Innovative Food Science and Emerging Technologies 80 (2022) 103076

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

# Formulation of germinated brown rice fermented products functionalized by probiotics

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ARTICLE INFO

*Keywords:*
Fermentation
Probiotics
Bioactive compounds
Anti-inflammatory properties
Plant-based product

ABSTRACT

Germinated brown rice is considered a functional food in relation to the presence of beneficial nutrients and bioactive compounds in considerable amounts. The present study, evaluated, for the first time, the suitability of germinated brown rice for the production of value-added fermented products functionalized by the addition of probiotics. Four different fermented products with (EYGG, EYBB536, EYBB12, and EYM) and without (EY) probiotics addition were formulated. Microbiological and chemical profiles, presence of bioactive compounds, antioxidant activity, sensory attributes, and shelf-life during refrigerated storage were evaluated. Results showed that the fermentation process determined the improvement of both bioactive compound profile and anti-inflammatory properties of germinated brown rice. All the fermented products were microbiologically stable during refrigerated storage at +4 °C for 28 days. In addition, a high count of both lactobacilli and bifidobacteria was achieved during the shelf-life, indicating the suitability of the germinated brown rice as a probiotic carrier. Based on the sensory profile, high acceptability scores were attributed by panelists to the germinated brown rice experimental products. Based on the aforementioned results, germinated brown rice can be processed as a new fermented formulation with potential health benefits.

## 1. Introduction

The increasing demand for high-quality foods by health-conscious consumers has managed both the scientific community and the food industry to develop new products with enhanced nutritional and functional properties along with appreciable sensory quality (Guiné, Florença, Barroca, & Anjos, 2020). In addition, due to both lactose intolerance and allergies to milk proteins as well as to the spread of people who follow vegetarian and vegan diets, an increase in the demand for vegetable-based products was registered. In this context, cereals are considered a promising alternative to milk and milk-based foods. Among these, increasing attention was paid to brown rice which is characterized by a balanced and complete nutritional profile due to the presence of essential fatty acids, proteins, fibres, vitamins, antioxidants, and key phytochemicals (Beaulieu, Reed, Obando-Ulloa, Boue, & Cole, 2020; Gong et al., 2017). In particular, tocopherols, tocotrienols, oryzanol, B vitamins, phytosterols (β-sitosterol, campesterol, and stigmasterol), carotenoids, and beneficial phenolics confer health-promoting relevance to brown rice. The aforementioned compounds boast antioxidant properties, the ability to scavenge free radicals, aptitude to improve the immune system and reduce the risk of both heart disease and cancer development (Liu, 2007; Okarter, Liu, Sorrells, & Liu, 2010; Qureshi, Mo, Packer, & Peterson, 2000; Xu, Hua, & Godber, 2001).

The germination treatment is one of the main technologies applied to brown rice to improve textural and organoleptic qualities, flavor components, and phytochemical bioavailability (Cáceres, Martínez-Villaluenga, Amigo, & Frias, 2014). Several studies had shown that the

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https://doi.org/10.1016/j.ifset.2022.103076
Received 23 December 2021; Received in revised form 5 July 2022; Accepted 11 July 2022
Available online 16 July 2022

germination process allows to an increase in the level of specific bio-functional components, such as γ- aminobutyric acid (GABA), lysine, B-group vitamins, and several antioxidants (e.g. γ-oryzanol, vitamin E, and phenolic compounds) (Cho & Lim, 2016; Han, Arijaje, Jinn, Maur-omoustakos, & Wang, 2016; Kim et al., 2012; Shao & Bao, 2015; Wu, Yang, Toure, Jin, & Xu, 2013; Zhang et al., 2014). In particular, GABA exerts health-promoting properties, including the regulation of blood pressure; the stimulation of immune cells; the alleviation of pain, anxiety, and sleeplessness; the reduction of cancer cell proliferation; the prevention of diabetes, which were demonstrated in animal feeding studies (Ardiansyah et al., 2006; Esa, Abdul-Kadir, Amom, & Azlan, 2013; Imam, Azmi, Bhanger, Ismail, & Ismail, 2012; Jung, Kim, Hwang, & Ha, 2007). In addition, brown rice phenols, such as ferulic and caffeic acids, can exert chemopreventive action preventing the growth of human breast and colon cancer cells (Hudson, Dinh, Kokubun, Simmonds, & Gescher, 2000).

Along with the enhancement of health-promoting properties, the germination process makes brown rice suitable for the production of different food products (e.g. gluten-free noodles, beverages, cookies, and bread) (Cáceres, Peñas, Martínez-Villaluenga, García-Mora, & Frías, 2019; Wang et al., 2020; Wu, Yang, Chen, Jin, & Xu, 2011; Zhang, Liu, Wang, Liu, & Lan, 2019). In particular, brown rice beverages represent one of the main commercially available and their consumption is increasing worldwide (Mäkinen, Wanhalinna, Zannini, & Arendt, 2016; Paul, Kumar, Kumar, & Sharma, 2020). Up to now, several studies were focused on the evaluation of different methods exploitable to make brown rice beverages as well as on their effects on physicochemical parameters (Beaulieu, Reed, Obando-Ulloa, & McClung, 2020). Even though the fermentation process, like germination, can improve nutritional value, sensory and functional properties of cereals, such as germinated brown rice, scant is the literature related to the formulation of fermented brown rice products (Montemurro, Pontonio, Coda, and Rizzello (2021); Cáceres et al., 2019). According to that, the present study aimed to formulate fermented brown rice products with enhanced nutritional and health-promoting properties starting from two commercially available brown rice drinks. Both drinks were obtained through two different technological processes and were analysed from both physicochemical and microbiological points of view.

## 2. Materials and methods

### 2.1. Germinated brown rice

Two different germinated brown rice drinks (GBR1 and GBR2), kindly provided by Mr. Bio Food srl (Crespadoro, Italy) were used in the present study. In detail, GBR1 was produced by sprouting brown rice seeds in water at 25 ± 2 °C for 48 h. The sprouted brown rice grains were steamed at 121 °C for 15 min, to reduce the microbial load present in the composition, and then subjected to micronization in a colloidal mill, to obtain particles with an average size between 10 and 100 μm. The micronization process was carried out in presence of water to limit the overheating of nutrients and, therefore, their thermo-degradation and/or oxidation. The obtained composition was incubated for 1 h at 37 °C in presence of 2 g/Kg of alpha- and beta-amylase enzymes to transform the starch into malts and simple sugars. GBR2 was obtained following the process previously described with the exception of steamed and incubation with enzymes, which were not applied.

### 2.2. Probiotic strains and starter cultures

*Bifidobacterium longum* BB536 (ATCC BAA-999), *Bifidobacterium bifidum* Bb-12 (kindly provided by Chr. Hansen Holding A/S (Denmark), and *Lacticaseibacillus rhamnosus* GG (ATCC 52103) were used in the present study as probiotic strains. The commercially available starter cultures Cryofast SST 31 (*Streptococcus thermophilus*) and Lyofast SY 1 (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), kindly provided by Sacco System srl (Cadorago, Italy), were used as deep-frozen and freeze-dried cultures, respectively. In addition, the YoFlex® YF-L02 DA, a thermophilic lactic acid culture, kindly provided by Chr. Hansen Holding A/S (Denmark), was used as frozen pellet.

### 2.3. Chemical characterization of germinated brown rice

GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), were subjected to chemical characterization. In detail, moisture, protein, fat, fiber, and ash were determined following AOAC (2016) methods. Sugars were detected by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD). Carbohydrates were calculated by difference. Energy was determined using standardized conversion factors (4.0 kcal/g for proteins and carbohydrates, and 9.0 kcal/g for fats) (FAO, 2002). pH was measured using a digital pH-meter (MettlerDL25, Mettler-Toledo International Inc.). Each analysis was performed in triplicate.

### 2.4. Detection of bioactive compounds

All reference standards were purchased from Sigma-Aldrich unless otherwise indicated. They were stored at −20 °C and kept in the dark until use. The samples were collected and stored at −20 °C until mass analysis. Selected compounds were: Butyric acid, Phytic acid, Oryzanol, GABA, Inositol, Lactic acid. Samples were prepared according to the protocol reported by Cáceres et al. (2019), Cáceres, Peñas, Martínez-Villaluenga, Amigo, and Frias (2017)). The active component was identified by Perkin Elmer – ABSciex ultra liquid chromatography-mass spectrometry (UPLC-Ms/Ms) system. The column used were an Phenomenex Luna 3 μm $NH_2$ 100A, LC Column 100 × 2 mm and an Phenomenex Luna C18, 5 μm, 15 × 0.1 cm. ESI-Ms/Ms. analysis was performed in negative and positive mode to find the best operative conditions. Negative polarity was used in full scan mode and the mass range was set at 100–1500 Da. The condition of UPLC were as follows: from 0 to 2 min at 60:40 *v*/v; from 2 to 30 min acetonitrile was changed to 100% linearly; from 30 to 40 min the system was reconnected to the initial conditions. A total volume of 10 μl was injected. The mass spectrometry settings are drying gas (N2) flow rate (10 l/min); Curtain gas 30; IonSpray Voltage (IS) ±4500; Ion Source Gas1 (GS1) 30.0; Ion Source Gas2 (GS2) 60.0 and Interface Heater ON; Declustering Potential (DP) ±50.0; Focusing Potential (FP) ±400.0 and Entrance Potential (EP) ±10.0.2.5.

### 2.5. Microbiological analysis of germinated brown rice

Germinated brown rice GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), were subjected to microbiological analysis. In detail, total mesophilic bacteria, lactic acid bacteria, lactococci, yeasts, Enterobacteria, faecal coliforms, staphylococci, *Escherichia coli*, and *Listeria* spp. counts were performed according to the method and using the culture media and conditions reported by Randazzo et al. (2021). In addition, Brilliance Salmonella agar (BSA), supplemented with Salmonella selective supplement, incubated at 37 °C for 24 ± 3 h, was used for *Salmonella* spp. count according to ISO 6579:2002 + A1:2007 standard. All media were purchased from Oxoid (Italy). Analyses were performed in triplicate and results were expressed as mean $\log_{10}$ cfu/ml and standard deviation.

### 2.6. Isolation and identification of autochthonous lactic acid bacteria

In order to in-depth study the autochthonous lactic acid population of GBR2 samples, 20% of the total number of colonies recovered on MRS and M17 agar plates were randomly selected. Isolates were purified by streaking three times and then subjected to microscopic observation, catalase, and Gram reaction before storing at −20 °C in liquid culture containing 20% of glycerol (*v*/v). Overall, 90 isolates were obtained and

80 of them were found gram-positive and catalase-negative. Isolates and reference strains, listed in Supplementary Table 1, were subjected to total DNA extraction following the protocol described by Pino et al. (2019) and Randazzo et al. (2015). DNA concentration was assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). The LAB isolates were clustered by PCR-RFLP analysis according to Pino et al. (2018). One representative member of each PCR-RFLP cluster was subjected to 16S rRNA gene sequencing. The identification at the species level was done by comparing the obtained sequences with known 16S rRNA gene sequences in the NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov) database and those of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) by using the BLAST algorithm. Sequences with a percentage identity of 98% or higher were considered to belong to the same species.

### 2.7. Abilities of starter cultures to acidify and coagulate germinated brown rice

To test acidifying activity, a 50% (v:v) mixture of both GBR1 and GBR2 was inoculated with YoFlex or with SST 31 and SY 1 starter cultures in a 1:1, 1:2, or 2:1 ratio and incubated at 42 °C. The pH changes were monitored at regular intervals up to 14 h using a pH meter (H19017, Microprocessor, Hanna Instruments, Ronchi di Villafranca, Padovana, Italy). Coagulation was visually evaluated till 8 h of incubation.

### 2.8. Manufacture of experimental fermented products

Based on previous results, the experimental fermented products were formulated using a 50%(*v*/v) mixture of GBR1 and GBR2 samples inoculated with the starter cultures SST 31 and SY 1 in a 1:1 ratio. After fermentation at 42 °C for 12 h, probiotic strains were inoculated at a final cell density of 9 log cfu/ml. Overall, five different fermentation batches were obtained namely: EYGG inoculated with *Lacticaseibacillus rhamnosus* GG (ATCC 52103); EYBB536 inoculated with *Bifidobacterium longum* BB536 *(*ATCC BAA-999*)*; EYBB12 inoculated with *Bifidobacterium animalis* subsp. *lactis* BB-12; EYM inoculated with a mixture of the aforementioned probiotic strains; and EY un-inoculated with probiotics. Each experimental fermentation was performed in triplicate.

### 2.9. Microbiological stability of the experimental fermented products during storage

Microbial stability and *shelf-life* of the experimental fermented products EY, EYGG, EYBB536, EYBB12, and EYM, stored at +4 °C (±1 °C), were weekly monitored for 28 days. Cell densities of yeasts and moulds, lactococci, Enterobacteria, faecal coliforms, staphilococci*, E. coli, Listeria* spp.*,* and *Salmonella* spp., were determined as previously described (Randazzo et al., 2021). In addition, BSM Agar (Oxoid, Italy), incubated at 37 °C for 48 h under anaerobic conditions, was used for bifidobacteria enumeration whereas lactobacilli were cultured on Rogosa Agar (Oxoid, Italy) incubated at 35 °C for 48-72 h under aerobic conditions.

### 2.10. Chemical and bioactive compounds analysis of fermented products

The EY, EYGG, EYBB536, EYBB12, and EYM fermented products were subjected to moisture, protein, fat, fiber, ash, and carbohydrates determination as described before. In addition, the profile of the bioactive compounds (GABA, butirric acid, phytic acid, inositol, and oryzanol) was investigated as previously described.

### 2.11. Anti-inflammatory properties

Human colon immortalized cell lines (Caco-2) were used to investigate the anti-inflammatory properties of EY, EYGG, EYBB536, EYBB12, and EYM fermented products. The cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). CaCo-2 cells were cultured in either 75 or 150 $cm^2$ flasks, in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (pen/strep), and 2 mM L-glutamine. Cells were cultured at 37 °C and 5% $CO_2$. To simulate the inflammation, the cells were further incubated with or without Lipopolysaccharides (LPS) at the concentration of 100 ng/ml for 2 h with the same medium (DMEM, 10% FBS) 34,308,769–34,269,367. Further, we treated the cells also with 10% of EY, EYGG, EYBB536, EYBB12, and EYM fermented products to test their effects. Cells were incubated for 6 h as described above. The inflammatory response and anti-inflammatory activity of the fermented products were determined by measuring the expression levels of inflammatory markers IL-1β, IL-6, and TNFα, through real-time PCR gene expression. In particular, after incubation, cells were collected by trypsinization, washed once with PBS (Phosphate-Buffered Saline), and then lysed for RNA extraction.

The quantitative analysis was performed with One-Step Fast Real-Time PCR System Applied Biosystem using the SYBR Green PCR master mix (Life Technology, Milan, Italy. The primer sequences used are shown in Supplementary Table 2. The mix for PCR analyses included previously synthesized cDNA, SYBR Green PCR master mix (Life Technology, Milan, Italy), primer mix (forward primer/reverse primer), and UltraPureTM Distilled Water DNase/RNase Free (Invitrogen by Life Technologies, Milan, Italy). PCR reactions were subjected to 40 cycles of 95 °C for 20s, 95 °C for 3 s, and 60 °C for 30s. The relative mRNA expression levels of each gene were determined by the threshold cycle (Ct) value of each PCR product and normalized with GAPDH (Glyceraldeyde-3-phosphate dehydrogenase) by using comparative 2-DDCt method. The analysis was performed in duplicates.

### 2.12. Sensory evaluation of fermented products

The experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM) were subjected to sensory evaluation by a panel of 15 judges (7 females and 8 males, aged between 24 and 40 years) recruited among the staff of Council for Agricultural Research and Economics, Research Centre for Olive, Fruit and Citrus Crops, (CREA-OFA) Acireale, Italy. For selection and training "general guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors" was used according to UNI EN ISO 8586:, 2014 rule. Judges were pre-screened based on (i) regular consumption of yogurt and non-dairy fermented products (at least once per month), and (ii) absence of taste/smell disorders. In addition, all panelists were informed about the possible presence of allergens (cereals containing gluten, peanuts, milk/dairy products, eggs, nuts, sesame, sulphites, and soybeans) in the experimental fermented sample. Fermented sample attributes were generated in consensus across eight 2-h sessions during which judges tasted an array of yogurts, fermented milk, and kefir. The sensory profile method (UNI EN ISO 13299:, 2016) was used for the sensory analysis. The standard UNI EN ISO provides guidelines on the global process of developing a sensory profile. Panelists were asked to evaluate the following descriptors: astringency, bitterness, sweetness, sourness, cereal-type flavor, fermented odour, creaminess, white color, off-odour, off-flavor, and overall acceptability, using a 10-point scale (ISO 4121:, 2003), ranging from 1 (absence of the sensation or extremely dislike) to 10 (extremely intense or extremely pleasant). All evaluations were carried out in the morning, at the sensory laboratory of the CREA-OFA conforming to the UNI EN ISO 8589:, 2014 standard. The order of presentation was randomized among judges and sessions. Data were acquired by Smart Sensory box a direct computerized registration system (Smart Sensory Solution, Sassari, Italy).

### 2.13. Statistical analysis

Microbiological and chemical data were analysed by ANOVA (One way-Analysis of Variance) followed by Tukey's post-hoc test, in order to assess the overall differences between germinated brown rice samples (GBR1 and GBR2) and among experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM). Statistical analysis was performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA) and the reference level of significance was 0.05 in all the assays.

## 3. Results

### 3.1. Chemical composition and bioactive compounds detected in germinated brown rice samples

The mean value of pH, proximate composition, and bioactive compounds profile of germinated brown rice samples GBR1 and GBR2, obtained from three different germination batches (a, b, and c), are reported in Table 1. No statistically significant differences were recorded among the same type of sample (GBR1 or GBR2) derived from different germination batches (a, b, and c) (data not shown). The technological process had a negligible impact on fat, fiber, and ash content; GBR2 samples exhibited higher moisture and protein content than GBR1 samples, whereas carbohydrates and sugar were detected at higher concentrations in GBR1 samples (Table 1). Regarding bio-active compounds profile, GABA, butyiric acid, phytic acid, and inositol were higher in GBR 2 than in GBR 1 samples, whereas oryzanol was detected at higher concentrations in GBR 1 samples.

### 3.2. Microbiological analysis of germinated brown rice samples

The mean values (expressed as $\log_{10}$ cfu/ml) and standard deviations of the main microbial groups detected in GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), are shown in Supplementary Table 3. Overall, yeasts and moulds, Enterobacteria, faecal coliforms, staphilococci, *E. coli*, *Listeria* spp., and *Salmonella* spp. were never detected in the analysed samples. In addition, within each GBR sample, no statistically significant differences were detected among the analysed germination batches (a, b, and c). Mesophilic aerobic bacteria were detected in all the analysed samples and the highest count was registered in GBR2 samples. Lactic acid bacteria and lactococci were found only in GBR2 samples with cell densities of about 5 log units and 2 log units, respectively (Supplementary Table 3).

Data are reported as mean value and standard deviation of three determinations. Between germinated brown rice samples and among experimental fermented samples, different lowercase letters (a–b) in the same row, indicate a significant difference at $p < 0.05$ (ANOVA with Tukey's post-hoc test), respectively.

### 3.3. Isolation and identification of autochthonous lactic acid bacteria

Eighty isolates, obtained from MRS and M17 agar plates, were considered LAB based on positive Gram reaction, nonmotility, absence of catalase activity and spore formation, and rod or coccal shape (Supplementary Table 4). The PCR-RFLP profiles of LAB isolates, in comparison to those obtained from reference strains, allowed to discriminate five clusters and to ascribe the isolates to the following species: *Lacticaseibacillus paracasei* (31), *Lacticaseibacillus rhamnosus* (26), *Limosilactobacillus fermentum* (11), *Lactococcus lactis* (7), and *Pediococcus pentosaceus* (5) (Supplementary Table 4). To confirm the species attribution, one representative member of each PCR-RFLP cluster was subjected to 16S rRNA gene sequencing. Details about Query cover (%), *E*-value, Identity (%), and Accession number of the sequenced strains (GBR 41, GBR 59, GBR 77, GBR 48, and GBR 7) are reported in Supplementary Table 4.

### 3.4. Acidifying and coagulation abilities of starter cultures

Fig. 1 shows the acidifying activity exhibited by both SST 31-SY 1 (inoculated in a 1:1, 1:2, and 2:1 ratio) and YoFlex starters. In detail, the YoFlex starter determined low acidification of the product. SST 31 and SY 1, combined in a 1:2 and 2:1 ratio, showed similar behavior determining a drop in pH value of 1.23 and 1.28, respectively. Differently, when SST 31 and SY 1 were combined in a 1:1 ratio, the starters showed higher acidifying activity reaching a pH of 4.38 after both 12 and 14 h of incubation (Fig. 1). Based on the visual evaluation of clot formation, no differences were observed about the coagulation ability exhibited by the SST 31 and SY 1 starters at 1:1, 1:2, and 2:1 ratio whereas YoFlex determined a poor clot formation.

**Table 1**
pH, proximate composition (g/100 ml), energy (Kcal/100 g), and bioactive compounds (μg/ml) of geminated brown rice GBR1 and GBR2 samples, obtained from three different germination batches (a, b, and c), and of experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM).

| | Germinated brown rice samples | | Fermented products | | | | |
|---|---|---|---|---|---|---|---|
| | GBR1 | GBR2 | EY† | EYGG† | EYBB536† | EYBB12† | EYM† |
| pH | $6.41^{a} \pm 0.11$ | $6.32^{a} \pm 0.11$ | $4.38^{a} \pm 0.05$ | $4.35^{a} \pm 0.07$ | $4.32^{a} \pm 0.04$ | $4.33^{a} \pm 0.03$ | $4.35^{a} \pm 0.05$ |
| Proximate composition (g/100 ml) | | | | | | | |
| Moisture | $75.25^{b} \pm 0.27$ | $81.22^{a} \pm 0.28$ | $78.91^{a} \pm 0.32$ | $77.44^{a} \pm 0.31$ | $77.93^{a} \pm 0.26$ | $78.11^{a} \pm 0.28$ | $78.83^{a} \pm 0.21$ |
| Protein | $1.81^{b} \pm 0.13$ | $2.23^{a} \pm 0.14$ | $1.64^{a} \pm 0.14$ | $1.61^{a} \pm 0.11$ | $1.68^{a} \pm 0.18$ | $1.65^{a} \pm 0.21$ | $1.64^{a} \pm 0.14$ |
| Fat | $0.75^{a} \pm 0.09$ | $0.78^{a} \pm 0.13$ | $0.58^{a} \pm 0.07$ | $0.64^{a} \pm 0.07$ | $0.63^{a} \pm 0.13$ | $0.69^{a} \pm 0.20$ | $0.66^{a} \pm 0.15$ |
| Fiber | $0.64^{a} \pm 0.24$ | $0.71^{a} \pm 0.28$ | $0.72^{a} \pm 0.34$ | $0.75^{a} \pm 0.20$ | $0.76^{a} \pm 0.12$ | $0.78^{a} \pm 0.26$ | $0.70^{a} \pm 0.18$ |
| Ash | $0.35^{a} \pm 0.06$ | $0.36^{a} \pm 0.05$ | $0.33^{a} \pm 0.02$ | $0.32^{a} \pm 0.18$ | $0.35^{a} \pm 0.09$ | $0.38^{a} \pm 0.08$ | $0.31^{a} \pm 0.09$ |
| Carbohydrates | $20.42^{a} \pm 0.41$ | $14.79^{b} \pm 0.38$ | $17.82^{a} \pm 0.49$ | $17.86^{a} \pm 0.26$ | $17.73^{a} \pm 0.24$ | $17.80^{a} \pm 0.26$ | $14.78^{a} \pm 0.21$ |
| Sugar | $12.39^{a} \pm 1.21$ | $0.53^{b} \pm 0.11$ | $7.57^{a} \pm 0.70$ | $7.43^{a} \pm 1.11$ | $7.49^{a} \pm 1.03$ | $7.52^{b} \pm 0.18$ | $7.46^{a} \pm 0.14$ |
| Energy (Kcal) | $95^{a} \pm 1.49$ | $51^{b} \pm 1.41$ | $104^{a} \pm 1.05$ | $106^{a} \pm 0.93$ | $106^{a} \pm 1.09$ | $109^{a} \pm 1.00$ | $104^{a} \pm 1.11$ |
| Bioactive compounds (μg/ml) | | | | | | | |
| GABA | $178.71^{b} \pm 4.47$ | $227^{a} \pm 4.80$ | $359^{a} \pm 8.0$ | $35.90^{e} \pm 1.5$ | $63.90^{d} \pm 1.6$ | $112^{c} \pm 0.3$ | $149^{b} \pm 0.7$ |
| Butyric acid | $27.04^{b} \pm 2.10$ | $94.10^{a} \pm 1.53$ | $5^{b} \pm 1.3$ | nd | nd | $9.49^{a} \pm 1.3$ | nd |
| Phytic acid | $0.83^{b} \pm 1.67$ | $73.70^{a} \pm 1.77$ | nd | nd | nd | nd | $1.76 \pm 1.6$ |
| Inositol | $27.29^{b} \pm 2.03$ | $58.51^{a} \pm 2.23$ | $18.1^{c} \pm 1.9$ | $18.1^{c} \pm 1.3$ | $12.3^{d} \pm 1.4$ | $22.9^{b} \pm 0.9$ | $31.5^{a} \pm 0.8$ |
| Oryzanol | $44.80^{a} \pm 1.65$ | $39.01^{b} \pm 1.60$ | $96.3^{a} \pm 1.2$ | $97.3^{a} \pm 1.6$ | $90.1^{b} \pm 1.1$ | $15.6^{d} \pm 2.3$ | $53.8^{c} \pm 1.3$ |

† EY: un-inoculated with probiotics; EYGG: inoculated with *Lacticaseibacillus rhamnosus* GG (ATCC 52103); EYBB536: inoculated with *Bifidobacterium longum* BB536 (ATCC BAA-999); EYBB12: inoculated with *Bifidobacterium animalis* subsp. *lactis* BB-12; EYM: inoculated with a mixture of the aforementioned probiotic strains. nd: below the detection limit.

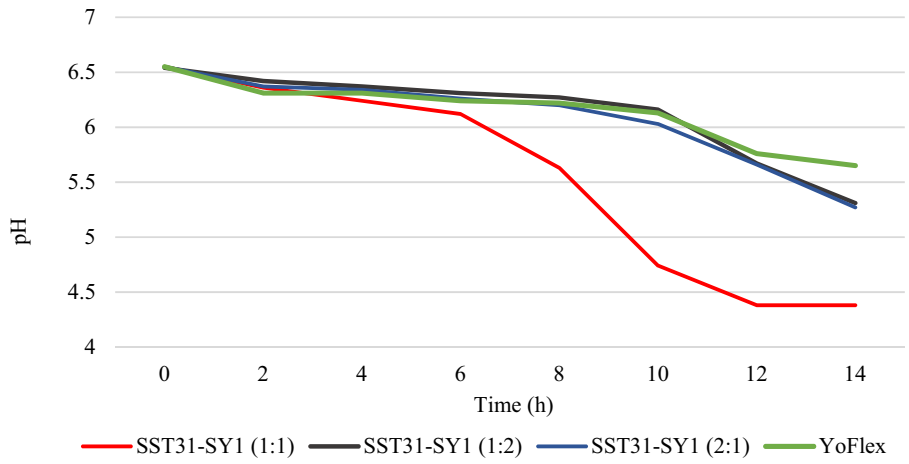


**Fig. 1.** Acidification profile during fermentation of germined brown rice at 42 °C by YoFlex and SST 31-SY 1 starters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### *3.5. Microbiological analysis of experimental fermented products during storage*

The microbiological stability of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM), stored at +4 °C, as well as the viability of the adjunct probiotic strains, were weekly monitored for 28 days and results are reported in Table 2. Overall, yeasts and moulds, Enterobacteria and faecal coliforms, staphylococci, *E. coli*, *Listeria* spp., and *Salmonella* spp. were never detected in all the analysed samples. Total mesophilic bacteria and lactococci decreased during the storage time reaching, in the fourth week, values of about 6 log units and 2 log units, respectively. A high count of lactobacilli and bifidobacteria was achieved, till the fourth week of storage under refrigerated conditions, in experimental fermented products inoculated with probiotics postulating their ability to survive during the shelf-life of the products.

### *3.6. Chemical composition and bioactive compounds profile of experimental fermented products*

Table 1 shows both the chemical composition and bioactive compounds profile of experimental fermented products EY, EYGG, EYBB536, EYBB12, and EYM. Overall, all the analysed samples showed similar proximate composition.

Regarding bioactive compounds profile, EY samples showed the highest concentration of both GABA and Oryzanol, whereas inositol was mainly detected in the EYM sample. Butyric acid was detected in both EY and EYBB12 samples, whereas the presence of phytic acid was revealed only in EYM sample.

### *3.7. Sensory evaluation of fermented products*

Fig. 2 shows the sensory profiles of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM) defined by fifteen trained judges. Sensory acceptability was high for all evaluated products. The sensory descriptors bitterness, fermented odour, and creaminess obtained similar scores in all samples. Astringency and sourness were mainly perceived in the EY sample whereas cereal-type flavor, white color, and sweetness descriptors were equally scored in EYGG, EYBB536, and EYBB12 samples. Samples EYGG and EYBB12 had the same ratings of sensory descriptors therefore their profiles overlapped. Off-flavor and off-odour were not perceived in any experimental fermented product.

### *3.8. Anti-inflammatory properties*

A human colon cell line, treated by LPS, was used as in vitro model to evaluate the anti-inflammatory activity of fermented products (EY, EYGG, EYBB536, EYBB12, and EYM). Fig. 3 shows that all fermented

**Table 2**
Microbiological stability of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM) during storage at +4 °C for 28 days.

| Micorbial groups | Time (Weeks) | Experimental fermented products | | | | |
|---|---|---|---|---|---|---|
| | | EY[†] | EYGG[†] | EYBB536[†] | EYBB12[†] | EYM[†] |
| Mesophilic aerobic bacteria | 1 | $7.15^{a} \pm 0.08$ | $7.28^{a} \pm 0.05$ | $7.17^{a} \pm 0.10$ | $7.23^{a} \pm 0.07$ | $7.21^{a} \pm 0.12$ |
| | 2 | $7.03^{a} \pm 0.10$ | $7.11^{b} \pm 0.08$ | $7.07^{a} \pm 0.06$ | $7.14^{a} \pm 0.02$ | $7.09^{b} \pm 0.04$ |
| | 3 | $6.74^{b} \pm 0.05$ | $6.81^{c} \pm 0.06$ | $6.83^{b} \pm 0.08$ | $6.82^{b} \pm 0.05$ | $6.78^{c} \pm 0.06$ |
| | 4 | $6.51^{c} \pm 0.03$ | $6.65^{d} \pm 0.09$ | $6.59^{c} \pm 0.09$ | $6.55^{c} \pm 0.10$ | $6.63^{c} \pm 0.06$ |
| Lactobacilli | 1 | $6.96^{a} \pm 0.06$ | $9.94^{a} \pm 0.08$ | $6.85^{a} \pm 0.05$ | $6.76^{a} \pm 0.05$ | $9.85^{a} \pm 0.07$ |
| | 2 | $6.52^{b} \pm 0.08$ | $9.46^{b} \pm 0.10$ | $6.63^{b} \pm 0.03$ | $6.78^{a} \pm 0.12$ | $9.33^{b} \pm 0.05$ |
| | 3 | $6.21^{c} \pm 0.03$ | $9.34^{c} \pm 0.08$ | $6.27^{c} \pm 0.06$ | $6.64^{b} \pm 0.09$ | $9.21^{bc} \pm 0.03$ |
| | 4 | $6.03^{d} \pm 0.05$ | $9.11^{d} \pm 0.06$ | $6.08^{d} \pm 0.08$ | $6.51^{b} \pm 0.06$ | $9.14^{c} \pm 0.11$ |
| Bifidobacteria | 1 | nd | nd | $9.85^{a} \pm 0.05$ | $9.76^{a} \pm 0.05$ | $9.77^{a} \pm 0.10$ |
| | 2 | nd | nd | $9.63^{b} \pm 0.03$ | $9.78^{a} \pm 0.12$ | $9.19^{b} \pm 0.05$ |
| | 3 | nd | nd | $9.27^{c} \pm 0.06$ | $9.64^{b} \pm 0.09$ | $9.11^{bc} \pm 0.03$ |
| | 4 | nd | nd | $9.08^{d} \pm 0.08$ | $9.51^{b} \pm 0.06$ | $9.00^{c} \pm 0.06$ |

nd: below the detection limit.
Data are reported as mean log value and stardard deviation of three determinations. For each microbial group different lowercase letters (a–b) in the same column, indicate a significant difference among samples at $p < 0.05$ (ANOVA with Tukey's post-hoc test).
[†] EY: un-inoculated with probiotics; EYGG: inoculated with *Lacticaseibacillus rhamnosus* GG (ATCC 52103); EYBB536: inoculated with *Bifidobacterium longum* BB536 (ATCC BAA-999); EYBB12: inoculated with *Bifidobacterium animalis* subsp. *lactis* BB-12; EYM: inoculated with a mixture of the aforementioned probiotic strains.

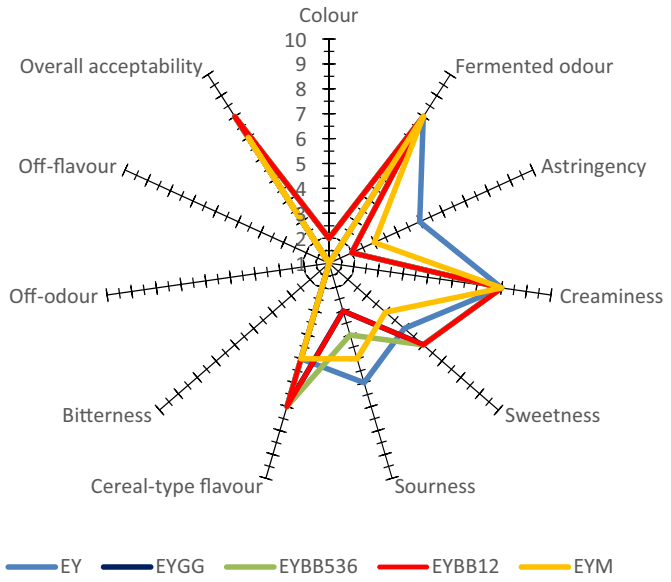


**Fig. 2.** Sensory Profile of the experimental fermented products (EY, EYGG, EYBB536, EYBB12, and EYM).

products did not induce significant changes in pro-inflammatory cytokines IL-1β, IL-6, and TNFα gene expression. Fig. 4 shows that, in cells treated with LPS, there was an increase in pro-inflammatory genes compared to the control group. The co-treatment with EY, EYGG, EYBB12, and EYM inverted these harmful outcomes downregulating genes expression at values close to control. Differently, the EYBB536 sample showed an increase in TNFα gene expression.

## 4. Discussion

In recent years, plant-derived foods and drinks, have gained more attention as valuable alternatives to animal-derived products. Consumer lifestyle choices and diet-related diseases (cardiovascular disease, elevated blood pressure, lactose intolerance, allergy to milk proteins) have led to higher demand, by both consumers and the food industry, for plant-based products (Montemurro et al., 2021). In addition, these products are considered an economic and biotechnological choice to increase nutritional and functional features, along with the technological and sensory properties of non-dairy products (Coda, Montemurro, & Rizzello, 2017; FONA International, 2018).

In the present study germinated brown rice (GBR) fermented product functionalized with the addition of probiotics was formulated. GBR boasts peculiar nutritional properties thanks to the presence of bioactive molecules able to exert nutrigenomic effect. In particular, blood pressure and type II diabetes regulation, as well as the reduction in the risk of some chronic conditions, such as cancer, cardiovascular, and Alzheimer's diseases, have been demonstrated (Ravichanthiran et al., 2018; Wu et al., 2013). Although, GBR is considered an interesting substrate, up to now few studies have been conducted to formulate novel GBR besed-foods, such as bread (Cornejo, Caceres, Martínez-Villaluenga, Rosell, & Frias, 2015), cookies (Chung, Cho, & Lim, 2014) and noodles (Gong et al., 2017). Only recently, Cáceres et al. (2019) investigated the suitability of different brown rice (BR) derivates, such as BR flour, socked and germinated BR, for the formulation of a yogurt-like product. However, to the best of our knowledge, the suitability of GBR for the formulation of yogurt-like brown rice products, functionalized with the addition of probiotics, has not been yet explored.

It is well known that the germination process enhances both the concentration and bio-availability of nutrients including γ-aminobutyric acid (GABA), lysine, vitamins, dietary fiber, niacin, magnesium, zinc, potassium, inositol, ferulic and phytic acids, tocotrienols, γ-oryzanol, and prolyl-endopeptidase inhibitor (Kim et al., 2020; Patil & Khan, 2011; Ravichanthiran et al., 2018). As well documented, the amount of bioactive compounds, accumulated during the germination process, vary greatly based on several factors such as pH, temperature, and time during soaking, germination, and post-germination (Cáceres et al., 2014; Watchararparpaiboon, Laohakunjit, & Kerdchoechuen, 2010). According to that, the highest amount of GABA, butyric acid, and inositol detected in GBR2 samples is probably due to the germination process applied.

GBR is a food matrix rich in several nutrients that can be used as growth substrates by starters. In addition, its suitability for fermentation is related to the presence of both carbohydrates and sugar. In the present study, GBR1 samples exhibited higher concentration of both carbohydrate and sugar than GBR2 ones which could be related to the germination processes. In fact, alpha- and beta-amylase enzymes, used for the production of GBR1 samples, are involved in the transformation of starch into malts and simple sugars, determining an increase in both carbohydrates and sugar content. According to the fermentability of GBR, although differences among the tested starter cultures were observed, the mixture of the SST31 and SY1 strains showed an optimal acidification rate, in line with previously reported data (Cáceres et al., 2019; Chekdid et al., 2021; Nionelli et al., 2014). In fact, when the strains SST31 and SY1 were used in a 1:1 ratio, a fast drop in pH was detected. This was probably due to their rapid metabolism, the nature of the substrates consumed, and the incubation temperature. In addition, in spite of the low amount of proteins, a good coagulum was visualized without the need for structuring agents and emulsifier additions. The optimal texture, of the fermented products formulated in the present study, could be attributed to the ability of starters to synthesise exopolysaccharides (EPS), which was confirmed by the creaminess descriptor and acceptability by panelists. It is well known that EPS improve rheological properties and enhance sensory and mouth-feel characteristics of fermented products (Ripari, 2019). No off-flavours and off-odours were perceived by panelists and very low scores were attributed to bitterness and sourness, which are related to the possible occurrence of lipid oxidation during the germination process (Kince et al., 2017). Interestingly, in discordance to previously reported data high perception of the fermented odour was revealed by pannelists (Cáceres et al., 2019; Chekdid et al., 2021).

In the present study, LAB strains were isolated from GBR samples and mainly genotypically ascribed to *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Limosilactobacillus fermentum*, *Lactococcus lactis*, and *Pediococcus pentosaceus* species. The dominance of these species in plant-derived products is in accordance with other studies (Ziarno & Cichońska, 2021). The isolated strains were also characterized for technological features in order to formulate a good candidate as a starter culture.

Based on the bioactive compounds profile of the formulated yogurt-like products, the present study highlighted that the fermentation process improved the nutritional value of GBR by increasing both GABA and oryzanol and by neutralizing the anti-nutritional phytic acid. In addition, both GABA and oryzanol contents increased in samples functionalized by the addition of probiotics indicating that GABA accumulation was initiated in the germination process and continued thought both the fermentation and the *shelf-life* of the product, according to previous studies (Cáceres et al., 2014; Cáceres et al., 2019; Sen, Tewu, Lijun, & Shanbai, 2008).

Notoriously, GABA, a bio-functional substance with several health-beneficial properties, is produced during the germination of brown rice (Cho & Lim, 2016; Ravichanthiran et al., 2018). The glutamate decarboxylase or the diamine oxidase of polyamines are responsible for GABA formation (Khwanchai, Chinprahast, Pichyangkura, & Chaiwanichsiri, 2014; Yang, Chen, & Gu, 2011). Although the GABA content change in relation to both rice variety and germination process parameters (e.g. pH, temperature, and time of soaking), the present study revealed higher GABA levels in both non-fermented and fermented

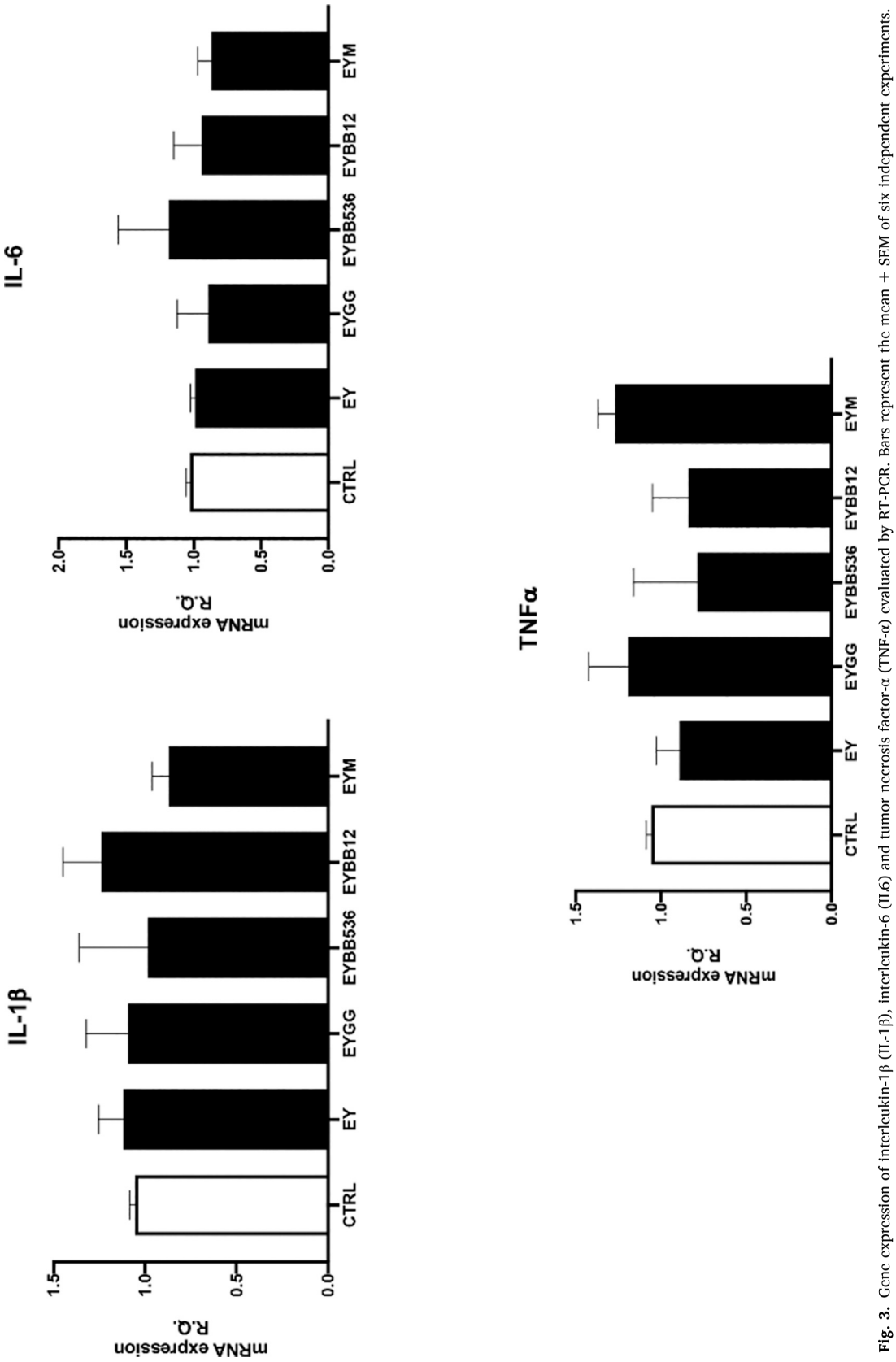


**Fig. 3.** Gene expression of interleukin-1β (IL-1β), interleukin-6 (IL6) and tumor necrosis factor-α (TNF-α) evaluated by RT-PCR. Bars represent the mean ± SEM of six independent experiments.

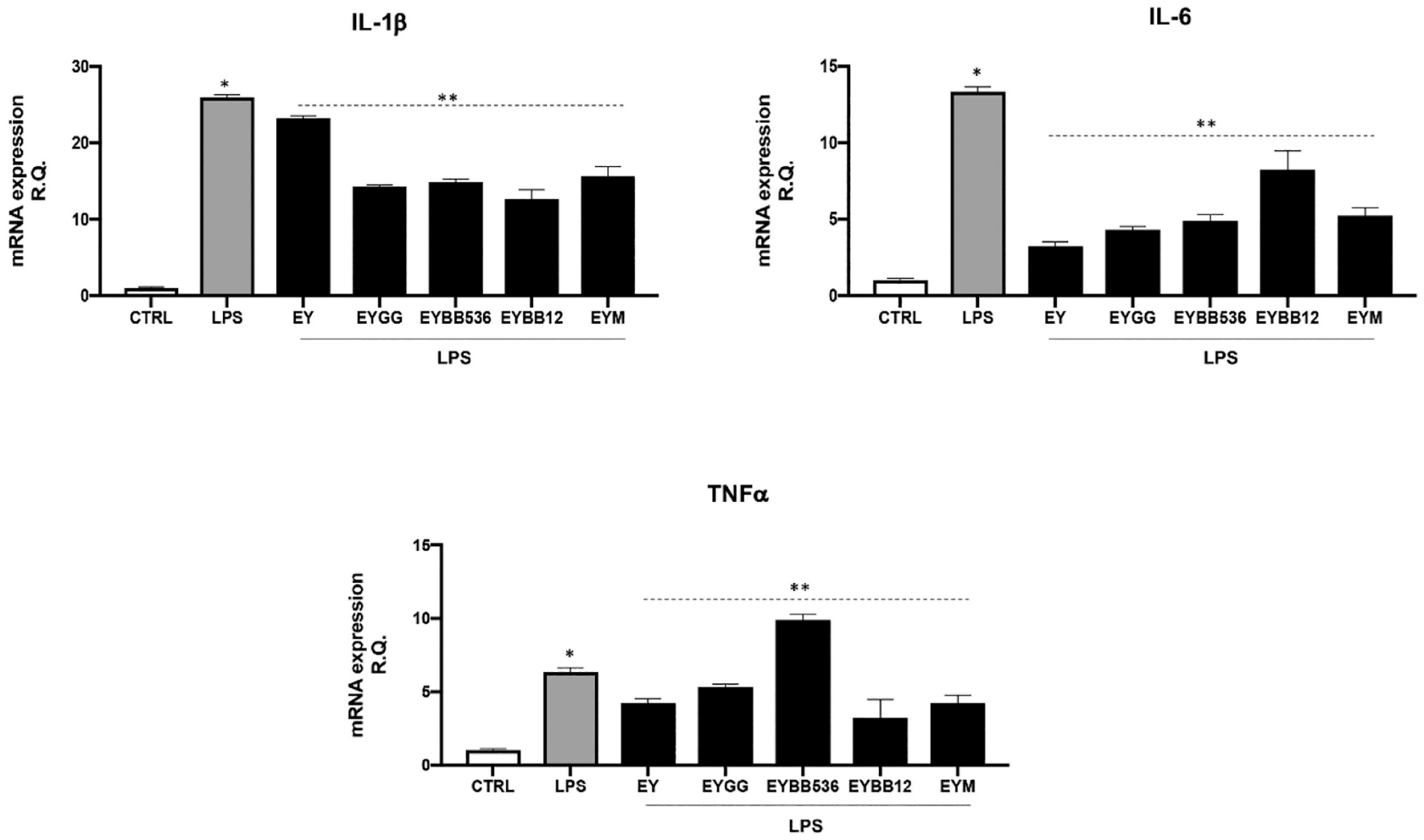


**Fig. 4.** Gene expression of interleukin-1β (IL-1β), interleukin-6 (IL6) and tumor necrosis factor-α (TNF-α) evaluated by RT-PCR. Bars represent the mean ± SEM of six independent experiments. * $p < 0.05$ versus CTRL cells; ** $p < 0.05$ versus LPS treatment.

products compared to previously published data (Cáceres et al., 2019; Chekdid et al., 2021; Cho & Lim, 2016). Interestingly, the fermentation process determined an increase in the GABA content of >40%. This effect can be attributed to the metabolic activity explicated by the used starter cultures in line with previously reported evidence suggesting that, among bacteria, lactobacilli, including *Lactobacillus delbrueckii* strains, are considered the main producers of GABA (; Dhakal, Bajpai, & Baek, 2012; Seok et al., 2008; Siragusa et al., 2020; Yunes et al., 2016).

Among nutrients, oryzanol is the most common sterol present in the bran of BR with documented physiological properties including antioxidant, anticarcinogenic, antihyperlipidemic, anti-inflammatory, and neuroprotective properties (Cicero & Gaddi, 2001; Francisqueti et al., 2017; Ravichanthiran et al., 2018). Our data revealed that the fermentation process determined a remarkable increase in oryzanol, in discordance with previously reported data (Cáceres et al., 2019; Chekdid et al., 2021), suggesting that the used starter stains could be able to synthetize oryzanol. This feature was previously demonstrated by Esteban-Torres, Reverón, Mancheño, de Las Rivas, and Muñoz (2013) for strains ascribed to the *Lactiplantibacillus plantarum* strains. In addition, our data revealed that, along with the enhancement of the bioactive compounds profile, the yogurt-like products, formulated in the present study, showed interesting anti-inflammatory properties on human colon cell line, treated by LPS, determining the reduction of proinflammatory cytokines IL-1β, IL-6 and TNF $\alpha$ gene expression.

Butyric acid was below the limit of detection in EYGG, EYBB536, and EYM samples. Its absence could be due to the ability of the used probiotic strains to metabolize butyric acid. The present study, investigated, for the first time, the suitability of GBR as a substrate for the formulation of fermented products functionalized by the addition of probiotic strains. As displayed by microbiological counts, all the fermented products, formulated in the present study, were stable and safe throughthe *shelf-life* of 28 days. In addition, based on the obtained results, both lactobacilli and bifidobacteria probiotic strains added to the yogurt-like products were able to survive, at cell densities higher than 9 log units, during storage at +4 °C for 28 days. This evidence is not in line with previous studies indicating that the growth of *Bifidobacteria* spp. in cereal substrates is difficult unless a growth promoter (milk or yeast extract) is added (Gupta & Abu-Ghannam, 2012).

## 5. Conclusions

The present study demonstrated the suitability of GBR for the formulation of a plant derived fermented product. The fermentation process improved the nutritional composition of GBR, increasing the bioactive compounds content. In addition, high acceptability scores by panelists was recorded and the high level of lactobacilli and bifidobacteria indicated that GBR is a suitable matrix for the development of healthy plant derived foods.

## Funding

This project was partially supported by Mr. Bio Food srl (Crespadoro, Italy).

## Author statement

All data, models, and code generated or used during the study appear in the submitted article.

## 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.

## Acknowledgements

Authors thanks Sacco System srl (Cadorago, Italy) and Chr. Hansen Holding A/S (Denmark) companies for providing starter cultures, as well as Mr. Bio Food srl (Crespadoro, Italy) for providing germinated brown rice samples.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2022.103076.

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