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Development of a synbiotic formulation based on germinated brown rice and probiotics suitable to support women's well-being

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ABSTRACT

The aim of the present study was to develop a synbiotic formulation based on germinated brown rice (GBR) and probiotics with health-promoting benefits. The GBR was selected based on its well-known role as a prebiotic substrate rich in bioactive compounds, particularly γ-oryzanol and γ-aminobutyric acid, able to exert antioxidant and anti-inflammatory activities. Four probiotic strains (*Lacticaseibacillus rhamnosus* CA15, *Limosilactibacillus fermentum* CS57, *Lactiplantibacillus plantarum* IMC510, and *Bifidobacterium animalis* subsp. *lactis* BLC1) were selected and *in vitro* tested, single and as a blend, for the ability to survive in the simulated gastrointestinal tract. Simultaneously, the GBR was investigated for antioxidant, anti-inflammatory, glycogen release, and cell proliferation abilities. Synbiotic formulations, based on a blend of the four selected probiotics and GBR at three different concentrations (GBR1, 40 %; GBR2, 30 %; GBR3, 20 %), were set up to evaluate the survivability of the probiotics and the antagonistic activity against pathogens. Results revealed a good survivability of the probiotic strains under *in vitro* simulated gastrointestinal transit and in presence of different GBR concentrations. The synbiotic formulation containing the lowest GBR concentration (GBR3) showed the broadest spectrum of antagonistic activity against the tested pathogens. The GBR exerted cell proliferation, glycogen release, and anti-inflammatory effect, confirmed by the decrease of IL-6 and the increase of both IL-10 and SIRT-1 expression levels. In conclusion, the present study provides new insight into developing a synbiotic formulation that could represent a promising supplement with health-promoting benefits suitable to support women's well-being.

1. Introduction

Recently, the interest in natural approaches to maintain overall health has brought nutraceuticals to the forefront of research (Bhoyar et al., 2025). Among these, prebiotics received increasing recognition for their role in modulating gut microbiota and supporting general well-being (Dasriya et al., 2024). Among cereal-derived prebiotics, brown rice (BR) is considered a healthy and sustainable choice due to a significantly richer nutritional profile than polished white rice (WR). The germination of BR grains in water is a technological process widely applied to improve the sensory characteristics and increase the bioavailability of health-promoting molecules (Gan et al., 2017). Germinated brown rice (GBR) exhibits a valuable content of nutrients and is rich in insoluble fibers, inositol, ferulic and phytic acid, minerals, tocotrienols, γ-aminobutyric acid (GABA), and γ-oryzanol (Cho & Lim, 2016). Moreover, studies suggest that GBR could be a prebiotic source supporting the growth of beneficial bacteria (Dangmane, 2023; Subhasree & Babu, 2013). Recently, Pino and co-workers (Pino et al., 2022a), evaluating the suitability of GBR to produce value-added fermented products, functionalized by probiotics, demonstrated that the fermentation process improved the nutritional composition of GBR, increasing the content of bioactive compounds. Based on available data, GBR has been identified as a valuable functional ingredient suitable to mitigate chronic and non-communicable diseases and promote health and well-being, mitigating hyperlipidemia and hypertension, as well as reducing the risk to develop cancer, diabetes, and cardiovascular diseases (Chinma et al., 2024; Ukpong & Onyeka, 2019; Wu et al., 2013). In addition, Lin and co-workers (2018) investigated the preventive effect of

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GBR, combined with *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis*, on colorectal carcinogenesis in rats. The authors highlighted inhibition in colorectal carcinogenesis and enhancement in antioxidative capacity while inducing apoptosis. Interestingly, although the use of GBR was mainly focused on gut health, emerging evidence suggests a positive role exerted by specific GBR-derived molecules in other health-related functions. Among these, γ-oryzanol has been reported to have estrogen-like activity, suggesting its suitability in maintaining women's well-being and preventing vaginal disorders (Muhammad et al., 2013). Given the established role of probiotics in maintaining vaginal health (Amabebe & Anumba, 2018), the combination of GBR and probiotic strains could represent a promising strategy to support the balance of the vaginal environment, especially relevant for postmenopausal women, who often experience microbiota dysbiosis, mucosal thinning, and increased susceptibility to infection. Although current evidence is limited, this potential synergism deserves further exploration, as this combination offers a compelling direction for non-pharmacological intervention by harnessing the dual action of nutraceuticals and microbiome-targeted strategies. Such a formulation not only aligns with the growing demand for personalized, natural solutions in women's health but also underscores the value of functional food-based innovation in preventive medicine. Further investigation into the efficacy and mechanisms of this approach could pave the way for the development of functional beverages or supplements specifically designed to address female intimate health challenges in aging populations.

The aim of the present study was to develop a synbiotic formulation based on GBR and selected probiotics with health-promoting features. Antimicrobial, antioxidant, and anti-inflammatory activities, as well as cell proliferation, exerted by the synbiotic formulation, were *in vitro* tested at different concentrations, to establish its potential beneficial effects on vaginal cell models.

2. Materials and methods

2.1. Microorganisms and culture conditions

The probiotic strains *Lacticaseibacillus rhamnosus* CA15 (DSM 33960), belonging to the culture collection of ProBioEtna srl (Catania, Italy); *Lactobacillus fermentum* CS57 and *Bifidobacterium animalis* subsp. *lactis* BLC1, kindly provided by Sacco System srl (Cadorago, Italy), and the *Lactiplantibacillus plantarum* IMC510 strain, provided by Synbiotec srl (Camerino, Italy), were used in the present study. All lactobacilli strains were routinely cultured overnight in de Man Rogosa Sharpe (MRS, Biolife, Italy) and anaerobically incubated at 37 °C overnight, whereas the *B. animalis* subsp. *lactis* BLC1 strain was cultured in Bifidobacteria Selective Media (BSM, Biolife, Italy) and anaerobically incubated at 37 °C overnight. *Escherichia coli* (ATCC 25922, ATCC 35219, and DSM 105393), *Enterococcus faecalis* DSM 2570, *Enterobacter cloaceae* DSM 30054, *Pseudomonas aeruginosa* (DSM 1117 and DSM 3227), *Staphylococcus aureus* DSM 1104, and *Gardnerella vaginalis* ATCC 14019 were used as reference pathogen strains in the antagonistic assay. Each pathogen was cultured using the medium and following the conditions suggested by ATCC or DSM. *Lacticaseibacillus rhamnosus* GG (ATCC 53103) was used as a standard strain for comparison.

2.2. Selected probiotic strains used in a blend formulation

The strains mentioned above were selected based on *in vitro* and *in vivo* probiotic potential (Azagra-Boronat et al., 2020; Coman et al., 2022; Di Bonaventura et al., 2021; Holkem, Favaro-Trindade, & Lacroix, 2020, Holkem, Neto, et al., 2020; Molina-Tijeras et al., 2020; Pagliai et al., 2023; Pino et al., 2022b; Rapisarda et al., 2023; Sabia et al., 2014; Šola et al., 2022). In the present study, a probiotic blend was set up by combining the *L. rhamnosus* CA15 strain, standardized to 2 billion CFU, and the *B. animalis* subsp. *lactis* BLC1, *L. fermentum* CS57, and *L. plantarum* IMC 510 strains, singularly standardized to 1 billion CFU, based on a previous study (unpublished data).

2.3. Probiotics tolerance to lysozyme, low pH, and bile salts

Each probiotic strain was evaluated singly and as a blend for tolerance to lysozyme (20.000 units/mg protein; 100 μg/mL), low pH (pH 3.0), and 0.5 % and 1 % of bovine bile salts (Oxgall), following the method previously described by Pino and co-workers (Pino et al., 2021). Each assay was performed using media opportunely modified as reported below: MRS broth or BSM broth was used to test single culture, while MRS medium, supplemented with 0.05 % (w/v) cysteine-hydrochloride (mMRS), was used to test the blend. Sterile electrolyte solution without lysozyme, media at pH 6.2, and media at pH 6.2 without bovine bile salts were used as controls. For each assay, three independent experiments were performed, and viable cells were enumerated by plate count after incubation at 37 °C for 48h. Results were expressed as survival rate (SR%) using the formula below:

$$SR\% = \frac{\log T}{\log C}\, x\, 100 \qquad (1)$$

Where log T represents the logarithmic number of colonies present after treatment and log C is the logarithmic number of colonies present in the control. All chemicals were purchased from Sigma-Aldrich (Milan, Italy).

2.4. Probiotics ability to survive during the simulated gastrointestinal transit

Each probiotic strain was tested, single and as a blend, for the ability to survive during the *in vitro* gastrointestinal transit. The survivability was evaluated on simulated gastric juice (SGJ) and simulated intestinal fluid (SIF) according to Pino and co-workers (Pino et al., 2021). In detail, SGJ was prepared using 0.3 % pepsin, 0.5 % NaCl, adjusted to pH 2.5 by adding 1 M HCl, SIF contained 0.1 % pancreatin, 0.5 % bile salt, 0.5 % NaCl, 0.4 % phenol, adjusted to pH 7.5 by adding 1 M NaOH. All chemicals were obtained from Sigma-Aldrich (Milan, Italy). SGJ and SGJ–SIF-treated cells were subjected to plate count for the detection of cell viability. The assay was performed in triplicate, and results were expressed as survival rate (SR%) using the formula below:

$$SR\% = \frac{\log N}{\log N_0}\, x\, 100 \qquad (2)$$

Where log N represents the logarithmic number of colonies present at the end of the test and log No is the logarithmic number of colonies present at the start of the test.

2.5. Germinated brown rice

Germinated brown rice (GBR), used in the present study, was kindly provided by Mr. Bio Food srl (Crespadoro, VI, Italy). Physico-chemical and nutritional profile of the GBR is reported in Table 1. Briefly, GBR was obtained by sprouting brown rice seeds in water (25 ± 2 °C for 48 h) and steaming at 121 °C for 15 min. To obtain particles with an average size between 10 and 100 μm, the micronization was performed in a colloidal mill and in the presence of water. Afterwards, to convert the starch into malts and simple sugars, the product was incubated for 1 h at 37 °C in the presence of 2 g/kg of alpha- and beta-amylase enzymes and then freeze-dried. The obtained product was stored under refrigerated conditions until use.

2.6. Antioxidant activity of GBR in cell-free system- DPPH inhibition

The free radical scavenging activity of GBR was evaluated performing a DPPH (2,2–75 diphenyl-1-picrylhydrazyl) assay. The reaction

Table 1
Proximate composition (g/100 g), energy (kcal/100 g), minerals (mg/100g), sugars (g/100g), amino acids and bioactive compounds (mg/kg) of geminated brown rice.

Proximate composition (g/100 g)	
Moisture	73.20 ± 2.08
Protein	2.25 ± 0.14
Total Fat	0.790 ± 0.09
Dietary Fiber	1.02 ± 0.16
Ash	0.37 ± 0.03
Carbohydrates	22.37 ± 2.09
Dry Matter	26.80 ± 0.79
Energy (kcal)	108 ± 9
Minerals (mg/100g)	
Calcium	93 ± 18
Iron	0.184 ± 0.073
Phosphorus	750 ± 170
Magnesium	328 ± 74
Potassium	540 ± 120
Selenium	0.0323 ± 0.007
Sodium	41.2 ± 9.6
Zinc	0.315 ± 0.07
Sugars (g/100g)	
Glucose	11.7 ± 1.70
Fructose	< LoQ
Lactose	< LoQ
Sucrose	0.165 ± 0.03
Maltose	3.72 ± 0.26
Total sugars	15.585 ± 1.72
Amino acids and bioactive compounds (mg/kg)	
Aspartic acid	58 ± 13
Glutamic acid	82 ± 18
Alanine	121 ± 27
Arginine	53 ± 12
Asparagine	63 ± 14
Cystine	<10
Proline	<10
Phenylalanine	19.3 ± 4.30
Glycine	33.8 ± 7.50
Glutamine	<10
Isoleucine	19.3 ± 4.30
Histidine	19.3 ± 4.30
Leucine	38.7 ± 8.50
Lysine	24.2 ± 5.40
Methionine	<10
Ornithine	<10
Serine	29.0 ± 6.40
Tyrosine	24.2 ± 5.40
Taurine	<10
Threonine	24.2 ± 5.40
Valine	24.2 ± 5.40
Gamma-aminobutyric acid (GABA)	121 ± 27
Citrulline	<10
Cysteine	<10
Alpha-aminobutyric acid	<10
Total free amino acids	754.2 ± 51.30
Gamma-oryzanol	113 ± 12

LoQ: limit of quantification corresponding to 10 mg/kg.

mixtures contained 86 μM DPPH, solubilized in ethanol, and different concentrations of GBR (0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15, 20, 30 %). After 10 min at room temperature (25 ± 2 °C) the absorbance at λ = 517 nm was recorded in a microplate reader (Biotek Synergy-HT, Winooski, VT, USA).

2.7. GBR effect on cell cultures and viability assays

Experiments were conducted on an epithelial cell line of vaginal mucosa (VK2/E6E7, ATCC-CRL-2616). Cells were cultured in Keratinocyte-Serum Free medium (GIBCO-BRL 17005-042) with 0.1 ng/ml human recombinant epidermal growth factor (EGF), 0.05 mg/ml bovine pituitary extract (BPE; Gibco), and calcium chloride 0.044.1 mg/ml (final concentration 0.4 mM). Cells were maintained at 37 °C under 5 % of CO_2. The effect of GBR on VK2/E6E7 cell viability was evaluated by MTT assay following 72h of treatment at different concentrations (0.1, 0.5, 1.0, 5.0,10 %). Cells were seeded into 96-well plates at a density of 7.0×10^3 cells/well in 100 μL of the culture medium. After 72h of treatment 100 μL/well of a 0.25 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the cells and incubated for 2h. Formazan crystals metabolized by the cells were then eluted with DMSO and absorbance read at λ = 570 nm. LPS (0.1, 1.0, 10.0, 25.0, 50.0 μg/mL) effect on VK2/E6E7 cell viability was also evaluated at 24 and 48h using Cell Counting Kit-8 (96992- Sigma-Aldrich) following manufacturer instructions.

2.8. Clonogenic assay

Cells (500 cells/well) were plated into 6-well plates and incubated with the selected non-toxic concentrations (0.1, 0.5, 1.0 %) of GBR for 14 days. Cells were then washed with PBS, fixed with 10 % formaldehyde for 20 min, and stained with 0.1 % (w/v) crystal violet solution for 10 min at room temperature (25 ± 2 °C). After three washes in PBS, cell colonies containing more than 50 cells were counted under an inverted light microscope. Subsequently, crystal violet was eluted with methanol and absorbance was read at λ = 590 nm in a microplate reader.

2.9. Cell proliferation assay

Muse® Ki67 Proliferation Kit (MCH100114, Luminex, USA) was used in order to determine the percentage of proliferating cells based on Ki67 expression after GBR treatment (0.1, 0.5, 1.0 %), following manufacturer's instructions. Briefly, cell suspensions (5×10^3 - 1×10^5 cells per sample) were fixed using Fixation Solution, permeabilized, and stained with Muse anti-Ki67-PE or IgG1-PE antibody as per the kit protocol. After sequential incubation and washes with Assay Buffer, samples were analyzed on the Guava Muse Cell Analyzer to assess the proportion of Ki67-positive proliferating cells. Two populations of cells were distinguished, Ki67(−) cells (not proliferating) and Ki67(+) cells (proliferating) respectively. The software provides percentages of both Ki67(+) cells and Ki67(−) cells.

2.10. Western Blotting

VK2/E6E7 cells were treated with the selected GBR concentrations (0.1, 0.5, 1.0 %) and harvested after 72h; pellets were sonicated and centrifuged at 2500 rpm for 10 min at 4 °C to extract proteins from the total lysate. The protein samples (70 μg) were diluted in 2x Laemmli Sample Buffer (#1610737, BioRad, Hercules, CA, USA) and heated at 85 °C for 5 min. Proteins were separated via electrophoresis and then transferred as previously reported (Consoli et al., 2023). Membranes were incubated overnight with Ki67 (ab16667, diluted 1:1000, Abcam, Cambridge, U.K.) and β-actin (GTX109639, diluted 1:7000, GeneTex, Irvine, CA, USA) primary antibodies. Appropriate secondary antibodies were used to detect blots (dil. 1:10.000). Then, the blots were scanned, and densitometric analysis was performed using the Odyssey Infrared Imaging System (LI–COR, Milan, Italy). Values were normalized to β-actin.

2.11. Glycogen release

Glycogen concentration was determined in conditioned medium of treated cells using a coupled enzymatic assay that produces a colorimetric product proportional to the glycogen content. Glycogen Assay Kit (no. MAK016, Sigma-Aldrich Pty. Ltd., Missouri, USA) was used following manufacturer's instructions and the absorbance was measured at λ = 570 nm in a microplate reader.

2.12. RNA extraction and quantitative real-time PCR analysis

Cells were pre-treated for 48h with the selected concentration of the extract (0.1 %) and then co-treated with LPS (10 μg/mL) for 24h. After treatments, cells were harvested for RNA extraction using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as previously reported (Vanella et al., 2023). Subsequently, qRT-PCR analysis was performed in a Step One Fast Real-Time PCR System Applied Biosystems using the SYBR Green PCR Master Mix (Life Technologies, Monza MB, Italy) to evaluate IL-6, IL-10 and SIRT-1 gene expression. Results were normalized with the housekeeping gene GAPDH using a comparative $2-\Delta\Delta Ct$ method.

2.13. Enzyme-linked immunosorbent assay (ELISA)

IL-6 levels were measured in the conditioned medium of VK2/E6E7 following 48h pre-treatment with the GBR (0.1 %) and subsequent co-treatment with LPS (10 μg/mL) for 24h using ELISA kit (BMS213INST, eBioscience, Vienna, Austria). The results are expressed as pg/mL.

2.14. Setup of experimental synbiotic products

Freeze-dried GBR was suspended in water at final concentrations of 40 % (GBR1), 30 % (GBR2), and 20 % (GBR3). After homogenization, different fermentation batches were setup, in triplicate, by inoculating overnight cells of each probiotic strain, single or as a blend, at a final cell density of 6 log cfu/ml. The survivability/growth of probiotic strains was evaluated till 12 h of incubation at 37 °C by monitoring the pH changes (T0, T6, T9, and T12) using a pH meter (BenchMeter, Violab srl, Italy). The viability of probiotics was evaluated by plate count in MRS agar, for lactobacilli, BSM agar, for bifidobacteria, and L-MRS to determine the cell density of the blend. Each assay was carried out in triplicate and results were expressed as mean log CFU/ml and standard deviation.

2.15. Antagonistic activity of the synbiotic against pathogens

The antimicrobial potential of the experimental synbiotic products was assessed using the agar spot method and following the protocol reported by Pino and co-workers (Pino et al., 2019). Synbiotic products, previously formulated, were collected after 12 h of incubation and tested against target pathogens. The antagonistic activity was ranked as follows: absent (−, no inhibition zone); low (+, inhibition zone <10 mm); intermediate (++, inhibition zone between 11 and 20 mm); and high (+++, inhibition zone >20 mm). The experiments were carried out in triplicate.

2.16. Synergistic effect of the synbiotic formulation on vaginal cell proliferation

The selected GBR concentration (0.1 %) and the probiotic blend was evaluated for the potential synergistic effect for vaginal cells proliferation by using the method previously described.

2.17. Statistical analysis

At least three independent experiments were performed for each analysis. The statistical significance ($p < 0.05$) of the differences between the experimental groups was determined by Fisher's method for analyses of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested via either a single-factor analysis of variance (ANOVA) for multiple groups or an unpaired *t*-test for two groups, and the data are presented as means ± SEM.

3. Results and discussion

3.1. Lysozyme tolerance

Lysozyme is a hydrolytic enzyme present in several secretions, such as saliva, that is able to exert antimicrobial properties (Vila et al., 2019). Probiotics for oral use, although transiently exposed to lysozyme, can exhibit varying degrees of tolerance depending on structural and physiological traits (Cele et al., 2022; Dias et al., 2015). According to that, in the present study, a strain-dependent lysozyme tolerance was detected. In detail, as displayed in Fig. 1, after 30 min of exposure to lysozyme, all tested strains showed SR% higher than 94 % with the exception of *B. animalis* subsp. *lactis* BLC1 (85 %) and LGG (92 %) strains. In particular, the *L. fermentum* CS57 and the *L. plantarum* IMC510 strains maintained the highest survival rate, showing values of 97.8 % and 97.7 %, respectively. After 120 min of exposure to lysozyme, both *L. fermentum* CS57 and *L. rhamnosus* CA15 strains, with values of 98.1 % and 93.0 %, retained high viability. Otherwise, the *L. plantarum* IMC510 and the *B. animalis* subsp. *lactis* BLC1 strains were most affected by the exposure to lysozyme, displaying SR% of 60.9 % and 87.9 %, respectively (Fig. 1). Interestingly, high tolerance to lysozyme was displayed by the blend, after both 30 and 120 min of exposure (Fig. 1). The obtained results suggest that the probiotics under study, when tested as a blend, can tolerate lysozyme contained in the oral cavity, since 100 μg/mL is the highest level of lysozyme that is normally used to simulate *in vivo* conditions (Cele et al., 2022; Turchi et al., 2013). This finding corroborates previously reported data, suggesting that, by combining strains in a blend, a protective or synergistic effect can be achieved. Consequently, more tolerant strains in the blend help stabilize or shield the more sensitive ones, determining the enhancement of survival under stress conditions (Kwoji et al., 2021; Mathipa & Thantsha, 2015).

3.2. Tolerance to low pH and bile salts

During the passage through the GIT, the first critical stage occurs in the stomach since acidity represents a detrimental condition for the viability of microorganisms. In particular, the low pH of the stomach can damage the composition, integrity, and functionality of plasma membranes, leading to cell death (Bustos et al., 2025; Guan & Liu, 2020). It is well known that the ability of probiotic bacteria to survive during the passage through the stomach is variable and strain-dependent (Dos Santos et al., 2021; Palaniswamy & Govindaswamy, 2016) however, as displayed in Fig. 2, all the tested strains were able to tolerate low pH. In particular, the *L. rhamnosus* CA15 strain, along with LGG, was able to maintain a high viability at pH 3 over time, showing SR% of 92.0 % and 86.0 % after 2 h and 4 h of exposure, respectively. This evidence confirms the ability of the *L. rhamnosus* CA15 strain to survive under acidic

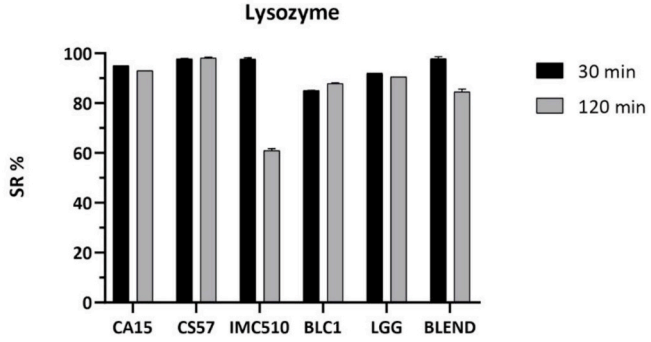

Fig. 1. Lysozyme tolerance displayed by *L. rhamnosus* CA15, *L. fermentum* CS57, *L. plantarum* IMC 510, and *B. animalis* subsp. *lactis* BLC1 probiotic strains singularly and as a blend.
Data are reported as survival rate (SR%) after lysozyme exposure for 30 and 120 min. Electrolyte solution without lysozyme was used as control.

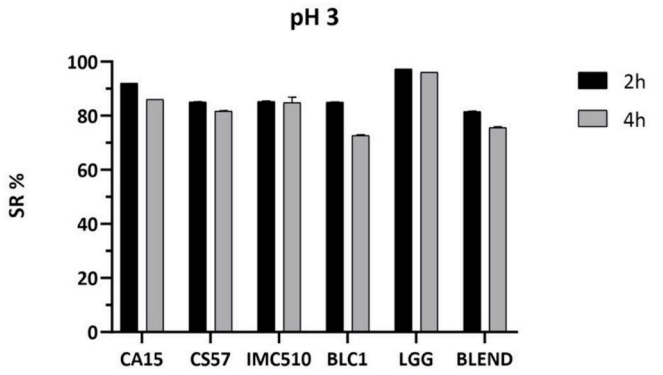

Fig. 2. Tolerance to pH 3.0 exhibited by *L. rhamnosus* CA15, *L. fermentum* CS57, *L. plantarum* IMC 510, and *B. animalis* subsp. *lactis* BLC1 probiotic strains singularly and as a blend.
Data are reported as survival rate (SR%) after 2 and 4 h of exposure to pH 3.0. MRS at pH 6.2 was used as control.

stress (Pino et al., 2022b). High sensitivity to pH 3.0 was displayed by the *B. animalis* subsp. *lactis* BLC1 strain after 4h of exposure (72.6 %), consistent with a higher tolerability of lactobacilli to low pH values than bifidobacteria (Rivera-Espinoza & Gallardo-Navarro, 2010; Moussavi & Adams, 2010).

Besides acidic pH, another important feature of probiotics is resistance to bile salts, representing the second challenge associated with the passage through the duodenal loop of the small intestine (Taranto, Perez-Martinez, & Font de Valdez, 2006). In fact, bile salts are amphipathic molecules with potent antimicrobial activity that act as a detergent in destabilizing biological membranes, affecting both cell permeability and interactions between the membrane and its environment (Taranto et al., 2006; van de Guchte et al., 2002). Although with high variability among strains, several LABs are bile tolerant, since they are able to exert bile salt hydrolysis capacity (Agolino et al., 2025; Bustos et al., 2025). In the present study, as reported in Fig. 3, all the tested strains exhibited tolerance abilities to both 0.5 % and 1.0 % of bile salts, higher than LGG. In detail, after 2h of exposure to both 0.5 % and 1.0 % of bile salts, SR% higher than 80 % were revealed. Differently, after 4h of exposure, the *L. plantarum* IMC510 exhibited the highest resilience in the presence of both 0.5 % and 1 % of bile salts (93.6 % and 94.4 %). Differently, the *B. animalis* subsp. *lactis* BLC1 strain was the worst performing, exhibiting SR% of 73.3 % and 75.1 % after 2h and 4h of exposure, respectively (Fig. 3). Previously reported data shown that the sensitivity to bile salts can increase based on bile salt concentrations and incubation time (Bustos et al., 2025; Dos Santos et al., 2021; Palaniswamy & Govindaswamy, 2016). In our study, except for the *L. fermentum* CS57 strain, a slight variation in survival rates were detected when 1.0 % of bile salts was tested (Fig. 3). Concerning the blend, as displayed in Fig. 3, in presence of both 0.5 % and 1.0 % of bile salts, SR% higher than 85 % was observed after both 2 and 4 h of exposure.

3.3. Survival during in vitro GI transit

Survival in the harsh conditions occurring during GI passage, due to the presence of digestive enzymes, bile, and acid, as well as persistence in the intestinal environment, are critical for probiotics since the health-promoting effect is dependent on both viability and activity in the intestine (Wendel, 2022). In the present study, by testing the ability of the probiotic strains and of the blend to survive during *in vitro* gastrointestinal transit, high tolerance to both SGJ and SIF was detected (Fig. 4). In fact, SR% higher than 80 % was registered after exposure to gastric juice and maintained after treatment with SIF. Notably, the *L. rhamnosus* CA15 and the *L. plantarum* IMC510 strains showed the highest survival after exposure to both SGJ and SIF, with cell densities comparable to LGG. Similar behaviour was displayed by the blend.

3.4. Antioxidant capacity of the GBR

Rice (*Oryza sativa* L.) serves as an excellent dietary source of natural antioxidants, notable for its enrichment in vitamin E homologs,

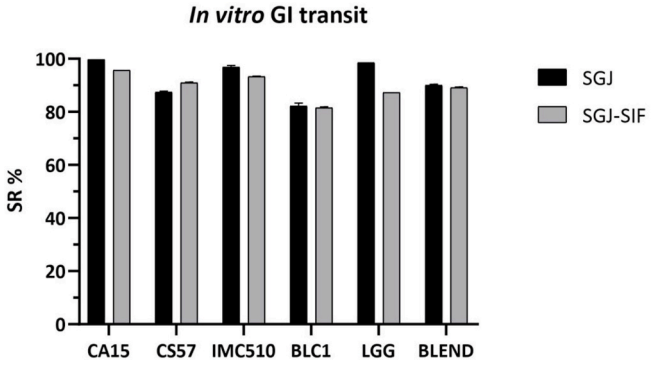

Fig. 4. Survivability of *L. rhamnosus* CA15, *L. fermentum* CS57, *L. plantarum* IMC 510, and *B. animalis* subsp. *lactis* BLC1 probiotic strains singularly and as a blend in simulated gastrointestinal transit.
Data are reported as survival rate (SR%) after exposure to simulated gastric juice (SGJ) and simulated intestinal fluid (SIF).

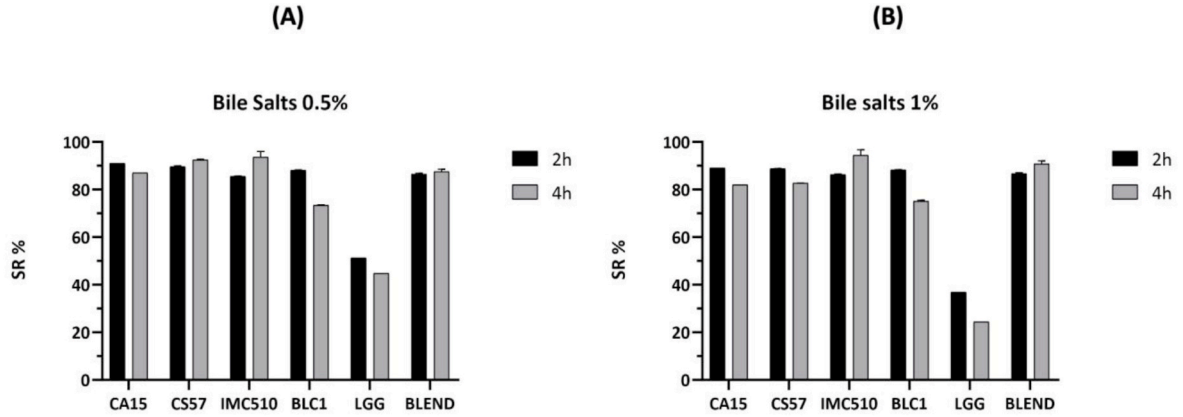

Fig. 3. Tolerance to bile salts at 0.5 % (panel A) and 1 % (panel B) exhibited by *L. rhamnosus* CA15, *L. fermentum* CS57, *L. plantarum* IMC 510, and *B. animalis* subsp. *lactis* BLC1 probiotic strains singularly and as a blend.
Data are reported as survival rate (SR%) after exposure to bile salts (0.5 % and 1 %) for 2 and 4 h. Growth media without bile salts were used as controls.

anthocyanins, and phenolic compounds (Soobrattee et al., 2005). Among these phytochemicals, γ-oryzanol, a complex of ferulic acid esters with phytosterols such as cycloartenyl, campesteryl, and 24-methylenecycloartanyl ferulates, stands out for its particularly potent antioxidant capacity (Goufo & Trindade, 2014; Rungratanawanich et al., 2018). The anti-oxidative activity of γ-oryzanol has been investigated *in vitro* and *in vivo* (Juliano et al., 2005; Spiazzi et al., 2013). Mechanistically, γ-oryzanol exhibits versatile radical-scavenging activities across both lipophilic and hydrophilic environments. In cellular models, pretreatment with γ-oryzanol derivatives significantly reduced hydrogen-peroxide–induced ROS generation and upregulated endogenous antioxidant defences, such as increased expression of SOD enzymes (Rungratanawanich et al., 2018). Further, γ-oryzanol activates the Nrf2–ARE signalling pathway, enhancing the expression of phase II antioxidant genes, due to its structure containing an α,β-unsaturated carbonyl moiety that may modify Keap1, facilitating Nrf2 nuclear translocation and protective gene induction (Ismail et al., 2014; Rungratanawanich et al., 2018; Xu et al., 2001). *In vivo* studies corroborate these findings: γ-oryzanol supplementation in animal models led to increased activity of antioxidant enzymes (SOD, CAT, GST), reduced lipid peroxidation, and improved redox balance in conditions such as ethanol-induced liver injury and high-fat diet–induced oxidative stress. Moreover, clinical assessments in hyperlipidaemic patients have shown that γ-oryzanol lowered oxidative stress markers and inflammatory cytokines (e.g., TNF-α, IL-1β, TXB_2) more effectively than vitamin E or omega-3 supplements (Araujo et al., 2015; Chotimarkorn & Ushio, 2008; Juliano et al., 2005). To assess the antioxidant capacity of GBR, which is rich in γ-oryzanol, a preliminary evaluation was conducted in a *cell-free* model using different concentrations (0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, and 30.0 %). As shown in Fig. 5, there was appreciable dose-dependent scavenger activity of the GBR, as previously reported for GBR bioactive compounds (Kang et al., 2022; Md Zamri et al., 2014; Rungratanawanich et al., 2018).

3.5. Effect of the GBR on VK2/E6E7 cell viability and survival

Identifying non-toxic doses in natural extracts is essential to ensure their safety and efficacy, preventing adverse health effects and ensuring consumer protection, given that the extracts themselves may contain chemicals that, if uncontrolled, can be harmful or even toxic, as required by food and supplement labelling and safety regulations (Dwyer, 2023; Ekor, 2014). In *in vitro* studies on non-tumor cell cultures, to evaluate the beneficial effect of natural extracts, it is essential to identify non-toxic doses. In order to evaluate the GBR effect, a preliminary step of incubation (72h) in complete cell culture media was necessary since bacterial proliferation was observed upon cells' exposure to GBR without pre-incubation, which compromises the reliability of the results. Thus, this experimental condition was maintained for all further experiments performed. The GBR effect on VK2/E6E7 cells was evaluated by MTT assay after 72h of treatment at different concentrations (0.1, 0.5, 1.0, 5.0, 10.0 % respectively). Highest concentrations (5 % and 10 %) resulted in a significant decrease in cell viability rate, thus none of those concentrations were further examined (Fig. 6, panel A). In perimenopausal and postmenopausal women, serum estrogen levels undergo a marked decline, leading to a series of physiological alterations, most notably vaginal atrophy. The current standard therapeutic approach for managing vaginal atrophy is estrogen replacement therapy (ERT), which restores estrogenic activity and thereby counteracts atrophic changes in the vaginal mucosa (North American Menopause Society, 2007). However, ERT is associated with side effects including tumorigenesis promotion and development of breast cancer, thickening of the endometrium, and increased risk of endometrial cancer (Davis et al., 2015).

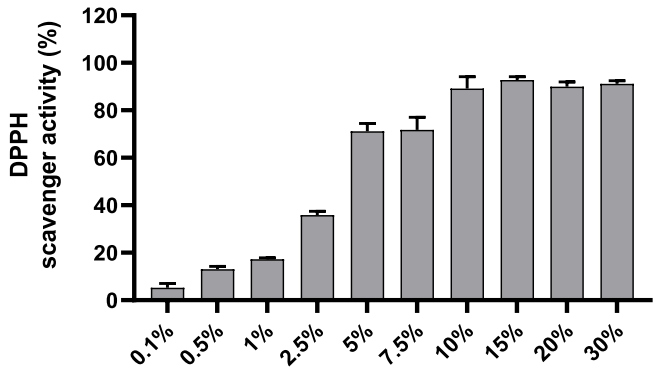

Fig. 5. Evaluation of radical scavenging activity of the GBR through DPPH assay in a cell-free model.

Therefore, alternative therapies with few side effects have been studied. Stem cell therapy has been shown to be highly effective for treating multiple conditions such as vaginal atrophy (Shimoyama et al., 2022). Moreover, estrogenic effects of Cell-Free Fat Extract have been reported. It was demonstrated that Cell-Free Fat Extract was able to prevent vaginal atrophy by promoting the proliferation and migration of VK2/E6E7 cells (Shimoyama et al., 2022). Selected non-toxic concentrations (0.1, 0.5, and 1 %) were used to perform the clonogenic assay, a cell survival assay used to evaluate the ability of a single cell to proliferate and form a colony, which is typically defined as a cluster of 50 or more cells. Cells (500 cells/well) were plated into 6-well plates and concomitantly incubated with the indicated concentrations (0.1, 0.5, 1 %) of GBR, which was maintained for 12 days to evaluate cells colony formation. As observed in Fig. 6 (panels B and C), only the lowest concentration tested showed a significant enhancement in terms of cell proliferation rate corresponding to a higher number of colonies counted, compared to the untreated control. GBR is known to contain both gamma-oryzanol and GABA (Pino et al., 2022a), the first being an estrogen-like compound, while the second has been shown to counteract cell proliferation (Wang et al., 2019). Indeed, γ-oryzanol has been shown to be able to modulate expression of metabolic regulators such as PPARγ, which can indirectly impact cell cycle progression and Ki67 expression in cellular models (Imam et al., 2013; Wu et al., 2022), while it is known the anti-proliferative effect of GABA through induction of apoptosis (Oh & Oh, 2004). Thus, we can hypothesize that the notable increase for the lowest concentration may be due to a lower concentration of GABA compared to higher concentrations (0.5, 1 %). Indeed, the final concentration of γ-oryzanol in the solution (0.1 %) is around 40 ng/mL, while GABA is around 200 ng/mL. Additionally, higher GBR concentrations showed aggregates in medium solutions which can impede cellular uptake of bioactive compounds.

3.6. Effect of the GBR on VK2/E6E7 cell proliferation

Cell proliferation profile was additionally examined through flow cytometry analysis using the MUSE cell analyzer. In particular, the Ki67 Proliferation kit (SKU MCH100114. Cytek) was used, allowing for the quantification of the percentages of proliferating and nonproliferating cells based on Ki67 expression. Results obtained highlighted the GBR capacity of increasing single-cell Ki67 expression at the lowest concentration tested (0.1 %) (Fig. 7, panels A and B), in accordance with colony formation observations. On the other hand, the same increase was not appreciable through Western Blot analysis of Ki67 expression in VK2/E6E7 whole cell lysates (Fig. 7, panels C and D). These findings suggest that flow cytometric analysis of Ki67 expression may offer superior sensitivity compared to the Western blot technique, potentially accounting for the observed differences in proliferative marker detection.

3.7. Effect of the GBR on VK2/E6E7 cells glycogen release

Estrogen has been postulated to influence glycogen levels by vaginal epithelial cells (Farage & Maibach, 2006). Furthermore, free glycogen

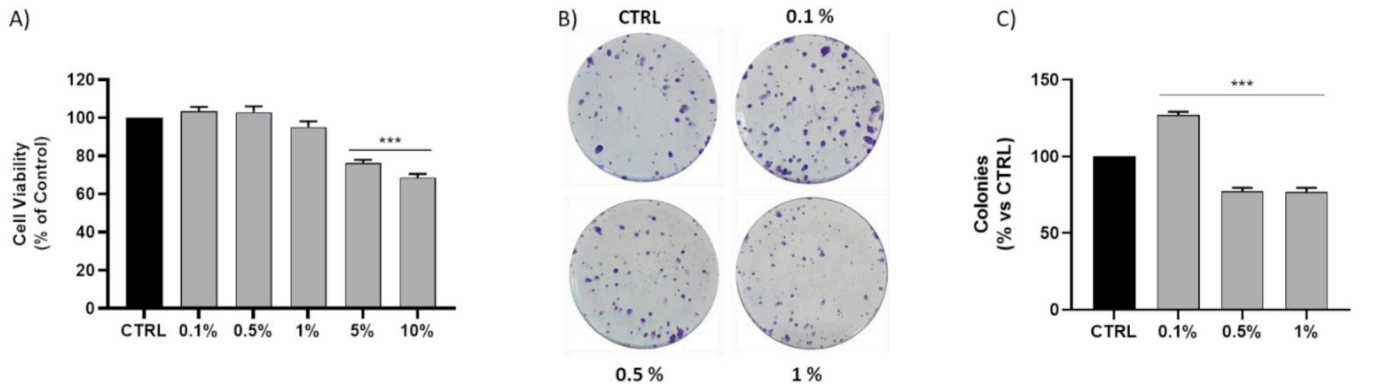

Fig. 6. Assessment of the GBR effect on VK2/E6E7 cell viability (A). Representative images of single-cell clone proliferation. stained with crystal violet and colony quantification (B and C). (***p < 0.0005 vs CTRL).

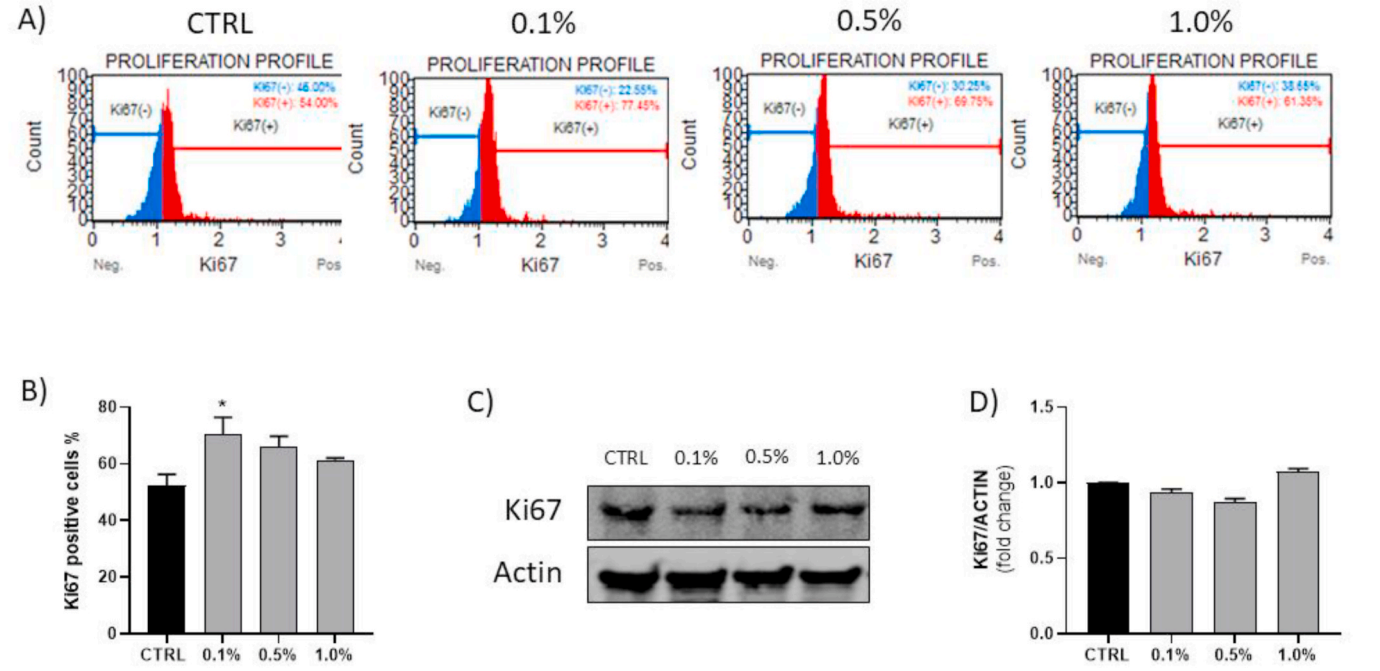

Fig. 7. Assessment of cells proliferation profile through Ki67 expression by flow cytometry (A and B) and Western Blot analysis (C and D).

was observed in both pre- and post-menopausal women, with post-menopausal women exhibiting significantly lower levels compared to pre-menopausal women (Mirmonsef et al., 2015). Interestingly, GBR was able to increase the release of glycogen into the conditioned medium (CM) of VK2/E6E7 cells with all tested concentrations, but once again highlighting the most significant effect at 0.1 % concentration (Fig. 8). The observed increase in glycogen levels may be attributed to the combined presence of γ-oryzanol and GABA, both of which have been reported to modulate glycogen metabolism (Adamu et al., 2017; Dastgerdi et al., 2021; Wang et al., 2015). Intracellular glycogen within vaginal epithelial cells is a critical source of lactic acid production, which helps maintain a protective acidic pH in the vaginal milieu (Navarro et al., 2023). Physiological estrogen stimulates glycogen deposition in these cells, and the ensuing anaerobic glycolysis yields lactic acid that diffuses into the vaginal fluid, contributing significantly to microbiota stability and mucosal health. Notably, the attenuated effect observed at higher concentrations could be explained by reduced cellular uptake of bioactive compounds, potentially resulting from solubility limitations or the formation of non-homogeneous solutions. In postmenopausal women, the decline in estrogen levels leads to a

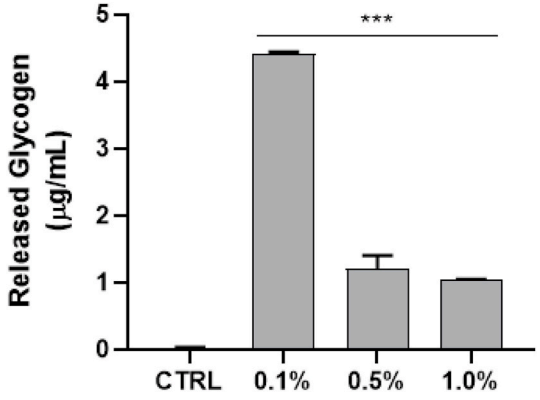

Fig. 8. Measurement of glycogen release in CM (*p < 0.05. ***p < 0.0005 vs CTRL).

reduction in vaginal epithelial glycogen, subsequently impairing lactic acid production and compromising its associated anti-inflammatory functions (Lehtoranta, Ala-Jaakkola, Laitila, & Maukonen, 2022).

3.8. Anti-inflammatory effect of the GBR on VK2/E6E7 cells

Vaginal epithelial cells exhibit the capacity to produce different cytokines that can recruit innate and adaptive immune responses. Estrogen can differentially regulate the expression of several genes affecting the vaginal epithelium homeostasis, including those involved in the pro- and anti-inflammatory response (Wagner & Johnson, 2012). Lactic acid, predominantly produced by Lactobacillus spp., plays a fundamental role in preserving vaginal homeostasis by maintaining a low pH that inhibits the growth of pathogenic microorganisms and reduces the activation of inflammatory pathways (Amabebe & Anumba, 2018; Jenkins et al., 2023). The anti-inflammatory potential of GBR was then evaluated following the establishment of an LPS-induced inflammation model on VK2/E6E7 cells. After a preliminary analysis of LPS effect at different concentrations (0.1, 1.0, 10.0, 25.0, 50.0 μg/mL) on cell viability after 24 and 48h, which showed no significant alterations (Fig. 9, panels A and B), cells were pre-treated for 48h with the selected concentration of the GBR (0.1 %) and then co-treated with LPS (10 μg/mL) for further 24h. Analysis of relative mRNA levels of interleukin 10 (IL-10), sirtuin-1 (SIRT-1), and IL-6 showed a modulatory effect of the GBR on different cytokines, reflecting a significant anti-inflammatory activity (Fig. 9 panels C–E). To further corroborate these data, measurement of released IL-6 levels in CM was performed using enzyme-linked immunosorbent assay (ELISA) (Fig. 9, panel F). Results obtained confirmed the anti-inflammatory effect of the GBR as it was able to significantly reduce IL-6 levels compared to LPS treatment group, in agreement with literature findings (Chung et al., 2019; Sun et al., 2018).

3.9. Viability of probiotics in experimental synbiotic products

GBR contains many essential nutrients to support the growth of probiotics and can directly be used as the substrate for microbial growth, as reported in Table 1. In line with this evidence, in the present study, both probiotics and the blend were tested for viability in experimental products (GBR1, GBR2, and GBR3) over 12 h of incubation (Fig. 10, panels A and B) and pH changes were detected (Fig. 10, panels A and B). Overall, all tested strains grew in each GBR formulation with different behavior. The GBR3, containing the lowest concentration of GBR (20 %), was the most effective formulation in supporting the growth of both probiotic strains and blend. Notably, after 9 and 12 h of incubation, the probiotic strains and the blend reached the highest cell density in GBR3. The only exception was represented by the *L. plantarum* IMC510 strain, which reached similar cell density in both GBR1 and GBR3 after 12 h of incubation (Fig. 10 panels A and B). Previous studies reported that the GBR supports the viability of lactobacilli in a dose-dependent manner. In particular, Dangmanee (2024), with the aim of producing a synbiotic dietary supplement, studied the prebiotic property of germinated Sang Yod brown rice powder. By testing three different concentrations of brown rice (1 %, 3 %, and 5 %), the authors showed that the concentration of 3 % better supported the viability of the *Lactobacillus pentosus* GP6 strain, up to 6 months of conservation under refrigerated conditions (Dangmanee, 2024). Similarly, Tayuan and collaborators (Tayuan et al., 2016), by evaluating the growth and viability of the *L. acidophilus* TISTR 450 and *L. plantarum* TISTR 875 probiotic strains in germinated Hang rice, showed that the rice powder at both 3 % and 5 %, supported the growth of *L. plantarum* TISTR 875 and its viability during the refrigerated storage period (22 days).

Concerning pH changes, as displayed in Fig. 11 (panels A and B), a constant decrease over time in all experimental conditions was observed, indicating active metabolism of the inoculated probiotic strains. It is well known that the fermentation of complex sugars by LABs determines the production of organic acids, mainly lactic acid, which is responsible for lowering the pH (de Mesquita et al., 2017). According to the results obtained, the *L. rhamnosus* CA15 and *L. fermentum* CS57 strains showed a more pronounced acidification in GBR3, reaching, after 12 h of fermentation, a pH value of 4.17 (Fig. 11 panel A). Similarly, the blend determined the highest acidification in GBR3, suggesting higher fermentative activity in the presence of the lowest concentration of GBR (20 %) (Fig. 11 panel B). This feature could be attributed to the lower concentration of fibrous matrix, which could improve the availability of fermentable sugars by accelerating the production of organic acids (Popova-Krumova et al., 2024).

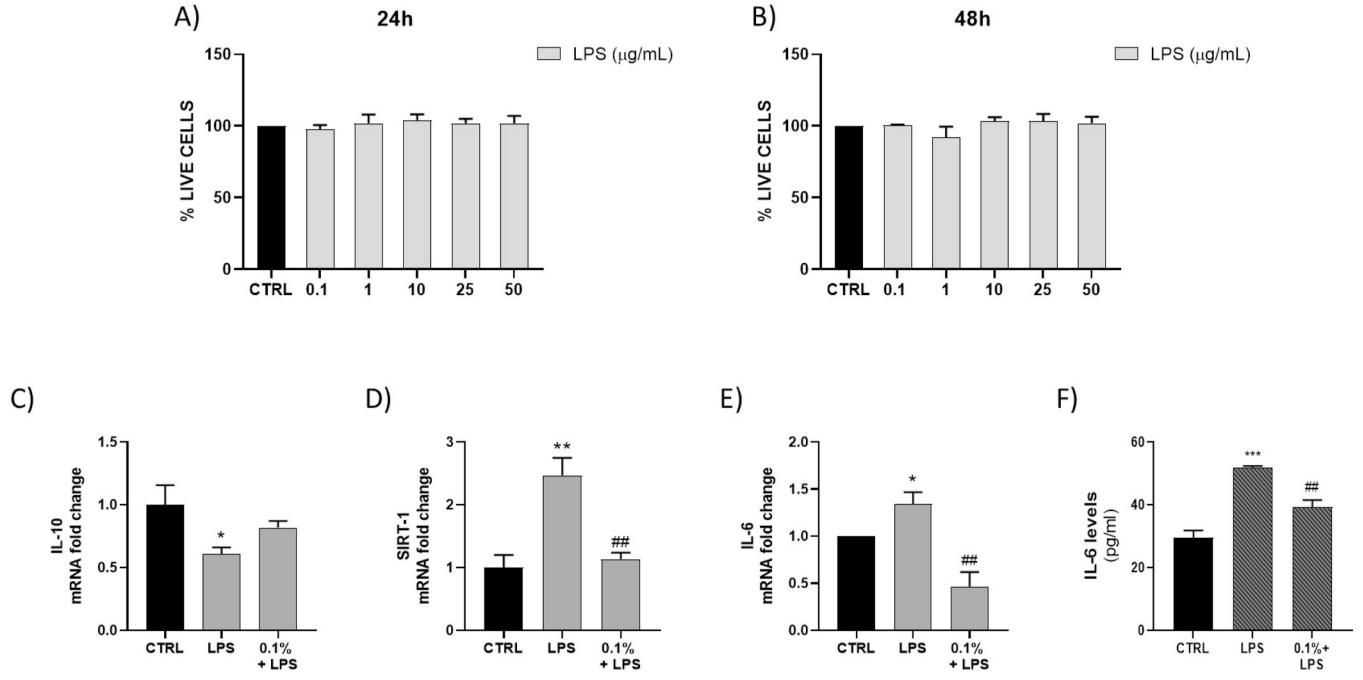

Fig. 9. Assessment of LPS effect on VK2/E6E7 cell viability after 24h (A) and 48h (B). Evaluation of IL-10, SIRT-1 and IL-6 gene expression levels (C–E). Expression levels of IL-6 in the CM (F). (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ vs CTRL; ##$p < 0.005$ vs LPS).

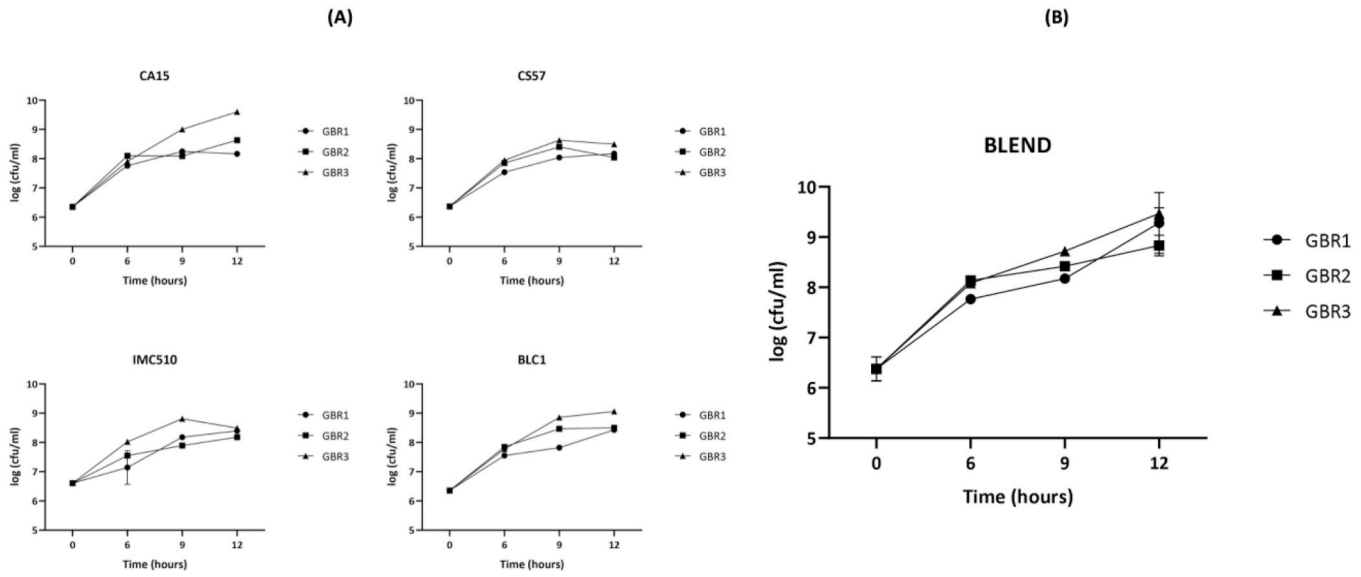

Fig. 10. Viability of *L. rhamnosus* CA15, *L. fermentum* CS57, *L. plantarum* IMC 510, and *B. animalis* subsp. *lactis* BLC1 probiotic strains, singularly (panel A) and as a blend (panel B), in experimental synbiotic products (GBR1, GBR2, and GBR3).
Data are reported as log cfu/ml after 0, 6, 9, and 12 h of fermentation in GBR1 (40 %), GBR2 (30 %) and GBR3 (20 %).

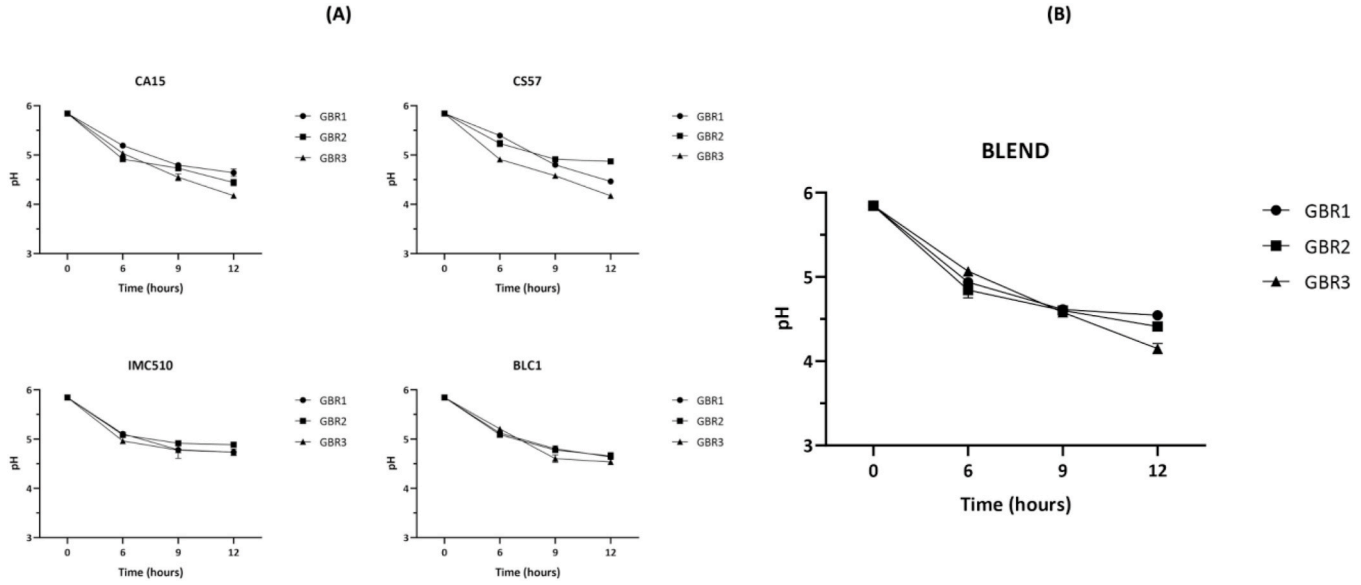

Fig. 11. Acidification profile during 12 h of incubation of experimental synbiotic products (GBR1, GBR2, and GBR3) inoculated with single probiotic strains (panel A) and with probiotic blend (panel B).

3.10. Antagonistic activity against pathogens

The antimicrobial activity against pathogens, exerted by the experimental synbiotic formulations (GBR1, GBR2, GBR3), inoculated with the tested probiotic strains, single and as a blend, are displayed in Fig. 12. Based on our knowledge, no previous studies evaluated the antimicrobial activity exerted by probiotics grown in GBR. The antimicrobial activity against pathogens is a key feature for probiotic selection, and, it is well established to be strain dependent. Our data confirmed a marked variability among both strains and target pathogens, revealing that the *L. rhamnosus* CA15 strain and the blend showed the broadest spectrum of antagonistic activity. The ability of the CA15 strain to counteract pathogens was previously reported by Pino and co-workers (Pino et al., 2022b). The authors observed that the tested strain was able to produce a high amount of lactic acid and, according to the absence of antimicrobial activity after CFS neutralization, hypothesized that the acid environment had a substantial action against the growth of the tested pathogens. In addition, the noticeable inhibitory activity shown by the blend, at almost all GBR concentrations, confirms a synergistic effect among the tested probiotic strains, resulting in improved antagonistic efficacy against several pathogens (Mathipa & Thantsha, 2015).

3.11. Cytocompatibility of Co-administration of GBR and blend in VK2/E6E7 cells

In order to assess a potential synergistic effect on cell proliferation between GBR and probiotic blend, *in vitro* co-treatments were administered. Preliminary evaluation of cytotoxicity was conducted for the probiotic blend alone at different concentrations (expressed as protein

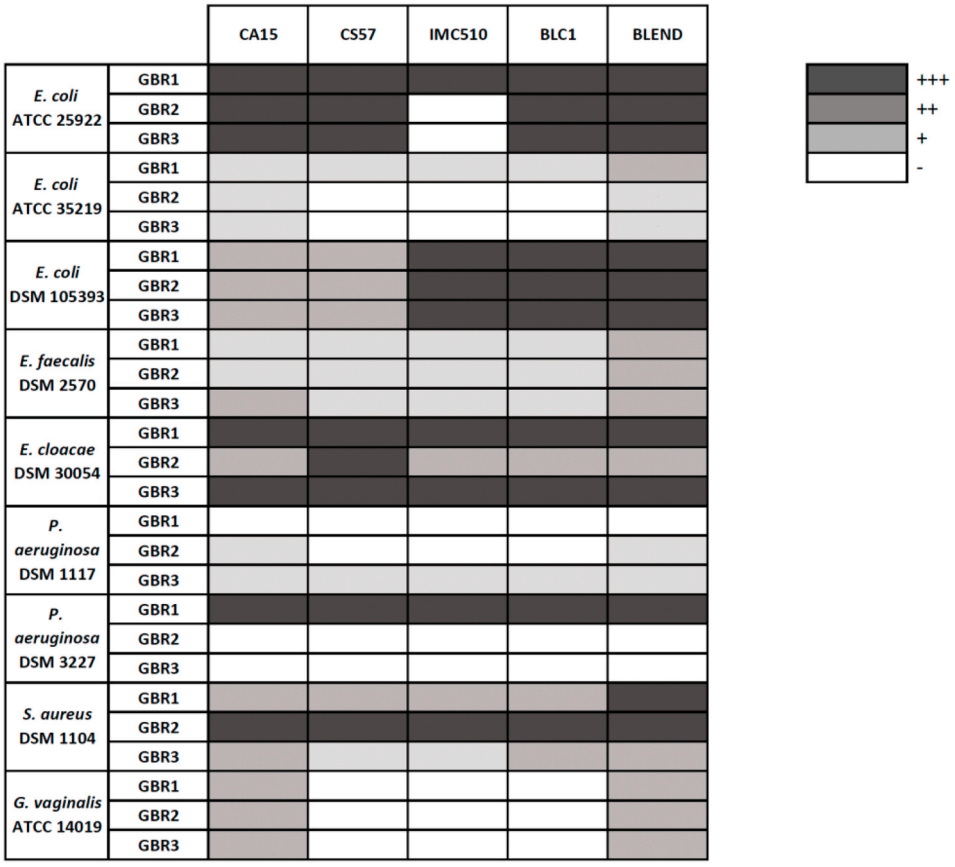

Fig. 12. Antimicrobial activity against pathogens.
Colors indicate the level of inhibition as follows: white, (−, no inhibition zone); light grey, (+, inhibition zone <10 mm); grey, (++, inhibition zone between 11 and 20 mm); dark grey (+++, inhibition zone >20 mm).

content in mg/mL) in VK2/E6E7 cells following 72h exposure. As shown in Fig. 13 (panel A), the highest concentrations resulted in a significant reduction of cell viability, thus only 0.1, 0.25, and 0.5 mg/mL were tested in combination with the previously selected concentration of GBR (0.1 %). MTT assay results showed a slight increase in cell proliferation only for the combination containing 0.25 mg/mL of the probiotic blend (Fig. 13, panel B). When the latter was combined with GBR, no enhanced proliferation, in terms of colony formation was detected (data not shown). Indeed, synbiotic formulation did not elicit a greater proliferative response than GBR (0.1 %) alone, suggesting the absence of a clear synergistic interaction under the present experimental conditions. This outcome may indicate that the effect on cell proliferation of GBR is possibly due to its content of bioactive compounds, such as γ-oryzanol, ferulic acid, and GABA, known to modulate cellular metabolism, antioxidant defense, and growth pathways.

GBR, owing to its rich composition of bioactive compounds emerges as a promising nutritional and phytotherapeutic intervention. In addition, as previously demonstrated, its potential to support viability of probiotics, which exert several mechanisms, including short-chain fatty acid production, immune signaling, and epithelial barrier reinforcement, and modulation of gut microbiota. Thus, while direct effects of probiotics on proliferation may not be additive, GBR and the probiotic blend could still act in concert to promote a general state of cellular and metabolic balance, supporting redox homeostasis, anti-inflammatory

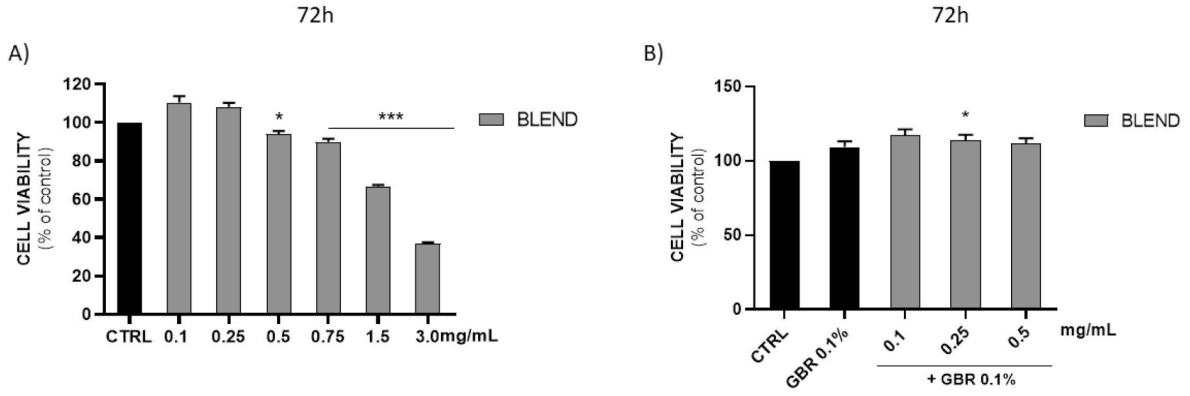

Fig. 13. Evaluation of blend effect on cell viability after 72h of treatment (A). Effect of co-administration of blend with selected GBR concentration after 72h of treatment (B). (*p < 0.05, ***p < 0.0005 vs CTRL).

regulation, and tissue integrity. However, it should be noted that this conclusion is limited to the specific concentrations, experimental model, and conditions tested in this study.

4. Conclusions

The synbiotic formulation, proposed in the present study, represents a novel and promising phytotherapeutic strategy, suggesting GBR as a valuable ally in promoting vaginal health and mitigating menopausal-associated dysbiosis, further corroborated by the positive potential effect of the selected probiotic strains. Nevertheless, in-depth studies would be required to determine whether a synergistic relationship could occur under alternative conditions, such as the potential enhancement of beneficial effects during functional food formulation or ingredients development, specifically for women's well-being.

CRediT authorship contribution statement

Georgiana Bosco: Writing – original draft, Formal analysis, Data curation. **Valeria Consoli:** Writing – original draft, Methodology, Formal analysis, Data curation. **Alessandra Pino:** Writing – review & editing, Visualization, Methodology, Data curation. **Amanda Vaccalluzzo:** Writing – review & editing, Formal analysis, Data curation. **Cinzia Caggia:** Writing – review & editing, Visualization. **Valeria Sorrenti:** Writing – review & editing, Validation, Supervision, Methodology. **Cinzia Lucia Randazzo:** Writing – review & editing, Supervision, Methodology, 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.

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Data availability

Data will be made available on request.

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