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Improved nutritional composition and *in vitro* protein digestibility of fermented soy beverages produced with vaginal probiotics as adjunct cultures

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ABSTRACT

Fermented soy beverages, made with yogurt starter cultures and formulated with functional vaginal bacteria, namely *Lactobacillus crispatus* BC4 and *Lactobacillus gasseri* BC9, were characterized in terms of centesimal, fatty acids, mineral, vitamins and amino acids composition. The products were subjected to simulated gastro-duodenal digestion to determine protein hydrolysis and protein bioaccessibility. Although a species-specific effect was osbserved, samples containing the vaginal strain mix presented lower carbohydrates but a higher protein, iron, magnesium, and essential amino acid content, compared to the product with only yogurt starters. Eventually, samples produced with the mix of vaginal strains showed lower lipid quality indices but higher protein hydrolysis and bioaccessibility (total protein content was 2-fold higher than the control at the end of duodenal phase). The use of vaginal probiotic strains as adjunct cultures represents a suitable vehicle to deliver probiotics and to improve the nutritional value of the final product.

1. Introduction

Soy beverage is a traditional protein-rich beverage made from soybeans. As a plant-based product, soy beverage is low in saturated fats and cholesterol, and it contains proteins, dietary fiber, and polyphenols that may be beneficial for human health (Wu et al., 2021). According to the *meta*-analysis performed by Hooper et al. (2009), soy proteins and isoflavones can potentially reduce the risk of hormone-associated health disorders in pre-and post-menopausal women. Lactose-free soy beverage is usually used as an alternative for cow's milk due to its lower allergenicity, and reduced environmental impacts (Singh-Povel et al., 2022; Wu et al., 2022a). However, it is less preferred by some consumers due to its beany flavor, flatulence factors, and high content of indigestible α-galactosyl oligosaccharides, such as raffinose and stachyose, which limit the consumption of soybeans as a raw food matrix. An exhaustive literature (Ghoneem et al., 2018; Ho et al., 2022; Wu et al., 2022b) suggests that fermentation of soy beverages with lactic acid bacteria (LAB) not only overcomes the limitations mentioned above but increases the nutritional value of soybean-derived products. The global fermented plant-based alternatives market is expected to have a compound annual growth rate of 5% from 2021 to 2026 (BIS Research, 2021). Plant-based milk substitutes, such as soy beverages, can preserve the viability of LAB during their storage (Cui et al., 2021; D'Alessandro et al., 2023); therefore, they can be considered promising carriers for probiotics. Probiotics are defined as living microorganisms that, when administered sufficiently, provide a health benefit to the host, starting from the intestinal tract and beyond (Sanders et al., 2018). In fact, Petricevic et al. (2008) has demonstrated that oral probiotic formulations and vaginal instillation can restore the healthy vaginal microbiome. On this regard, Parolin et al. (2015) isolated vaginal strains with functional features. For

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instance, *Lactobacillus crispatus* BC4 and *Lactobacillus gasseri* BC9 showed an antagonistic activity towards several uro-genital pathogens, such as *Candida* spp. (Calonghi et al., 2017; Parolin et al., 2015), *Chlamydia trachomatis* (Nardini et al., 2016; Parolin et al., 2018), *Neisseria gonorrhoeae* (Foschi et al., 2017), Group-B *Streptococcus* (Marziali et al., 2019) and HIV1 (Ñahui Palomino et al., 2017). At the same time, other works evaluated their safety (antibiotic resistance), probioticity (such as hydrophobicity, auto-aggregation, bile salt hydrolase (BSH) activity, resistance after simulated gastric acidity) and technological features (acidification kinetic, survival at 4 °C, inhibition of foodborne pathogenic species) (D'Alessandro et al., 2021a; D'Alessandro et al., 2021b; Siroli et al., 2017). The same strains were applied as functional cultures in food products (Siroli et al., 2017) and the fate of *L. crispatus* BC4, incorporated in a soft cheese, was investigated using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) (Patrignani et al., 2020). Besides supplying extra probiotic functions, LAB can degrade soy proteins into smaller peptides and amino acids, increasing protein bioavailability (Cui et al., 2021; Ghoneem et al., 2018; Wu et al., 2022b). Moreover, processing (including formulation and fermentation) deeply modifies nutrients' content and bioaccessibility. In particular, modifications to the supramolecular architecture, interactions between molecules, and the placement of nutrients within compartments can all affect bioaccessibility, which is defined as the portion of a substance's total amount that is released from the food matrix during digestion and potentially becomes available for absorption (Rodrigues et al., 2022).

Considering the growing demand for non-dairy alternatives as probiotic carriers, the main aim of this study was to characterize fermented soy beverages obtained with the addition of yogurt starter cultures and supplemented with functional vaginal strains (*L. crispatus* BC4 and *L. gasseri* BC9), alone or in combination. Survival of probiotics and starter cultures, physical and sensory characteristics of these fermented beverages were already analyzed by D'Alessandro et al. (2023). In this work, the nutritional profiles of the formulated products were evaluated with a specific focus on centesimal composition, minerals, vitamins, fatty acids, lipid quality indices and amino acids content. Moreover, fermented soy beverages were subjected to *in vitro* static gastrointestinal digestion according to the INFOGEST protocol, and the kinetics of protein hydrolysis and release from the food matrix were followed by sampling at the end of the gastric phase, as well as in the middle and at the end of the intestinal phase.

2. Materials and methods

2.1. Materials

Unless otherwise specified, chemicals and solvents were from Sigma-Aldrich (St. Lou-is, MO, USA), and were of the highest available analytical grade.

2.2. Bacterial strains

Lyofast Y450B (Sacco srl, Italy) was used as yogurt starter cultures and it contains strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. The vaginal strains used in this work (*Lactobacillus crispatus* BC4 and *Lactobacillus gasseri* BC9) belong to the collection of the Department of Pharmacy and Biotechnology (FABIT, University of Bologna, Italy) and were isolated from the vagina of premenopausal Caucasian women (aged 18–45 years), with no symptoms of vaginal or urinary tract infections, in accordance with the Ethics Committee of the University of Bologna (52/2014/U/Tess). Fresh cultures of the vaginal strains were obtained from frozen stocks by two consecutive transfers in MRS broth (Oxoid, Basing-stoke, United Kingdom) using a 1% (v/v) inoculum and incubated overnight at 37 °C in aerobic conditions.

2.3. Preparation of fermented soy beverages

The production of fermented soy beverages was carried out in lab conditions as described by D'Alessandro et al. (2023) using Lyofast Y450B (yogurt starter cultures) and the vaginal probiotics according to the following plan:

Control: fermented soy beverage containing yogurt starter cultures only;

BC4: fermented soy beverage containing yogurt starter cultures and *L. crispatus* BC4;

BC9: fermented soy beverage containing yogurt starter cultures and *L. gasseri* BC9;

BC4 + BC9: fermented soy beverage containing yogurt starter cultures, *L. crispatus* BC4 + *L. gasseri* BC9.

2.4. Nutritional composition of the different fermented soy beverages

Fermented soy beverages were analyzed for the following parameters: energy values, fat, carbohydrates, proteins, dietary fibers, salt, moisture, and ashes, using the methods of the Association of Official Analytical Chemists (2000) (Obadina et al., 2013). The carbohydrates content was determined by difference between 100 and total sum of the percentage of moisture, proteins, fats and ashes (Association of Official Analytical Chemists, 1990). The quality and quantity of total fatty acids (FAs) were determined according to (Suzzi et al., 2015). Analyses were performed using an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Al-to, CA, USA) coupled to an Agilent 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) operating in electron impact mode (ionization voltage 70 eV). To separate sample FAs, a fused silica capillary column (30 m × 0.32 μm) with 0.2 μm film of Carbowax (Supelco, Bellefonte, PA, USA) as stationary phase was used. The GC/MS parameters were the following: injection temperature, 220 °C; detector temperature, 220 °C; carrier gas (helium) flow rate, 1.5 mL * min^{-1}; split ratio, 1:50 (v/v). The oven temperature was programmed from 60 to 220 °C with an increase of 4 °C/min. FAs present in the fermented soy beverage were identified by according to the retention time of a known FAs standard and by comparing their mass spectra with those present in the available database (NIST version 2011).

2.4.1. Lipid quality indices

The ratio of polyunsaturated/saturated fatty acids (PUFA/SFA) was calculated from the fatty acid profile of the fermented soy beverages. Furthermore, in order to link the fatty acid profiles with the risk of cardiovascular disorders, the Index of Atherogenicity (IA), Index of Thrombogenicity (IT) and the Hypocholesterolemic/Hypercholesterolemic (HH) ratio were calculated according to Chen & Liu (2020), using the following equations:

a) $$IA = \frac{[C12{:}0 + (4 \times C14{:}0) + C16{:}0]}{\sum UFA}$$

b) $$IT = \frac{(C14{:}0 + C16{:}0 + C18{:}0)}{[(0.5 \times \Sigma MUFA) + (0.5 \times \Sigma n - 6\ PUFA) + (3 \times \Sigma n{-}3\ PUFA) + (n{-}3/n{-}6)]}$$

c) $$HH = \frac{(cis{-}C18{:}1 + \Sigma PUFA)}{(C12{:}0 + C14{:}0 + C16{:}0)}$$

2.4.2. Minerals and vitamins content

Minerals (Calcium, Iron, Potassium, Magnesium, Sodium) were determined in fermented samples using the induced coupled plasma optical emission spectroscopy (ICP-OES; ICAP 6500 Duo ICP, Thermo Scientific, UK) method. The results are expressed as mg of mineral per 100 g of fermented soy beverage. Determination of selected vitamins, such as B12 (as Cyanocobalamin), B2 (Riboflavin), and D (D2 + D3), was carried out following the method of Zhao & Shah, (2014) with some modifications, using high-performance liquid chromatography. The results are expressed as mg or μg of vitamin per 100 g of fermented soy beverage.

2.4.3. Amino acids composition

The quantitative analysis of total amino acids in fermented soy beverage was carried out according to the method of Wu et al., (2022a). Briefly, fermented soy beverage (0.5 g) was hydrolyzed in hydrochloric acid (6 M, 6 mL) at 110 °C for 24 h. Subsequently, the hydrolysate was vacuum dried at 50 °C to remove the hydrochloric acid, and then redissolved in loading buffer (0.01 M HCl). The sample solution was filtrated (0.22 μm) and analyzed with an amino acid analyzer (L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan).

2.5. In vitro digestion

Fermented soy beverages were subjected to *in vitro* digestion according to the INFOGEST protocol (Minekus et al., 2014). In each formulation, digestion was performed in triplicate (n = 3). *In vitro* digestion lasted for 242 min consisting in 2 min of oral digestion, 120 min of gastric digestion, and 120 min of intestinal digestion, at 37 °C. During the process, several consecutive enzymatic reactions occurred by adding simulated saliva, simulated gastric juice (at pH 3, containing 2000 U/mL pepsin), and simulated pancreatic juice (at pH 7, including 10 mM bile and 100 U/mL pancreatin). Samples were taken at the end of the gastric phase (G120), after 60 min (D60), and 120 min (D120) of the duodenal phase. In G120 samples, the pH was increased to 7 with 35% NaOH to stop the pepsin hydrolytic action and reported to 3 with 37% HCl. Samples at D60 and D120 were acidified to pH 3 with 37% HCl to stop pancreatic hydrolysis and reported to 7 with 35% NaOH (Di Nunzio et al., 2022b). Digested samples were centrifuged at 50,000 g for 15 min, and the supernatants were stored at −80 °C until further analysis.

2.6. SDS-PAGE

A 0.1 mL volume of fermented soy milk or *in vitro* digested was incubated in an equal volume of 0.125 M Tris-HCl, pH 6.8, 50% glycerol, 1.7% SDS, 0.01 % bromophenol blue, 0.5% 2-mercaptoethanol and heated at 95 °C for 5 min. Sodium dodecyl-sulfate–polyacrylamide gel electrophoresis analysis was performed using handcast 16% polyacrylamide gels prepared according to Ferranti et al. (2014). Gel preparation and vertical electrophoresis were carried out using Bio-Rad (Bio-Rad Laboratories, California) equipment (Mini-PROTEANW system). Gels were stained in a solution containing 0.05% w/v Coomassie Blue, 50% v/v methanol, 10% v/v acetic acid, and water at around 50 °C. Finally, the gels were destained in water at 50 °C, and images were acquired using a benchtop scanner.

2.7. Protein hydrolysis

Protein hydrolysis in the digested samples was determined spectrophotometrically by o-phthaldialdehyde (OPA) reagent, according to Church et al. (1985). Values were normalized for the dilution factor due to the addition of digestive fluids and expressed as percent respect to the control at the end of gastric digestion (assigned as 100).

2.8. Soluble protein content

In digested samples, protein/peptide concentration was determined spectrophotometrically by measuring the absorbance at 280 nm (Di Nunzio et al., 2022a) and by the Bradford dye-binding assay (Ogihara & Haley-Vicente, 2002) using non-fatty dry milk powder as standard. Protein content from enzymes added during *in vitro* digestion was subtracted, values were normalized for the dilution factor due to the addition of digestive fluids, and data were expressed as milligrams of protein in digested fluid/gram of product.

2.9. Statistical analysis

The obtained data, intended as the mean of three repetitions, were analyzed by Statistica software (version 8.0; StatSoft, Tulsa, OK, USA), adopting the analysis of variance (ANOVA) and Tukey's test for data comparisons.

3. Results

3.1. Nutritional values of the different fermented soy beverages

Commercial soy beverage was fermented with yogurt starter cultures supplemented with two functional vaginal strains, *L. crispatus* BC4 and *L. gasseri* BC9, alone (BC4 and BC9) or in combination (BC4 + BC9). The centesimal composition of the different fermented soy beverages is reported in Table 1.

The addition of probiotic vaginal strains as adjunct cultures did not impact the content of fats and fibers, but enhanced the consumption of carbohydrates. Moreover, the addition of strain BC9, alone or mixed with BC4, produced a fermented soy beverage with a higher protein and lower salt content.

Concerning FA composition (Table 2), the level of saturation was lower in samples supplemented with *L. crispatus* BC4 (BC4) compared to control while it was higher in those containing the mix BC4 + BC9. The presence of BC9 alone determined an increase in the abundance of oleic acid (16.5% instead of 15.1% in the control), and a reduction of the polyunsatured linoleic acid (60.1 instead of 61.4%). On the other hand, fermented product containing BC4 showed a higher abudance of α-linolenic acid (8.4%) compared to the control (7.9%) and reduction of stearic acid (3.5 instead of 4.1%). The use of the mix showed both the reduction in linoleic acid and the increase in α-linolenic and palmitic acid, compared to control. Based on these results, PUFA/SFA ratio, Index of Atherogenicity (IA), Index of Thrombogenicity (IT), Hypo/hypercholesterolemic ratio (h/H) were calculated for the different samples (Chen & Liu 2020). Compared to control (PUFA/SFA = 4.65, AI = 0.0126, IT = 0.234 and h/H = 6.528), sample containing BC4 showed higher PUFA/SFA and h/H ratio (4.86 and 6.545, respectively) and

Table 1
Centesimal composition of the different samples of fermented soy beverage in terms of energy values (kJ/100 g), Fat, carbohydrates, proteins, dietary fibers, salt, humidity, ashes (all expressed as g/100 g of product). Control: soy beverage fermented with yogurt starter cultures; BC4: soy beverage fermented with yogurt starter cultures and supplemented with BC4; BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC9; BC4 + BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC4 and BC9. Differences reported as lowercase letters are significant within each parameter ($p < 0.05$).

Nutritional values	Unit	Control	BC4	BC9	BC4 + BC9
Energy values	kJ/100 g	150	147	149	155
Energy values	kcal/100 g	36	35	35	37
Fat	g/100 g	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
of which saturated FAs	g/100 g	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
Carbohydrates	g/100 g	2.8^{a} ± 0.1	2.4^{b} ± 0.1	2.5^{b} ± 0.1	2.6^{b} ± 0.1
of which sugars	g/100 g	2.1^{a} ± 0.1	1.8^{ab} ± 0.2	2.0^{a} ± 0.1	1.7^{b} ± 0.1
Proteins	g/100 g	2.76^{a} ± 0.02	2.79^{a} ± 0.01	2.96^{b} ± 0.07	2.90^{b} ± 0.07
Dietary fibers	g/100 g	< 0.1 ± 0.01	< 0.1 ± 0.01	< 0.1 ± 0.01	< 0.1 ± 0.01
Salt	g/100 g	0.14^{a} ± 0.01	0.11^{ab} ± 0.02	0.08^{b} ± 0.01	0.09^{b} ± 0.01
Moisture	g/100 g	91.9^{a} ± 0.1	92.4^{b} ± 0.2	92.2^{b} ± 0.1	91.9^{a} ± 0.1
Ashes	g/100 g	0.99^{a} ± 0.01	0.78^{b} ± 0.01	0.79^{b} ± 0.01	0.78^{b} ± 0.01

Table 2
Fatty acids composition of the different samples of fermented soy beverage reported as relative percentages (%) and corresponding lipid quality indices. Control: soy beverage fermented with yogurt starter cultures; BC4: soy beverage fermented with yogurt starter cultures and supplemented with BC4; BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC9; BC4 + BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC4 and BC9. Differences reported as lower case letters are significant within each parameter ($p < 0.05$).

	Control	BC4	BC9	BC4 + BC9
Fatty acids composition				
Saturated FAs (SFAs)	$14.9^{a} \pm 0.1$	$14.3^{b} \pm 0.2$	$14.9^{a} \pm 0.1$	$15.5^{c} \pm 0.3$
Unsaturated FAs (UFAs)	$85.1^{a} \pm 0.1$	$85.7^{b} \pm 0.1$	$85.1^{a} \pm 0.1$	$84.5^{c} \pm 0.2$
Monounsatered FAs (MUFAs)	$15.9^{a} \pm 0.2$	$16.3^{a} \pm 0.2$	$17.1^{b} \pm 0.2$	$15.8^{a} \pm 0.2$
Polyunsatured FAs (PUFAs)	$69.2^{a} \pm 0.2$	$69.4^{a} \pm 0.2$	$68.0^{b} \pm 0.4$	$68.7^{b} \pm 0.2$
Trans-unsaturated FAs	$<0.1 \pm 0.01$	$<0.1 \pm 0.01$	$<0.1 \pm 0.01$	$<0.1 \pm 0.01$
FAs Profile				
C16:0 (Palmitic acid)	$10.8^{a} \pm 0.2$	$10.8^{a} \pm 0.2$	$10.8^{a} \pm 0.2$	$11.4^{b} \pm 0.2$
C18:0 (Stearic acid)	$4.1^{a} \pm 0.3$	$3.5^{b} \pm 0.2$	$4.1^{a} \pm 0.3$	$4.1^{a} \pm 0.3$
C18:1 n9 (Oleic acid)	$15.1^{a} \pm 0.6$	$15.7^{a} \pm 0.2$	$16.5^{b} \pm 0.4$	$15.1^{a} \pm 0.5$
C18:1 (n7) (*cis*-Vaccenic acid)	0.8 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
C18:2n6c (Linoleic acid)	$61.4^{a} \pm 0.3$	$61.0^{a} \pm 0.3$	$60.1^{b} \pm 0.2$	$60.4^{b} \pm 0.2$
C18:3n3 (α-Linolenic acid)	$7.9^{a} \pm 0.2$	$8.4^{b} \pm 0.2$	$8.0^{a} \pm 0.1$	$8.3^{a,b} \pm 0.2$
Lipid quality indices				
PUFA/SFA ratio	$4.65^{a} \pm 0.012$	$4.86^{b} \pm 0.014$	$4.59^{c} \pm 0.013$	$4.47^{d} \pm 0.052$
Index of Atherogenicity (IA)	$0.0126^{a} \pm 0.002$	$0.0124^{a} \pm 0.001$	$0.0124^{a} \pm 0.001$	$0.0135^{b} \pm 0.004$
Index of Thrombogenicity (IT)	$0.234^{a} \pm 0.001$	$0.220^{b} \pm 0.001$	$0.232^{a} \pm 0.003$	$0.243^{c} \pm 0.004$
hypo/hyper-cholesterolemic ratio (HH)	$6.528^{a} \pm 0.001$	$6.545^{b} \pm 0.002$	$6.438^{c} \pm 0.001$	$6.067^{d} \pm 0.004$

lower IT index (0.0124 and 0.220, respectively). On the contrary, BC9 alone presented lower PUFA/SFA and h/H ratio (4.59 and 6.43, respectively). The use of the mix showed lower PUFA/SFA and h/H ratio (4.47 and 6.06, respectively) but also a higher IA (0.0135) and IT (0.243).

To assess more in depth the nutritional value of the different samples of fermented soy beverages, the content of selected minerals (like Ca, K, Fe, Mg, Na,) and vitamins (such as B12, B2, and D) was measured (Table 3). Based on the results shown in Table 3, it can be stated that potassium (309.1 mg/100 g) was the most represented mineral in the control followed by calcium (133.2 mg/100 g), sodium (55.7 mg/100 g), magnesium (12.2 mg/100 g), and iron (0.32 mg/100 g). Sample containing vaginal probiotics presented higher iron (between 0.36 and 0.40 mg/100 g) and magnesium (between 12.9 and 13.9 mg/100 g) content and reduced potassium (between 255.1 and 269.3 mg/100 g) and sodium (between 33.1 and 45.1 mg/100 g) amount. For what concerns vitamins content, no significantly different were observed among samples.

3.2. Total amino acids content

The amino acid composition was determined in all the samples of fermented soy beverage and reported in Fig. 1. Vaginal strains, in all the formulations tested, increased the amount of threonine, while phenylalanine and tryptophan were significantly higher only in samples containing BC4, alone or in mix. The use of the mix (BC4 + BC9) also increased the amount of glutamic acid and valine. Instead, no differences were observed for all the other amino acids when compared to control.

Table 3
Minerals and vitamins compositions of the different samples of fermented soy beverage: calcium, iron, potassium, magnesium, sodium, B12 vitamin (as Cyanocobalamin), B2 vitamin (Riboflavin) (all expressed as mg/100 g of product), D vitamins (D2 + D3) (μg/100 g of product). Control: soy beverage fermented with yogurt starter cultures; BC4: soy beverage fermented with yogurt starter cultures and supplemented with BC4; BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC9; BC4 + BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC4 and BC9. Differences reported as lower case letters are significant within each parameter ($p < 0.05$).

Minerals and vitamins	Unit	Control	BC4	BC9	BC4 + BC9
Calcium (Ca)	mg/100 g	$133.2^{a} \pm 0.1$	$143.3^{b} \pm 0.1$	$132.9^{c} \pm 0.1$	$132.6^{c} \pm 0.1$
Iron (Fe)	mg/100 g	$0.32^{a} \pm 0.01$	$0.40^{c} \pm 0.01$	$0.36^{b} \pm 0.01$	$0.37^{b} \pm 0.01$
Potassium (K)	mg/100 g	$309.1^{a} \pm 0.1$	$269.3^{b} \pm 0.1$	$255.1^{c} \pm 0.1$	$256.1^{c} \pm 0.1$
Magnesium (Mg)	mg/100 g	$12.2^{a} \pm 0.1$	$13.9^{b} \pm 0.1$	$12.9^{c} \pm 0.1$	$13.1^{c} \pm 0.1$
Sodium (Na)	mg/100 g	$55.7^{a} \pm 0.1$	$45.1^{b} \pm 0.1$	$33.1^{c} \pm 0.1$	$34.2^{c} \pm 0.1$
B12 Vitamin (as Cyanocobalamin)	μg/100 g	$< 0.01 \pm 0.01$	$< 0.01 \pm 0.01$	$< 0.01 \pm 0.01$	$< 0.01 \pm 0.01$
B2 Vitamin (Riboflavin)	mg/100 g	0.18 ± 0.02	0.16 ± 0.01	0.20 ± 0.01	0.19 ± 0.01
D Vitamins (D2 + D3)	μg/100 g	$< 0.20 \pm 0.02$	$< 0.01 \pm 0.01$	$< 0.20 \pm 0.01$	$< 0.20 \pm 0.01$

3.3. SDS-PAGE of undigested and digested fermented soy beverage products

Protein profiles of all the formulated soy beverages were evaluated by SDS-PAGE (Fig. 2). According to their sedimentation coefficient, soybean proteins can be divided into 2S, 7S, 11S, and 15S. β-Glycinin, the main polypeptide of 7S and the major allergen in soybean is composed of three subunits: α' (~72 kDa), α (~68 kDa) and β (~52 kDa) (Peng et al., 2022). 11S can be divided into acidic subunit A (~35 kDa) and alkaline subunit B (~20 kDa). The subunits mentioned above (α′, α, β of 7S and A, B of 11 S) were observed in Fig. 2, examining the different bands ranging approximately from 14 to 97 kDa. According to the profiles generated, no remarkable differences were observed among the samples' band intensities, except for those of sample BC4 + BC9 that were less intense.

Protein profiles of the samples were also evaluated upon *in vitro* digestion. In particular, all the samples were run on SDS-PAGE after 120 min of simulated gastric phase (G120), and 60 (D60) or 120 min (D120) of simulated duodenal phase (Fig. 3).

The SDS-PAGE performed with samples collected during *in vitro* digestion (Fig. 3) showed that at the end of the gastric phase (G120) (lane 2, 5, 8, and 11), there were no longer high and medium molecular weight protein bands but only protein fragments weighing<14 kDa. Duodenal digestion, on the other hand, determined the complete hydrolysis of the low molecular weight protein fragments generated during gastric digestion. Formation of lower MWs fragments cannot be detected with the SDSPAGE technique (lane 3, 4, 6, 7, 9, 10, 12 and 13) due to the lack of Coomassie blue stain to detect peptides below 3 kDa (Egger et al., 2017). The medium molecular weight bands present in the samples at the middle (D60) and at the end (D120) of the duodenal digestion may correspond both to the degradation of the 50 kDa bands detectable during the gastric phase and to the digestive enzymes added during the duodenal phase.

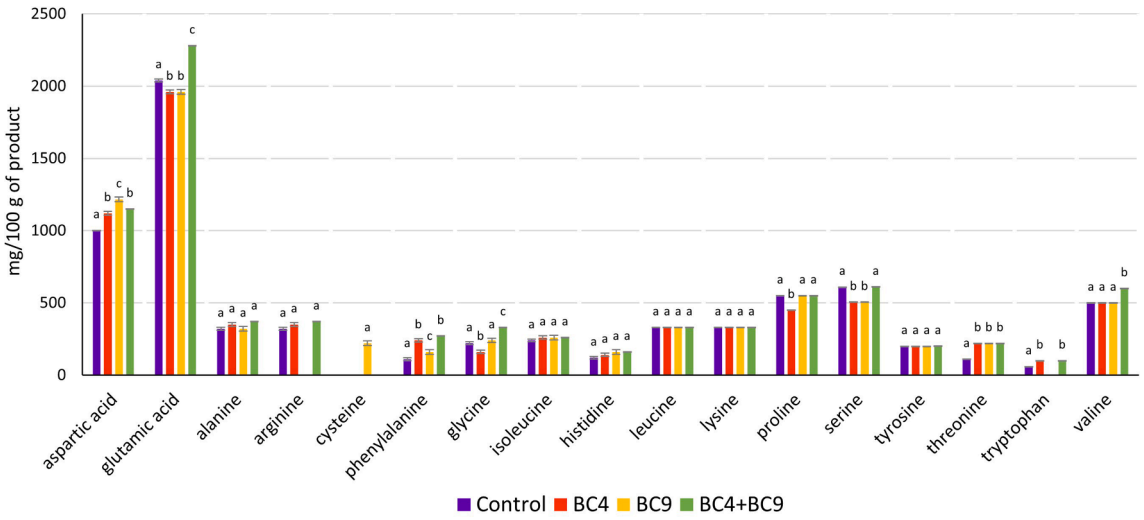

Fig. 1. Total amino acids content (mg/100 g of product) of the different samples of fermented soy beverage (control, BC4, BC9 and BC4 + BC9). Differences reported as lower case letters are significant within each parameter ($p < 0.05$).

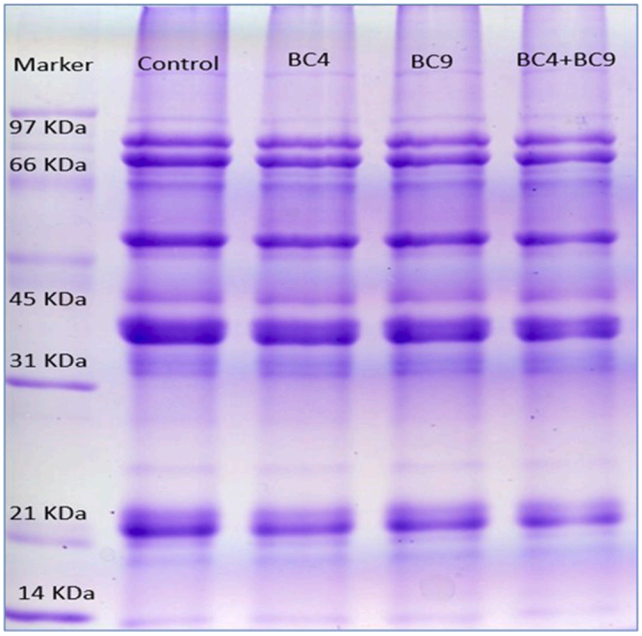

Fig. 2. SDS-PAGE of the different samples of fermented soy beverage containing vaginal probiotics. Marker (lane 1); Control: soy beverage fermented with yogurt starter cultures (lane 2); BC4: soy beverage fermented with yogurt starter cultures and supplemented with BC4 (lane 3); BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC9 (lane 4); BC4 + BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC4 and BC9 (lane 5).

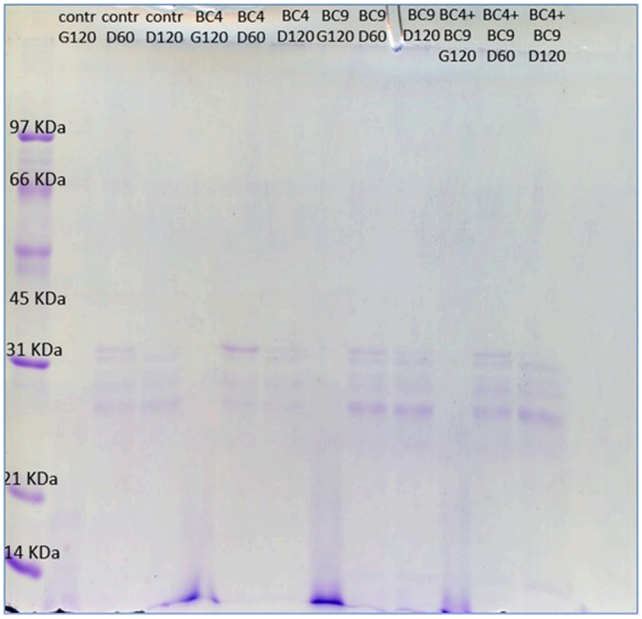

Fig. 3. SDS-PAGE of the different samples of fermented soy beverage, supplemented with vaginal probiotics, obtained after *in vitro* digestion at selected time points: end of the gastric phase (G120), after 60 (D60) and 120 min (D120) of duodenal phase. Control: soy beverage fermented with yogurt starter cultures; BC4: soy beverage fermented with yogurt starter cultures and supplemented with BC4; BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC9; BC4 + BC9: soy beverage fermented with yogurt starter cultures and supplemented with BC4 and BC9.

3.4. Protein hydrolysis

As can be seen in Fig. 4, at the end of *in vitro* gastric phase, protein hydrolysis showed no differences among all samples. Duodenal digestion determined a significant increase of protein hydrolysis up to six folders. Although no differences among experimental samples were detected at the end of duodenal digestion (D120), a significant rise in the degree of hydrolysis was observed for the sample containing the mix of BC4 and BC9 passing from D60 (423.8%) to D120 (592.1%).

3.5. Soluble protein content

The content of proteins with molecular weight higher than 3 kDa was determined in the digested products with the Coomassie blue assay (Fig. 5). Protein concentration increased overtime during *in vitro* digestion in all the samples, except for control at D120. This means that control showed the maximum release of proteins already in the middle of the duodenal phase (D60) while all the other samples, containing the vaginal strains, presented a full release only at the end of the duodenal digestion (D120). Within timepoint, sample with BC9 showed the highest content of soluble proteins compared to the control, while

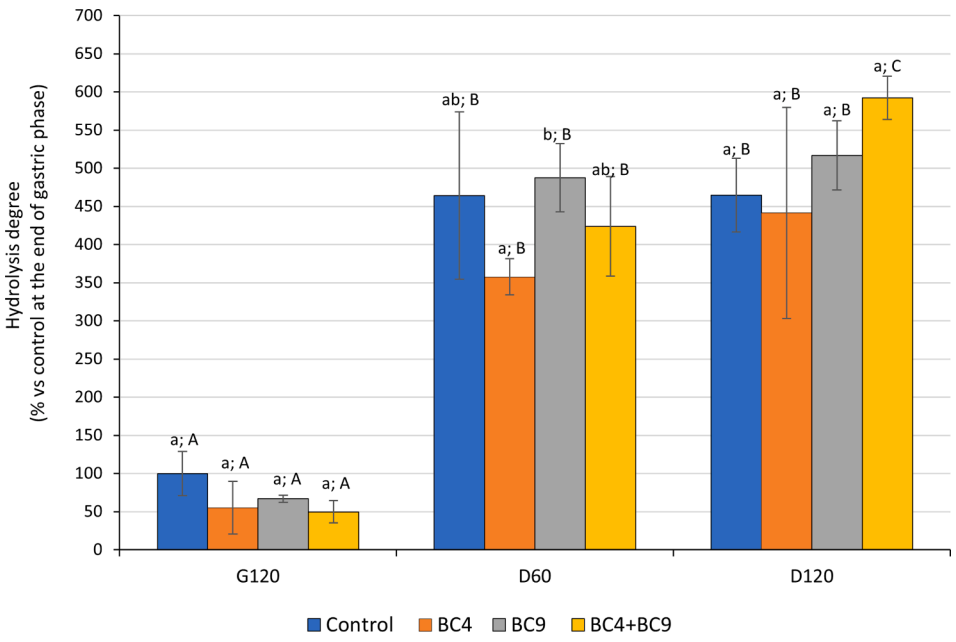

Fig. 4. Hydrolysis degree assessed by OPA assay of the different fermented soy beverages (control, BC4, BC9, BC4 + BC9,) after *in vitro* digestion, at selected time points (end of the gastric phase: G120, after 60 min: D60, and at the end of the duodenal phase D120). Hydrolysis degree was expressed as % vs control at the end of gastric phase. Statistical analysis was performed by a one-way ANOVA with Tukey's post-hoc test comparing the different formulations within each digestion time point (different lowercase letters indicate significant differences) and each formulation at the three digestion time points (different uppercase letters indicate significant differences) ($p < 0.05$).

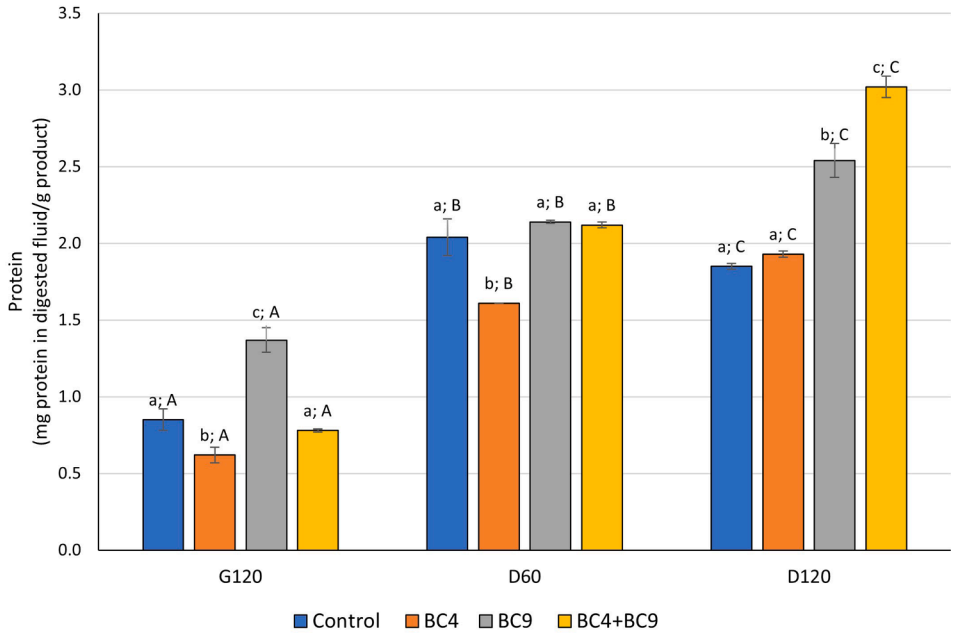

Fig. 5. Protein content (fragment > 3 kDa) measured by Coomassie assay of the different digested supernatant of fermented soy beverages (control, BC4, BC9, BC4 + BC9) after *in vitro* digestion, at the end of the gastric phase (G120) or after 60 (D60) and 120 min (D120) of duodenal phase. The obtained values are expressed as (mg protein in digested fluid/g of fermented soy beverage). Statistical analysis was performed by a one-way ANOVA with Tukey's post-hoc test comparing the different formulations within each digestion time point (different lowercase letters indicate significant differences) and each formulation at the three digestion time points (different uppercase letters indicate significant differences) ($p < 0.05$).

samples with BC4 was the lowest at the end of grastric phase (G120). In the middle of the simulated duodenal phase (D60), no differences were observed among control, BC9 and BC4 + BC9, while a lower amount of proteins was determined for BC4. At D120, sample fermented with BC9 or BC4 + BC9 showed the highest content of soluble proteins.

The total content of proteins and amino acids (regardless of the molecular weight) in the digestates was evaluated by measuring aromatic amino acid content at 280 nm (Fig. 6). Also in this case, total proteins increased over time during *in vitro* digestion, except for control and BC9 that reached their maximum level after D60. Looking at each timpoint, BC9 possessed the highest amount of total proteins after G120 while the other samples were not different. On the middle of the *in vitro* duodenal phase (D60), BC9 and the mix of vaginal strains (BC4 + BC9) showed a significantly higher content of total proteins than the control. This higher amount was also maintained at the end of the duodenal phase, with the mix having the highest amount of total proteins (26.12 mg protein in digested fluid/g fermented soy beverage).

4. Discussion

Fermentation with LAB has been proposed as an efficient tool to improve the nutritional bioavailability of food macronutrients, enhancing physicochemical and sensory properties and supplying health benefits for the consumers (Kumari et al., 2022). Functionality and probioticity of *L. crispatus* BC4 and *L. gasseri* BC9 were already assessed *in vitro* and in real food systems by several authors (Parolin et al., 2015, Nardini et al., 2016, Foschi et al., 2017, Siroli et al., 2017, Marziali et al., 2019, Patrignani et al., 2019, Patrignani et al., 2020, D'Alessandro et al., 2021a; D'Alessandro et al., 2021b, Parolin et al., 2022, D'Alessandro et al., 2023, Giordani et al., 2023). In our work, the addition of these

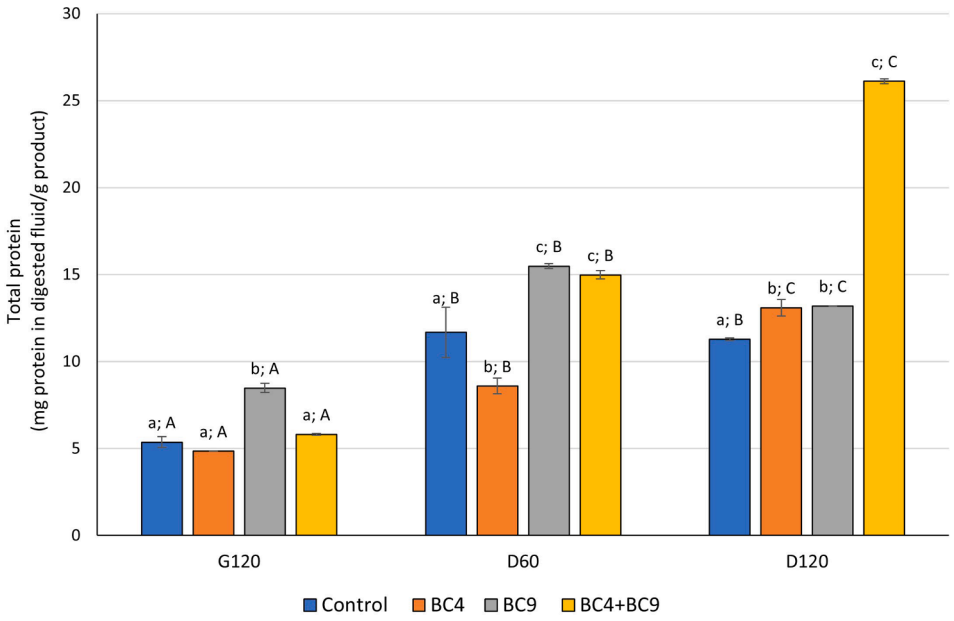

Fig. 6. Total protein content at 280 nm of each fermented soy beverage product (control, BC4, BC9, BC4 + BC9,) after *in vitro* digestion, at selected time points (end of the gastric phase: G120, after 60 min: D60, and at the end of the duodenal phase D120). The obtained values are expressed as (mg protein in digested fluid/g of fermented soy beverage). Statistical analysis was performed by a one-way ANOVA with Tukey's post-hoc test comparing the different formulations within each digestion time point (different lowercase letters indicate significant differences) and each formulation at the three digestion time points (different uppercase letters indicate significant differences) ($p < 0.05$).

vaginal bacteria into soy beverage prior fermentation with yogurt starter cultures impacted the final nutritional value of the products and the resulting protein bioaccessibility. Regarding the centesimal composition, carbohydrates decreased in all the fermented samples containing the probiotics. Several authors reported a reduction in carbohydrates during fermentation of soy beverages (Ahsan et al., 2020; Obadina et al., 2013; Osundahunsi et al., 2007). Sugars are fermented by microorganisms for cellular activities and growth. On the other hand, protein content was higher in soy beverages inoculated with BC9 (alone or in mix), suggesting that the strain of *L. gasseri* positively affected the amount of total proteins. Although fermentation is usually associated with protein hydrolysis, Obadina et al. (2013) described an increase in protein content during natural fermentation of nono, a fermented soy beverage. The increase may depend on several factors. From one hand, microbial cell proliferation can increase the final bacterial biomass resulting in a higher protein content. On this regard, D'Alessandro et al. (2023) reported that the addition of *L. gasseri* BC9 determined a decrease in *L. delbrueckii* subsp. *bulgaricus* but also a significant increase in the other starter, *S. thermophilus*. On the other hand, protein content may be affected by fermentation processes because it favours aggregation of proteins and/or polypeptides into higher molecular weight structure (Bonczar et al., 2016).

Lipid content was not impacted by the addition of functional probiotics. However, the presence of BC9 determined a significant increase in the relative abundance of oleic acid. In contrast, the relative abundance of α-linolenic acid and palmitic acid were higher in samples prepared with BC4 or BC4 + BC9, respectively. The capability of impacting fatty acid profile was evaluated by Ziarno et al. (2020) in bean-based fermented beverages. Depending on the species tested, fermentation increased palmitic, stearic, and oleic acids. The FA composition was used to determine specific lipid quality indices. PUFA/SFA ratio is commonly used to assess the impact of diet on cardiovascular health (CVH). It is known that PUFAs can lower low-density lipoprotein cholesterol (LDL-C) and serum cholesterol levels, while SFAs contribute to high serum cholesterol levels. Therefore, the higher the ratio, the better for consumers (Chen & Liu, 2020). Other indices used to better assess the atherogenicity and thrombogenicity of foods are IA and IT index. Both IA and IT can be used to assess the potential impact of FA composition on CVH. A FA composition with a lower IA and IT has better nutritional quality and its consumption may reduce the risk of coronary heart disease (CHD) (Chen & Liu, 2020). Eventually, h/H index has been proposed to assess the effects of FA content on cholesterol levels. Among the tested samples, fermented soy beverages containing BC4 showed the best healthy lipid indices (PUFA/SFA = 4.47, AI = 0.124, IT = 0.220 and HH = 6.545) while the lowest ones were observed for the product made with the probiotic mix. However, it is important to mention that although some values were lower than others, they were all better that those reported for fermented cow's milk-based beverages (Šertović et al., 2019).

For what concerns minerals, samples containing BC4 showed the highest level of calcium, iron and magnesium compared to control, which on the contrary, showed the highest amount of potassium and sodium (309.12 and 55.65 mg/100 g product, respectively). Potassium is one of the most important microelements fundamental for membrane transport, energy metabolism, and normal cell functioning. Therefore, its reduction in samples containing the vaginal probiotics is supported by the increased cell activity of the inoculated bacteria. Indeed, according to D'Alessandro et al. (2023), addition of vaginal strains promoted the growth of *S. thermophilus* as compared to control. The use of vaginal strains had also an impact on amino acid composition. Threonine increased with all the strains tested, while phenylalanine and tryptophan were specifically higher in samples with BC4. The use of the mix had the strongest impact on the overall amino acid profile when compared to the control, since it showed a higher content of some essential amino acids (es. valine, threonine, phenylalanine and tryptophan) and glutamic acid. An increase in essential amino acids has been already reported for fermented soy beverages produced with probiotic lactobacilli by Li et al. (2012). Instead, glutamic acid, the most abundant one, is a multifunctional amino acid involved in taste perception, excitatory neurotransmission, and intermediary metabolism (Kondoh et al., 2009). It plays an important role during gastric phase digestion by enhancing gastric exocrine secretion (Zolotarev et al., 2009). Furthermore, glutamic acid is a specific precursor for other amino acids, such as arginine and proline, and for bioactive molecules, like γ-amino butyric acid (GABA) and glutathione.

Eventually, fermented soy beverages were assessed for protein digestibility, which can be improved upon fermentation (Ketnawa & Ogawa 2021). On the other hand, Rui et al. (2016) reported that fermented soy beverages had lower protein solubility and degree of hydrolysis during each phase of gastrointestinal digestion compared to unfermented products. In our work, the degree of hydrolysis showed no differences between control and other samples within each time point of

the *in vitro* digestion. However, both Bradford assay and readings at 280 nm, which measure proteins above 3 kDa and total proteins, respectively, showed that especially samples incubated with the vaginal strains BC9 presented a higher amount of these compounds already after the simulated gastric phase and during the whole duodenal phase. The use of the mix determined a higher amount of soluble proteins only at the end of the duodenal phase. These aspects are crucial since it implies that proteins, amino acids or peptides can become more bioaccessible during the passage through the upper gastrointestinal tract when the vaginal strains are present. For instance, the total protein was 2-fold time higher than the control at the end of duodenal phase in samples containing the mix of probiotics.

5. Conclusions

The results of the present study complete the characterization of fermented soy beverages, made with yogurt starter coltures and vaginal strains, already described by D'Alessandro et al. (2023). Other than be more acceptable from a sensory point of view and providing living functional bacteria upon consumption, fermented soy beverages made with vaginal strains BC4 and BC9 presented an improved nutritional profile (es. lower carbohydrates, higher essential aminoacids). Moreover, a higher protein content and more bioaccessibile was observed when using BC9 or the vaginal mix. Further studies are required to understand the effect of the digested samples on a simulated human gut microbiome.

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CRediT authorship contribution statement

Margherita D'Alessandro: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft preparation. Writing – review and editing, Visualization. **Davide Gottardi:** Validation, Investigation, Data curation, Writing – original draft preparation, Writing – review and editing, Visualization, Supervision. **Mattia Di Nunzio:** Writing – review & editing, Supervision, Data curation. **Sara Margherita Borgonovi:** Formal analysis, Investigation. **Carola Parolin:** Writing – review & editing. **Beatrice Vitali:** Writing – review & editing. **Rosalba Lanciotti:** Writing – review & editing, Supervision, Resources, Conceptualization. **Lorenzo Siroli:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Francesca Patrignani:** Writing – review & editing, Supervision, Resources, Project administration, 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

All the data are available in the manuscript

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