54.5%, and 70.5%, respectively. Hereby, it is shown that the antioxidant activity is directly correlated with the TPC, as the higher the content of phenolic compounds, the higher the antioxidant activity (Nurhayati et al., 2022).

Furthermore, within the same cocoa content, the plain chocolate (control) and both probiotic-incorporated chocolates showed similar values for the antioxidant activity ($p > 0.05$) and did not present

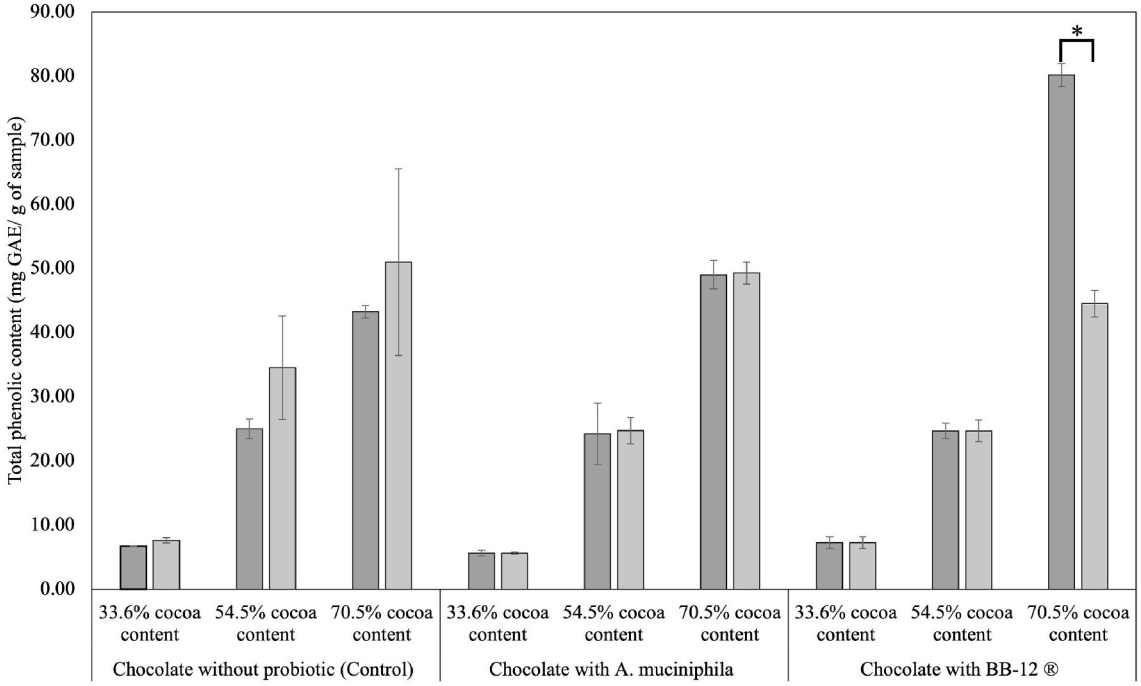


Fig. 3. Total phenolic compounds content between 0 (dark grey bars) and 28 days (light grey bars) of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *Akkermansia muciniphila* DSM 22959 and chocolate incorporating *Bifidobacterium animalis* subspecies *lactis* BB-12®. Statistical differences between storage day 0 and day 28 are marked with *.

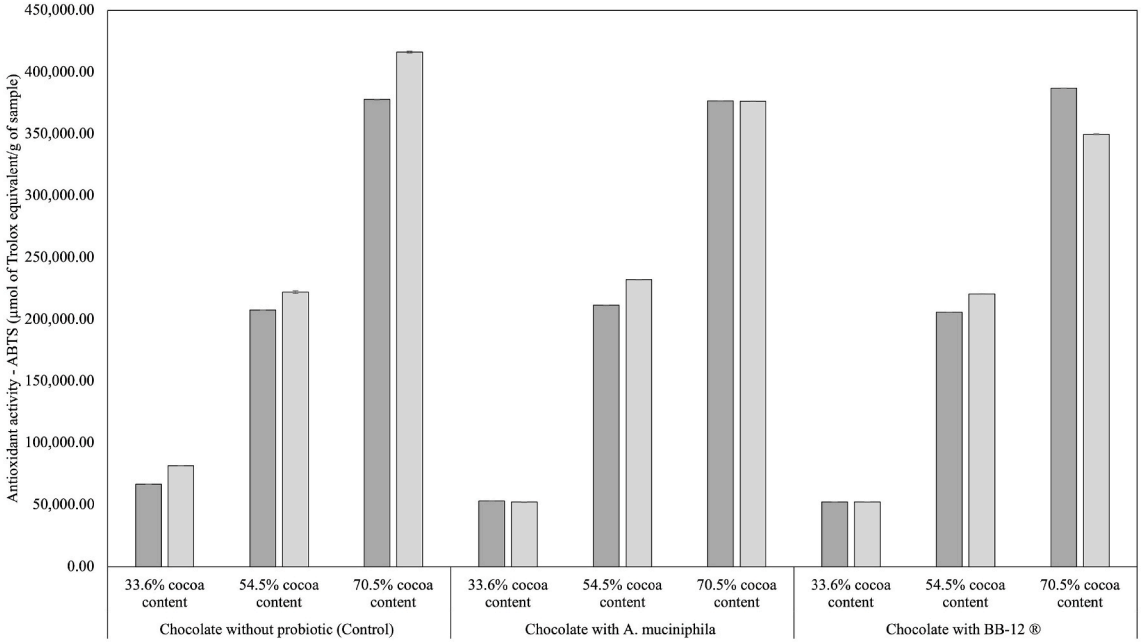


Fig. 4. Antioxidant activity between 0 (dark grey bars) and 28 days (light grey bars) of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *Akkermansia muciniphila* DSM 22959 and chocolate incorporating *Bifidobacterium animalis* subspecies *lactis* BB-12®. Standard deviation has been calculated, however the values are too low to be clearly visible in the figure.

statistical differences throughout 28-day aerobic storage at room temperature ($p > 0.05$, Fig. 4). Therefore, it was concluded that the probiotics addition does not affect the natural antioxidant potential of the chocolate.

Despite the difference in the TPC of the 70.5% cocoa content BB-12® incorporated chocolate (Fig. 3), the same effect is not observed for antioxidant activity (Fig. 4). Typically, the values of TPC and antioxidant activity are correlated, i.e., it was expected that the antioxidant activity varies with the loss of phenolic compounds. The TPC may be influenced by factors such as extraction technique, used solvents and interference with other compounds within the matrix (Urbańska & Kowalska, 2019). Therefore, the value obtained for the TPC of chocolate with 70.5% cocoa incorporated with *B. animalis* subsp. *lactis* BB-12® at day 0 seems to be inconsistent with the observed antioxidant activity and may have occurred due to the limitation of the determination technique.

3.5. Antidiabetic activity

Worldwide, type-2 diabetes is recognized as a serious public health problem with a substantial impact on human life and economy (Khan et al., 2019). A meta-analysis of prospective studies conducted by Yuan et al. demonstrated that moderate consumption of chocolate (1–6 servings/week) was related with a decreased risk of diabetes (Yuan et al., 2017). In this scope, the antidiabetic activities of probiotic and control chocolates were determined at day of production and after 28-days of aerobic storage. As shown in Fig. 5, an increasing trend in antidiabetic activity was observed with the increase of the chocolate matrix cocoa content.

Specifically, the probiotic chocolates did not present statistically different values during aerobic storage ($p > 0.05$, Fig. 5). For the control chocolates, statistical differences throughout storage were observed only in the chocolate with 54.5% cocoa ($p < 0.05$, Fig. 5). These observations show that the control chocolate may lose activity during storage, which is less probable in probiotic chocolates. Nonetheless, it was perceived that within the same cocoa content, the control and both probiotics incorporated into chocolates showed similar values and did not present statistical differences ($p > 0.05$). This indicates that predominantly the addition of probiotic does not impact the α-glucosidase inhibitory activity either positively or negatively. Importantly, *in vivo* studies point out that *A. muciniphila* has the potential to reduce body mass gain and plasma TNF-α levels, increase the number of goblet cells and mucin secretion, and restore gut barrier function (Deng et al., 2022; Zhang et al., 2021). All these effects contribute to the amelioration of diabetes related symptoms. Also, the assay performed on antidiabetic activity does not target insulin sensibility, instead it determines the inhibition of α-glucosidase. Therefore, a correlation between the incorporation *A. muciniphila* in a chocolate matrix and increase on antidiabetic activity cannot be inferred, as two different parameters are being evaluated. Although, the addition of *A. muciniphila* did not affect the α-glucosidase inhibitory activity in the chocolate matrices, the incorporation of the probiotic in chocolate might still be valuable regarding this property, as the bacterium might act against diabetes in the human system after consumption.

3.6. Antihypertensive activity

Hypertension is a medical condition characterized by elevated blood pressure that has been pointed as a major cause of premature death worldwide, since this disorder significantly increases the risks of heart, brain, kidney diseases (World Health Organization, 2021). A recent systematic review and meta-analysis performed by Amoah et al. showed that cocoa and dark chocolate consumption may contribute for lowering blood pressure (Amoah et al., 2022). Given the high potential for antihypertensive effects of chocolate matrices, this bioactivity was assayed for the different chocolates (probiotic and control) at day of production and after 28-days of aerobic storage. As observed in Fig. 6, an increased cocoa content is related with increasing antihypertensive activity. Moreover, it was observed that within the same cocoa content, the control and both probiotics incorporated into chocolates showed similar values and did not present statistical differences throughout the 28-day aerobic storage period at room temperature ($p > 0.05$). These results suggest that probiotics addition does not affect either positively or negatively the antihypertensive properties of the three tested chocolate matrices. However, the dark chocolate (matrices with 54.5 and 70.5% cocoa content) by itself is already responsible for an almost 100% ACE inhibition. It is important to note, that previous work reported that *A. muciniphila* may be involved in the modulation of metabolic pathways linked with the regulation of blood pressure (Lakshmanan et al., 2022) and therefore this bacterium may enhance chocolate antihypertensive effects.

4. Conclusions

The present study was the first to evaluate the effect of three different chocolate matrices (with 33.6%; 54.5% or 70.5% cocoa content) on the viability of *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* DSM 22959 during aerobic storage at room temperature for 28-days. Indeed, dark chocolates with 70.5% and 54.5% of cocoa seem to be a promising food carrier to deliver probiotic *B. animalis* BB-12® and *A. muciniphila* DSM 22959, respectively. Specifically, a chocolate matrix containing 70.5% cocoa allowed the survival of *B. animalis* BB-12® at viability level

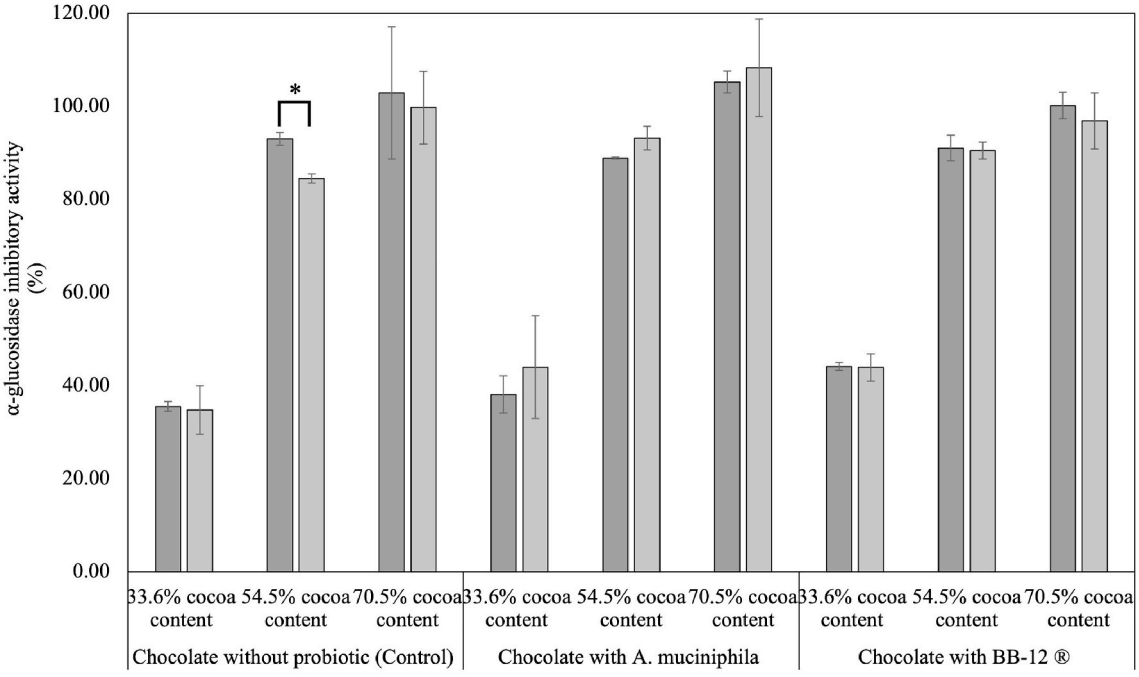


Fig. 5. α-glucosidase inhibitory activity between 0 (dark grey bars) and 28 days (light grey bars) of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *Akkermansia muciniphila* DSM 22959 and chocolate incorporating *Bifidobacterium animalis* subspecies *lactis* BB-12®. Statistical differences between storage day 0 and day 28 are marked with *.

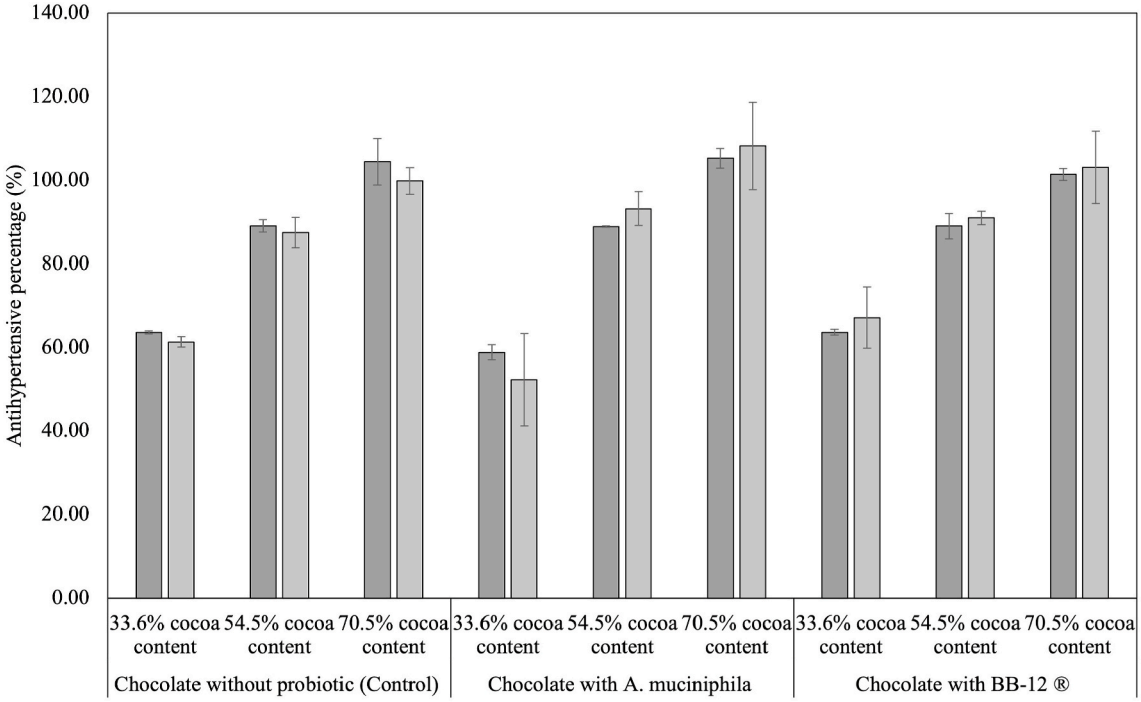


Fig. 6. Antihypertensive activity between 0 (dark grey bars) and 28 (light gret bars) days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *Akkermansia muciniphila* DSM 22959 and chocolate incorporating *Bifidobacterium animalis* subspecies *lactis* BB-12®.

at least 10^8 CFU/g, after a 28-day aerobic storage. Besides that, chocolate with 70.5% of cocoa exhibited the highest quantity of phenolic compounds as well as the most representative antioxidant, antidiabetic, and antihypertensive activities. Regarding *A. muciniphila* DSM 22959, the most appropriate chocolate matrix was the one with 54.5% cocoa, as cell viability was kept at levels equal or higher than the threshold (10^6 CFU/g) throughout aerobic storage and concurrently it showed interesting bioactivities. Concerning the perspective future works, the next step on this study should focus on the analysis of probiotic viability when submitted to the harsh conditions of the gastrointestinal tract. The incorporation in a chocolate matrix should ensure that the probiotic reach the action site in values above the minimum threshold, namely 10^6–10^7 CFU/g or CFU/ml, to guarantee that the probiotic exerts its beneficial effect. Due to the commercialization potential of this novel food product, further studies evaluating the nutritional composition of probiotic chocolates, their certain physicochemical parameters (such as water activity and rheological properties), as well as consumer acceptance testing focused on specific sensory attributes should be performed.

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CRediT authorship contribution statement

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

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115361.

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