Highlights

- There is a growing demand for healthy fermented vegetal foods as dairy alternatives.
- Soy is a vegetal food rich in nutrients and a source of isoflavones.
- *Leuconostoc* strains selected from kefir efficiently grew in soymilk.
- The resulting fermented product was creamy and free of flatus-causing sugars.
- Furthermore, the resulting fermented product had enhanced estrogenic activity.

Use of kefir-derived lactic acid bacteria for the preparation of a fermented soy drink with increased estrogenic activity

Running Title: Kefir LABs to ferment soy drink

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Abstract

Fermented foods are receiving growing attention for their health promoting properties. In particular, there is a growing demand for plant-based fermented foods as dairy alternatives. Considering that soy is a vegetal food rich in nutrients and a source of the phytoestrogen isoflavones, the aim of this study was to select safe food microorganisms with the ability to ferment a soy drink resulting in a final product with an increased estrogenic activity and improved functional properties. We used milk kefir grains, a dairy source of microorganisms with proven health-promoting properties, as a starting inoculum for a soymilk. After 14 passages of daily inoculum in fresh soy drink, we isolated four lactic acid bacterial strains: *Lactotoccus lactis* subsp. *lactis* K03, *Leuconostc pseudomesenteroides* K05, *Leuconostc mesenteroides* K09 and *Lentilactobacillus kefiri* K10. Isolated strains were proven to be safe for human consumption according to the assessment of their antibiotic resistance profile and comparative genomics. Furthermore, functional characterization of the bacterial strains demonstrated their ability to ferment sugars naturally present in soybeans and produce a creamy texture. In addition, we demonstrated, by means of a yeast-based bioluminescence reporter system, that the two strains belonging to the genus *Leuconostoc* increased the estrogenic activity of the soybean drink. In conclusion, the proposed application of the bacterial strains characterized in this study meets the growing demand of consumers for health-promoting vegetal food alternatives to dairy products.

Keywords: isoflavones, *Leuconostoc*, kefir, dairy alternative, *L. kefiri*, *L. lactis*, estrogen biosensor

1. Introduction

According to the "biodiversity hypothesis", during urbanization, widespread antibiotic use, westernization of diet, and improved hygiene practices drastically reduced contact between humans and microorganisms, resulting in the taxonomic impoverishment of the microbiotas associated to the human body (Haahtela, 2019). Increasing evidence supports the notion that a reduced biodiversity in human-associated microbial ecosystems generates improper immune system functionality, with a consequential increased incidence of autoimmune, allergic and, in general, noncommunicable diseases (Blaser & Falkow, 2009; Haahtela, 2019). In this context, fermented foods (i.e., "foods made through desired microbial growth and enzymatic conversions of food components" (Marco et al., 2021)) received growing attention as a source of live microbial cells that can positively modulate the composition of the intestinal microbiota and benefit host health (Rezac, Kok, Heermann, & Hutkins, 2018).

Fermentation is one of the oldest techniques adopted to preserve and modify food. Besides improving shelf-life, safety and sensory characteristics, fermentation may also enhance the nutritional and health-promoting properties of foods (Marco et al., 2017; Rezac et al., 2018). In fact, during fermentation, microorganisms may produce vitamins and bioactive molecules, and increase the bioavailability of food constituents (Şanlier, Gökcen, & Sezgin, 2019). Numerous fermented food products have been demonstrated to confer health benefits, such as sauerkraut (Yu et al., 2013), kombucha (Aloulou et al., 2012), and also novel products created using selected bacteria with proven beneficial properties (Plé et al., 2016).

A category of food-associated molecules that attracted great attention for its impact on human health are phytoestrogens (PEs), bioactive compounds naturally present in several vegetal foods that are structurally and/or functionally similar to mammalian estrogens (Patisaul & Jefferson, 2010). These compounds have been studied for more than 40 years for their potential effects in numerous hormone-associated conditions such as breast cancer (Cohen, Zhao, Pittman, & Scimeca, 2000; Martin, Horwitz, Ryan, & McGuire, 1978), prostate cancer (Adlercreutz et al., 1995), cardiovascular disorders (Baum et al., 1998; Potter et al., 1998), and menopausal symptoms (Jayachandran & Xu, 2019; Kurzer, 2000; Potter et al., 1998). A rich source of PEs are plants belonging to the *Fabaceae* family, including soy, green peas, and red clover, which contain isoflavones, a subclass of flavonoids that are among the first PEs discovered (Rossiter & Beck, 1966). Isoflavones are naturally present in plants in the form of β-glycosides, acetyl glycosides and malonyl glycosides, which are much less estrogenic than their respective aglycones (Křížová, Dadáková, Kašparovská, & Kašparovský, 2019; Landete et al., 2016). Once ingested, isoflavones glycosides can be hydrolyzed by β-glycosidases and further modified by intestinal bacteria, producing PE molecules such as equol, dihydrodaidzein and o-desmethylangolensin (Lampe, 2009). The deglycosylation of isoflavones can also occur in food through the fermentation of starter microorganisms possessing the β-glucosidase activity such as lactic acid bacteria (e.g., *Lacticaseibacillus casei*; (Matsuda et al., 1994)) or filamentous fungi like *Rhizopus oryzae* and *Mucor racemosus*, which are used to produce the soybean fermented product tempeh (He & Chen, 2013; Nakajima, Nozaki, Ishihara, Ishikawa, & Tsuji, 2005).

In this study, we used kefir grains as a source of microorganisms to ferment a water infusion of soya beans (commercially known as soymilk or soy drink). Then, four lactic acid bacterial strains were isolated from the fermented soy drink and characterized for their potential use in the preparation of a fermented soy product with improved functional properties and increased estrogenic activity.

2. Materials and methods

2.1. Adaptation of kefir microbial consortium to soy drink

Five grams of granules derived from a domestic (noncommercial) milk kefir collected in Bogogno (northeast Piedmont, Italy) were inoculated into 50 ml of a commercially available soy drink (infusion of 7% decorticated soya beans in water, pH 7.3) and incubated at 25 °C. After 24 h, the fermented product was homogenized and 1 ml of it was used to inoculate a further 50 ml of fresh soy drink. This operation was repeated daily for two weeks. The final product was observed in bright field optic microscopy under oil immersion at 1000X magnification after staining with 1% (w/v) methylene blue.

2.2. Isolation and identification of microbial strains in the fermented soy drink

Aliquots of the fermented product obtained after 2 weeks of subculturing were sown by spread-plating on two agar culture media: (i) deMan Rogosa Sharpe (MRS; Difco Laboratories Inc., Detroit, MI, USA) at pH 5.5, and (ii) M17 (Difco) supplemented with 1% (w/v) glucose and 1% lactose (w/v) (Sigma-Aldrich S.r.l., Milano, Italy). Then, Petri dishes were incubated at 25 °C for 48 h. Colonies with different

110 morphologies observed on agar plates were selected and transferred by streak-plating
111 to fresh agar media. This passage was repeated five times in order to obtain pure
112 cultures. After cultivation in liquid medium, genomic DNA was extracted from all
113 bacterial isolates using DNEasy® Ultraclean® Microbial® Kit (Qiagen, Hilden,
114 Germany). Then, isolates were grouped by molecular fingerprinting through BOX-
115 PCR with BOXA1 primer as in (Guglielmetti et al., 2010), obtaining four genotypic
116 groups. Two representative strains from each group were taxonomically identified by
117 sequencing the 16S rRNA gene. The 16S rRNA gene was amplified through PCR with
118 panbacterial primers (Suzuki & Giovannoni, 1996) and the amplicon was sequenced.
119 Finally, BLASTN program was used to search sequence similarity within the "*16S*
120 *ribosomal RNA sequences (Bacteria and Archaea)*" database in GenBank. The
121 identification of the subspecies within the species *Lactococcus lactis* was performed
122 via PCR with primers targeting the *his* operon (Corroler, Desmasures, & Gueguen,
123 1999).

124 *2.3. Cultivation of and soy drink fermentation with the isolated bacterial strains*
125 The four selected bacterial isolates were cultivated in the following growth media:
126 M17 + 2% (w/v) sucrose for strain *Lactococcus lactis* subsp. *lactis* K03, and MRS for
127 strains *Leuconostoc pseudomesenteroides* K05, *Leuconostoc mesenteroides* K09 and
128 *Lentilactobacillus kefiri* K10. The same agar media supplemented with 2% (w/v)
129 sucrose were also used for the visualization of extracellular capsular polysaccharides
130 on agar plates through the addition of Aniline blue or Congo red (Hawkins, Geddes, &
131 Oresnik, 2017). For the preparation of soy drink fermentates, a pre-inoculum was

prepared as indicated above. Then, cell viability was assessed by flow cytometry in accordance with the ISO 19344 protocol. In brief, the fluorescent dyes SYTO24™ (Thermo Fisher Scientific Inc., Monza, Italy) and propidium iodide (Sigma-Aldrich) were added to a diluted cell suspension in saline solution (0.9% NaCl) at a final concentration of 0.1 μM and 0.2 μM, respectively. The samples were then incubated at 37 °C for 15 min in the dark before analysis by flow cytometer (BD Accuri™ C6 Plus Flow Cytometer, BD Biosciences, Milan, Italy). Subsequently, bacterial cells were recovered by centrifugation from the broth culture, washed once with saline, and diluted in saline to 1×10^8 Active Fluorescent Unit (AFU)/ml. Then, each strain was individually inoculated at a concentration of 1×10^6 AFU/ml in the commercial soy drink. An inoculum composed of an equal part of the four strains at a final concentration of 10^6 AFU/ml (2.5×10^5 AFU/ml for each strain) was also used to ferment the soy drink. In addition, a strain of *Streptococcus thermophilus* was isolated from a commercial soy-based yogurt-like product prepared with the same quantity of decorticated soybeans (7%) (strain SY). This strain was cultivated in M17 medium + 2% (w/v) sucrose and then inoculated in soy drink as per the other strains. For subsequent experiments, each combination was cultivated in triplicate at 30 °C for 24 h.

2.4. Viable bacterial count of fermented soy products

The viable count of bacterial cells in the soy drink fermented with single strains or their combination was determined by plating in triplicate 10-fold serial dilutions prepared in saline. The following two agar media were used: (i) brain hearth infusion

(BHI; Difco) agar supplemented with 2% (w/v) glucose and 0.3 % (w/v) yeast extract (gyBHI), and (ii) homofermentative-heterofermentative differential (HHD) agar medium (McDonald, McFeeters, Daeschel, & Fleming, 1987). Colonies were analyzed and counted after incubating the plates at 30 °C for 48 h in aerobiosis.

2.5. Analysis of texture of the fermented soy products

For the texture analysis, fermentation (24 h at 30 °C) was carried out in 120 ml plastic caps, starting from 100 ml of soy drink; fermented products were then stored for 12 h at 4 °C. The texture of unfermented and fermented soy drinks was then assessed by means of a TA.HDplus Texture Analyzer (Stable Micro Systems, Surrey, UK) equipped with a 10-N load cell and a cylindrical probe of 35 mm diameter. A back-extrusion test was carried out, with a trigger force of 0.03 N, at a penetration speed of 2 mm/s up to a depth of 15 mm. The Texture Exponent TEE32 V. 3.0.4.0 software (Stable Micro System, Surrey, UK) was used for instrument control and data acquisition. As a comparison, a commercial yogurt-like soy product fermented by *Streptococcus thermophilus* was analyzed under the same conditions. Results are expressed as firmness (maximum load) and stiffness (slope of the initial part of the force-deformation curve) and are the average of five replicates for each sample.

2.6. Antibiotic resistance profiles

The bacterial strains isolated from fermented soymilk were tested for their sensitivity to a panel of nine antibiotics as suggested by EFSA (EFSA, 2012) as described in the ISO 10932 IDF 223 document. In details, the Minimum Inhibitory Concentration (MIC) values were assessed for each antibiotic within different ranges, as follow:

ampicillin (from 0.5 to 16 μg/ml), vancomycin (1-32 μg/ml), gentamicin (8-256 μg/ml), kanamycin (from 16 to 512 μg/ml), streptomycin (from 8 to 256 μg/ml), erythromycin (from 0.25 to 8 μg/ml), clindamycin (from 0.25 to 8 μg/ml), tetracycline (from 1 to 32 μg/ml) and chloramphenicol (from 2 to 64 μg/ml). All antibiotics were purchased from Sigma-Aldrich. The MICs were determined by micro-dilution method, using a media made up of ISO sensitest broth (Oxoid, Fisher Scientific Italia, Rodano, Italy) 90% (w/w) and MRS (Difco) 10% (w/w) (ISO-MRS), and MIC tests were performed in 384-well plates, filled with an automatic liquid handling system (EpMotion, Eppendorf, Milan, Italy) to a final volume of 80 μl. Each strain was exposed, in duplicate, to each antimicrobial concentration, starting from overnight cultures in ISO-MRS. For each strain, a positive control (inoculated medium without antibiotic) and a negative control (medium without inoculum) were included. Bacterial cells were precultured in ISO-MRS, quantified by flow cytometry and inoculated at a concentration of 1×10^5 AFU/ml. *Lacticaseibacillus paracasei* LMG12586 was used as reference strain according to ISO10932. The 384-well plates were incubated 48 h at 30 °C for the four isolated strains and 37 °C in anaerobiosis for the reference strain, and the cell density evaluated by $O.D._{600nm}$ measurement using a spectrophotometer (MicroWave RS2, Biotek, USA) and the Gene5 software (Biotek, USA). The MIC was determined as the lowest antibiotic concentration that inhibited bacterial growth and the results were interpreted according to the EFSA Guidance on the assessment of bacterial antimicrobial susceptibility (EFSA, 2012).

2.7. Genome sequencing, annotation, and comparative analysis

The draft genome of the four bacterial strains isolated from the fermented soy infusion was determined using an Illumina Hiseq 2500 system with paired-end and shotgun libraries. From each strain, we obtained reads length of 151 nucleotides for both R1 and R2. The number of high-quality paired-end reads (quality Phred score > 30) obtained per strain was: K03 = 5'949'531; K05 = 6'532'359; K09 = 5'331'816; K10 = 6'158'418. The SPAdes version 3.14.1 (Bankevich et al., 2012) algorithm was used for assembling reads into contigs and then in scaffolds. The success of the assembly was tested with Bandage version 0.8 (Wick, Schultz, Zobel, & Holt, 2015). General information on the obtained draft genomes is shown in **Supplementary Table S1**. Draft genome annotation was carried out by means of the automated pipeline RAST (Rapid Annotations using Subsystems Technology (Aziz et al., 2008). Putative antibiotic resistance genes were searched using two different tools: (i) the antimicrobial resistance gene detection tools of AMRFinderPlus (Feldgarden et al., 2021) and (ii) the Resistance Gene Identifier (RGI) on Comprehensive Antibiotic Resistance Database (CARD; updated April 2022; (Alcock et al., 2020)). Concerning RGI, "Perfect" and "Strict" algorithms were used to detect perfect match and previously unknown AMR genes variants, respectively. The "Strict" algorithm uses detection models with CARD's curated similarity cut-offs to ensure the detected variant is likely a functional AMR gene. Sequencing data were deposited in the European Nucleotide Archive of the European Bioinformatics Institute under the accession code PRJEB52922.

2.8. Sugar and isoflavone determination in soy drink

Samples were diluted 1:1 in methanol for the analysis isoflavones, and between 1:50 a and 1:1000 in HPLC-grade water for sugars. Then, samples were stirred at 10000 rpm for 30 s with a bench vortex and centrifuged at 11200 rcf for 5 min. Finally, the resulting supernatant was used. Isoflavones were analyzed using an Alliance chromatographic system mod. 2695 (Waters, Milford, MA, USA) with a diode array detector mod. 2996 (Waters). A 5 μm C_{18} Symmetry column (250×4.6 mm, Waters) was used at a flow rate of 1.5 ml/min. The eluents were (A) 0.1% HCOOH and (B) acetonitrile. The analysis was performed using the following linear gradient: from 10 to 20% B in 10 min, from 20 to 35% B in 10 min and then from 35 to 90% B in 10 min. The column and sample were maintained at 30 and 20°C, respectively. Injection volume was 50 μl. Mother solution of daidzin, genistin, daidzein, genistein, equol and dihydrodaidzein at 1 mg/ml was prepared in methanol and calibration range was 2-50 μg/ml. Data was acquired in the range 220-450 nm and chromatograms were integrated at 254 nm by Empower software (Waters).

Sugar analysis was performed by an UHPLC mod. Flexa (Thermo) coupled to a High-Resolution MS Spectrometry model Exactive (Thermo) equipped with an ESI interface operating in negative mode. A 1.7 μm BEH Amide column (150x2.1 mm, Waters, Milford, MA, USA) was used in isocratic mode for the separation at a flow/rate of 0.2 ml/min. The eluent was 0.02% NH_4OH in acetonitrile: 0.02% NH_4OH in water (65:35, v/v). The column and the sample were maintained at 35 and 20°C, respectively. The Mass conditions were the following: spray voltage -3 kV, sheath gas 35, auxiliary gas 10, capillary temperature 275°C, heather 120°C, capillary voltage -

241 37.5 V, tube lens -80 V, skimmer -16 V. All data was acquired by Xcalibur software
242 (Thermo Scientific). The acquisition was carried out in scan mode in the range of 100-
243 600 u. Calibration curves were obtained from glucose and fructose stock solutions
244 prepared by dissolving 20 mg of standard powder in 20 ml of water. The working
245 solution of sucrose, verbascose, raffinose and stachyose were prepared in the eluent
246 solution in the range of 2-50 µg/ml. Two µl was the volume injected in the UPLC
247 system per analysis.

248 *2.9. Estrogenic activity measurement through the* Saccharomyces cerevisiae
249 *BMAEREluc/ERα reporter system*
250 The estrogenic activity of soy drinks and controls was assessed by means of the
251 reporter yeast strain *S. cerevisiae* BMAEREluc/ERα, which expresses the human
252 estrogen receptor alpha (ERα) (Leskinen, Michelini, Picard, Karp, & Virta, 2005). In *S.*
253 *cerevisiae* BMAEREluc/Erα, ERα acts as a nuclear transcription factor that upon
254 binding with the ligand undergoes dimerization and binds the estrogen response
255 elements in the reporter vector triggering the expression of the luciferase (*luc*) gene.
256 Reporter yeast cells were prepared as previously described with little modification
257 (Leskinen et al., 2005; Välimaa, Kivistö, Leskinen, & Karp, 2010). In brief, the
258 reporter yeast strain was cultivated in synthetic dextrose (SD) medium composed of
259 yeast nitrogen base medium (6.7 g/l) (Difco) supplemented with ammonium sulfate (5
260 g/l), glucose (20 g/l), adenine (0.1 g/l), L-histidine (0.1 g/l) and L-leucine (0.10 g/l)
261 (Sigma-Aldrich) incubated at 30 °C. After 24 h of aerobic incubation on a rotative
262 shaker (230 rpm), yeast broth culture was diluted to $OD_{600\ nm}$ 0.6 and incubated again

263 until $OD_{600\ nm}$ 0.8 was reached. Then, 90 µl of yeast broth culture were aliquoted in a
264 96-well, white, flat-bottomed microtiter plate (Optiplate-96 culture plate; PerkinElmer
265 Inc., Waltham, MA, USA) and supplemented with 10 µl of sample. For the
266 measurement of the estrogenic activity, the pH of fermented soy drinks was corrected
267 to 7 and directly added to the microtiter plate after extensive mixing. Subsequently, the
268 microtiter plate was incubated at 30 °C for 2.5 h. After incubation, 100 µl of D-
269 luciferin (Sigma-Aldrich) in 0.1 M citrate buffer pH 5.0 was added to each well and the
270 emitted luminescence was immediately registered using a PerkinElmer Wallac
271 VICTOR3 1420 (PerkinElmer, Monza, Italy) luminometer. Bioluminescence
272 measurements were carried out in triplicate for each sample. Each sample was tested in
273 at least three independent experiments. The estrogenic mycotoxin zearalenone (ZEN,
274 Sigma-Aldrich) was used as reference since it was previously demonstrated to be an
275 effective activator of the biosensor (Välimaa et al., 2010). ZEN was used at 10 µM in
276 1% ethanol solution because we found that this concentration corresponds to the
277 plateau of light emission by the biosensor (**Supplementary Figure S1**). For each
278 sample, the fold of induction (FOI) was calculated as the ratio between the mean
279 emitted luminescence (expressed as relative luminescence units, RLUs) of the triplicate
280 of the sample and the mean RLUs of the triplicate of the unfermented soy drink in the
281 same experiment. Then, the estrogenic activity of each sample under investigation was
282 reported as the ratio between the FOI of the sample and the FOI of ZEN (FOI/FOI_{ZEN}
283 ratio); therefore, a value of 1 corresponds to an estrogenic activity equal to that of 10
284 µM ZEN in the adopted experimental setting.
285

3. Results

3.1. Subculturing of kefir grains in soy drink and microbial composition of the resulting fermented product

Artisanal kefir grains were inoculated into a commercial soy drink and propagated through a daily subculturing (1:50 inoculum) for 2 weeks. After a few days, kefir grains were no longer visible, and after 2 weeks a homogeneous creamy product was obtained (**Fig. 1**).

The microscope examination with methylene blue staining of the fermented soy drink obtained after two weeks of subculturing revealed the exclusive presence of bacterial cells, whereas cells/structures ascribable to fungi were not observed (**Supplementary Fig. S2**). Most of the bacterial cells had a coccoid morphology; nonetheless, we also found rod-shaped bacteria, which were rarely observed in aggregates of a few tens of cells (Supplementary Fig. S2C).

Dilution plating of the fermented product on gyBHI revealed a viable microbial count of 2×10^9 CFUs per ml. Similar microbial cell count was calculated when the MRS and gM17 media were used. On the differential medium HHD, four different types of colonies were observed (**Supplemetary Fig. S2D**), accounting collectively for a viable count not dissimilar from that calculated with the other agar media.

Several colonies, representative of the four morphologies observed on HHD agar, were isolated and characterized by BOX-PCR genetic fingerprinting. The isolates were clustered into four genotypic groups (**Supplementary Fig. S3**), which matched with the colony morphologies observed on HHD agar. Two representative isolates for each group were chosen to perform the taxonomic assignment by sequencing of the 16S

rRNA gene. A BLAST search revealed sequence similarities higher than 99% with the following bacterial species:

- genotype I, *Leuconostoc pseudomesenteroides*
- genotype II, *Lactococcus lactis*
- genotype III, *Leuconostoc mesenteroides*
- genotype IV, *Lentilactobacillus kefiri*.

The *his*-PCR experiment evidenced that the *Lactococcus lactis* isolates belonged to the *lactis* subspecies.

Then, one representative isolate for each identified bacterial taxon was chosen and used in the subsequent experiments: *L. pseudomesenteroides* K05, *L. lactis* subsp. *lactis* K03, *L. mesenteroides* K09, and *L. kefiri* K10.

3.2. Characterization of soy drink fermented with the selected bacterial strains

The four selected strains were cultivated singularly or in combination in the commercial soy drink for 24 h at the optimal temperature of the isolated bacteria, i.e., 30 °C. Viable count and pH of the resulting fermented products are reported in **Table 1**. Viable count on HHD agar plates revealed that strain *L. kefiri* K10 has a limited ability to grow in the soy infusion compared to the other strains, either as inoculated alone or in combination. Accordingly, strain K10 only marginally reduced the pH (from 7.3 to 6.5), whereas the other strains acidified the soy drink to pH < 5 (Table 1).

As expected, the fermentation induced drastic changes in the texture of the soy drink due to acid coagulation of the proteins (Zhang, Li, Feng, & Dong, 2013). In fact, all strains, used alone or in combination, except for *L. kefiri* K10, significantly

increased firmness (**Fig. 2**). *L. lactis* K03 determined the significantly highest values for both firmness and stiffness, while the effect of the other strains and their mix on stiffness was lower. Another factor that could influence texture during fermentation is the bacterial production of a polysaccharide capsule (Zeidan et al., 2017). For this reason, we assessed the ability to synthesize exopolysaccharides by the three bacterial isolates that modified soy drink firmness, i.e., strains K03, K05 and K09. The use of Aniline blue or Congo red revealed the presence of abundant EPS production by *Leuconostoc* strains (K05 and K09), but not for *L. lactis* K03 (**Supplementary Fig. S4**).

The same fermented samples were also used to define the sugars utilized by bacterial cells during the fermentation. The main sugar in the unfermented soy drink was sucrose, at a concentration of 4.1 g/l. In addition, stachyose and raffinose were detected at a concentration of 3.3 and 1.1 g/l, respectively. On the contrary, verbascose, a penta-saccharide commonly found in soy (Ibrahim, 2018), was not detected. After fermentation with all strains, sucrose was not detected anymore, suggesting that it was completely utilized in the bacterial metabolism (**Fig. 3**). Only strain *L. pseudomesenteroides* K05 consumed all stachyose and raffinose. A partial reduction of these two sugars was also observed after fermentation with *L. mesenteroides* K09 and the mix of bacterial strains. On the contrary, On the contrary, stachyose and raffinose were only marginally affected after fermentation with *L. lactis* K03, *L. kefiri* K10 and *S. thermophilus* SY (Fig. 3).

352 *3.3. Safety assessment of the selected bacterial strains*

353 To assess the safety of the four selected bacterial isolates, the presence of potential
354 acquired antibiotic resistances was tested according to the micro-dilution protocol
355 recommended by EFSA (EFSA, 2012). Compared to EFSA breakpoints, *L. lactis* K03,
356 *Leuconostoc mesenteroides* K09, and *L. kefiri* K10 showed a reduced susceptibility to
357 one antibiotic, *viz*. streptomycin (MIC = 128 µg/ml *vs.* breakpoint = 32 µg/ml),
358 kanamycin (MIC = 128 µg/ml *vs.* breakpoint = 16 µg/ml) and tetracycline (MIC = 16
359 µg/ml *vs.* breakpoint = 8 µg/ml), respectively (Table 2). Also, *Leuconostoc*
360 *pseudomesenteroides* K05 displayed reduced susceptibility toward kanamycin (MIC =
361 64 *vs.* breakpoint = 16 µg/ml) and, to a lesser extent, clindamycin (MIC = 2 µg/ml *vs.*
362 breakpoint = 1 µg/ml).

363 Subsequently, the draft genome of the four selected bacterial strains was studied
364 through comparative genomics to identify genes putatively coding for acquired
365 antibiotic resistance genes. This analysis did not reveal any known transmissible
366 antibiotic resistance genes for all tested strains (Supplementary Table S2).

367 *3.4. Isoflavones in soy drink before and after fermentation*

368 Isoflavones, which are the best-know bioactive compounds of soy, were quantified by
369 UPLC-MS/MS in the soy drink before and after fermentation with the selected
370 bacterial strains. This analysis showed that the o-glycosides daidzin and genistin were
371 the main isoflavones in the unfermented soy drink, with a mean concentration of 104
372 and 83 mg/l, respectively. Conversely, the corresponding aglycons daidzein and
373 genistein were found at a much lower concentration (0.8 and 1.1 mg/l, respectively)

374 (**Fig. 4A**). The fermentation with *L. pseudomesenteroides* K05 determined the
375 strongest conversion of glycosides into aglycons (more than 90 %). A conversion
376 between 20 and 25 % was observed after the fermentation with *L. mesenteroides* K09
377 and the mix of the four strains. The conversion of glycosides into aglycons after the
378 fermentation with *L. lactis* K03 was about 10 %, whereas that obtained for *L. kefiri*
379 K10 and *S. thermophilus* SY was negligible (**Fig. 4B**).

380 *3.5. Estrogenic activity of fermented soy drink*

381 The potential estrogenic activity of the soy drink before and after fermentation with the
382 selected bacteria was assessed by means of a luminescent biosensor based on a
383 recombinant bioluminescent yeast constitutively expressing a hormone receptor that
384 recognizes estrogenic ligands (Leskinen et al., 2005). This experiment showed that the
385 estrogenic activity of the unfermented soy drink was only marginally higher than the
386 background and was not significantly affected by the fermentation with strains *L. kefiri*
387 K10 and *S. thermophilus* SY (**Fig. 5**). On the contrary, a significant increase in the
388 estrogenic activity was observed after fermentation with *L. lactis* K03 (3.3-fold
389 increase over unfermented soy drink), *L. pseudomesenteroides* K05 (5.5-fold increase),
390 *L. mesenteroides* K09 (5.0-fold increase), and the mixture of the four strains (4.7-fold
391 increase) (Fig. 5).
392

393 **4. Discussion**

394 The integration of a diet including fermented foods was suggested as a potential
395 effective strategy to deliver health-promoting microbial cells to the gastrointestinal

tract, counteracting the detrimental consequences of bacterial deprivation that occurs in the environment and food of industrialized societies (Allaerts & Chang, 2017; Şanlier et al., 2019). In Western countries, most of the fermented products available on the market are dairy (milk-based) foods. Nonetheless, the animal rights aware choice of a vegan lifestyle, intolerances/allergies to milk-based products, and the general perception on the sustainability of dairy farming are increasingly orienting consumers towards plant-based dairy alternatives. One well-known and widely diffused example of plant-based dairy alternative is represented by yogurt-like fermented soy infusions (soy yogurt), which is conventionally produced at the industrial level through the direct fermentation of a soy infusion with the conventional dairy starter *Streptococcus thermophilus*.

In this context, we carried out this study to generate a novel vegan (non-animal) fermented product that could enhance the health-promoting properties of soy by means of unconventional microorganisms (*i.e.*, different from yogurt starters or commonly used probiotics), which can provide additional functionalities to the product. To this aim, we used milk kefir grains as the initial source of microorganisms, because this fermented product possesses a complex consortium of microorganisms including lactic acid bacteria, yeasts, and acetic bacteria (Garofalo et al., 2015; Prado et al., 2015) with demonstrated health-promoting properties (Hertzler & Clancy, 2003; Jeong et al., 2017; Merenstein, Foster, & D'Amico, 2009; Silva, Rodrigues, Filho, & Lima, 2009; Turan, Dedeli, Bor, & İlter, 2014; Yılmaz, Dolar, & Özpınar, 2019).

The subculturing of kefir grains in a commercial soy drink generated a fermented product with a creamy texture and induced the selection of four bacterial strains

belonging to four species of lactic acid bacteria reported to be common members of the kefir microbiota (Korsak et al., 2015; Kotova, Cherdyntseva, & Netrusov, 2016; Leite et al., 2012). The absence of a strain capable to produce the kefiran exopolysaccharide such as *Lactobacillus kefiranofaciens* among our isolates could explain the absence of granules in the fermented soy drink (Wang, Ahmed, Feng, Li, & Song, 2008).

All four isolated bacterial species, *viz. Lactococcus lactis*, *Lentilactobacillus kefiri*, *Leuconostoc mesenteroides*, and *Leuconostoc pseudomesenteroides*, possess the "qualified presumption of safety" (QPS) status, which allows the deliberate introduction of these microorganisms into the food chain in the European Union (EFSA, 2007, 2020). In addition, these LAB species are listed in the "*Inventory of microbial food cultures with safety demonstration in fermented food products*" of the International Diary Federation (IDF; Bulletin N° 514/2022). The safety of the isolated bacteria was also evaluated at strain level by the characterization of their antibiotic resistance profile. MIC analysis revealed a reduced susceptibility to one or two antibiotics for each strain, in particular for aminoglycoside antibiotics: kanamycin for *Leuconostoc* strains, and streptomycin for the *L. lactis* strain. The profiles of kanamycin sensitivity in *Leuconostoc* spp. were shown to vary largely among strains (Adimpong, Nielsen, Sorensen, Derkx, & Jespersen, 2012; Florez et al., 2016), and the reduced susceptibility to streptomycin was reported in several *L. lactis* strains (Toomey, Bolton, & Fanning, 2010). However, specific genes supporting these phenotypes have not been reported for these bacteria (Salvetti, Campedelli, Larini, Conedera, & Torriani, 2021) and have not been found for the strains here investigated according to a search in the antibiotic databases. Therefore, it appears plausible that the

442 observed reduced sensitivity towards some of the tested antibiotic could be associated
443 to intrinsic non-transmissible genetic features, such as the presence of an
444 exopolysaccharide capsule, as observed for the two *Leuconostoc* strains. Therefore,
445 considering that they belong to QPS species and are plausibly free from transmissible
446 antibiotic resistance genes, we can conclude that the four LAB strains here investigated
447 can be used in fermented food products intended for human consumption.
448 All four bacterial strains were able to grow in the soy infusion, reaching, within 24
449 h, a viable count ranging between 0.5 and 4 billion CFUs per ml of fermented product.
450 Hypothesizing the consumption of about 100 g of fermented soy product, the number
451 of bacterial cells that would be ingested is consistent with human intervention trials
452 that report a positive effect on host health upon administration of probiotic or
453 fermented food products (Derrien & van Hylckama Vlieg, 2015).
454 The fermentation of soy drink by the selected lactic acid bacteria determined
455 several desirable effects, including a creamy texture, and the removal of raffinose and
456 stachyose. In particular, firmness increased in all the fermented samples with respect to
457 soy milk, with the exception of K10, as soy protein coagulation due to cross-linking is
458 affected by pH and a value below 6 is necessary to induce gelation (Zhang et al.,
459 2013). Indeed, the sample fermented by *L. kefiri* K10 reached a pH value of 6.5 and it
460 didn't show any difference in texture parameters compared to soy milk. A higher
461 gelation effect is obtained by reaching the isoelectric point of soy protein, which is
462 around 4.5 (Hefnawy & Ramadan, 2011); in fact the lowest pH values associated to
463 samples K03 (4.3) and MIX (4.2) resulted in the highest texture changes, with a
464 particularly high stiffness for sample K03, indicating the production of a very compact

gel. The mix of the four strains produced a structure more similar to the commercial reference, with a significantly lower stiffness in comparison with K03, accounting for a creamier and more cohesive structure. Strains K05 and K09 produced a weaker gel, probably linked to the slightly higher pH values reached during fermentation and the abundant exopolysaccharide capsule, contributing to make the fermented product creamier and less brittle.

As for the raffinose and stachyose removal, notably, *Leuconostoc paramesenteroides* K05 removed completely these oligosaccharides, which are the major contributors of flatus and abdominal symptoms that represent the most important factor deterring many people from consuming soy products (Elango et al., 2022; Suarez et al., 1999).

Isoflavones are phytoestrogens with a structure resembling that of the human female hormone 17-β-estradiol. After binding to the estrogen receptors, isoflavones can exert a potent estrogenic activity that can provide many health benefits related to breast and prostate cancer prevention, postmenopausal symptoms, osteoporosis, and cardiovascular diseases (Alshehri et al., 2021; Boutas, Kontogeorgi, Dimitrakakis, & Kalantaridou, 2022; Chen & Chen, 2021; Vitale, Piazza, Melilli, Drago, & Salomone, 2013). Isoflavones are present in natural sources primarily as glucose-conjugates. According to literature, here we found that the main isoflavones in the investigated soy drink were the glucose-conjugated forms daidzin and genistin, whereas the corresponding aglycons daidzein and genistein were detected at 100 times lower concentration. However, glycosylated isoflavones are difficult to absorb by the intestinal epithelium and exert weaker biological activities than the corresponding

488 aglycones (Vitale et al., 2013). In our study, we showed that the fermentation of the
489 soy drink by the selected bacterial strains affected isoflavones, potentially influencing
490 their phytoestrogenic activity. In specific, fermentation increased the deglycosylated
491 forms of isoflavones in soy drink fermented with *L. lactis* K03, *L.*
492 *pseudomesenteroides* K05*, L. mesenteroides* K09 and the mix of the four strains.
493 Notably, the conversion of glycosylated isoflavones into aglycons was almost complete
494 after the fermentation with *L. pseudomesenteroides* K05 (more than 90% conversion),
495 whereas it was negligible for strains *L. kefiri* K10 and *S. thermophilus* SY. Reportedly,
496 the formation of deglycosylated forms of isoflavones derives from the β-glucosidase
497 enzymes (Ismail & Hayes, 2005), which are widely spread in lactic acid bacteria
498 (Michlmayr & Kneifel, 2014; Yuksekdag, Cinar Acar, Aslim, & Tukenmez, 2017).
499 Accordingly, the analysis of the draft genomes of the bacterial strains under
500 investigation revealed the presence of putative β-glucosidase coding genes in strains
501 K03, K05, and K09 but not in *L. kefiri* K10.
502 According to the ability of the bacteria under study to convert natural soy
503 isoflavones into aglycons, the K05-fermented soy drink displayed the highest ability to
504 activate the human estrogen receptor, whereas the estrogenic activity of the soy drink
505 fermented with strains K10 and SY was not significantly dissimilar from that of the
506 unfermented product. Nonetheless, strain K09, although converted glycosylated
507 isoflavones into aglycons much less than K05 (about 20 % conversion), increased the
508 estrogenic activity similarly to strain K05, suggesting that the reported system was
509 potentially saturated by the quantity of estrogenic molecules used in the experiment.
510

5. Conclusion

In this study we presented new lactic acid bacterial strains that are safe for human consumption and can be used to produce a novel soy-based fermented product with enhanced functional properties. In particular, we selected a strain, *Leuconostoc pseudomesenteroides* K05, which, once used for the fermentation of a soy drink, generated a product with a creamy consistency, free of the flatus-causing sugars, and with an enhanced estrogenic activity. The proposed use of the bacterial strains characterized in this study meets the growing demand of consumers for foods alternative to dairy products and with a high profile of health promotion and sustainability.

Authorship Contribution Statement

SG conceived and supervised the study. ADV performed the experiments of kefir bacteria selection by soy drink, LAB isolation and taxonomic identification with the assistance of VT, and GM. CG performed UPLC-MS/MS experiments for the quantification of sugars and isoflavones in soy drink. AL and CA set up and carried out the analysis of texture of the fermented soy products. SA carried out the antibiotic resistance experiments with the contribution of VT. GG performed the experiment of genome sequencing, annotation, and comparative analysis with the contribution of ADV and GM. MK, GM, ADV and RD set up and performed the experiments with the estrogenic reporter system. ADV and GM contributed to the realization of all laboratory experiments. SG wrote the original draft with the contribution of ADV and

GM, and revision from all authors. All authors read and approved the final version of the manuscript for publication.

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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751

752 **Tables**

753 **Table. 1**. Viable count and pH of the soy drink fermented with the four lactic acid
754 bacterial isolates (alone and in combination) after 24 h of incubation at 30 °C. Mix, all
755 strains inoculated simultaneously in the soy drink. AFU, active fluorescent units as
756 determined through flow cytometry.

Inoculum (AFU/ml)	**Bacterial strain**		**Viable count (CFU/ml)**	**pH**
10^6	*L. lactis* K03		1.7×10^9	4.3
10^6	*L. pseudomesenteroides* K05		1.1×10^9	4.4
10^6	*L. mesenteroides* K09		4.7×10^9	4.7
10^6	*L. kefiri* K10		5.7×10^8	6.5
10^6 (2.5×10^5 each strain)	Mix	*L. lactis* K03	1.2×10^9	4.2
		L. pseudomesenteroides K05	8.0×10^7	
		L. mesenteroides K09	4.8×10^8	
		L. kefiri K10	2.0×10^7	

757

Table 2. Antibiotic sensitivity of the bacterial strains isolated from the fermented soy drink determined according to the microdilution assay recommended by EFSA (EFSA, 2012). Data are reported as µg/ml. Minimum inhibitory concentrations (MICs) of the *L. paracasei* strain LMG12586 are shown with reference to the ISO10932 values used to validate the test. EFSA breakpoints for the corresponding bacterial group are reported on a grey background. Values exceeding the EFSA breakpoints are shown on darker orange background. *n.r.*, not required.

	ampicillin	vancomycin	gentamycin	kanamycin	streptomycin	erythromycin	clindamycin	tetracycline	chloramphenicol
Cut-off values for *Lactococcus lactis*	2	4	32	64	32	1	1	4	8
***Lactococcus lactis* K03**	0.5	0.5	8	64	**128**	0.125	0.25	<2	4
Cut-off values for obligate heterofermentative Lactobacillus	2	*n.r.*	16	32	64	1	1	8	4
***Lentilactobacillus kefiri* K10**	2	>16	<2	4	4	0.125	0.125	**16**	4
Cut-off values for *Leuconostoc*	2	*n.r.*	16	16	64	1	1	8	4
***Leuconostoc mesenteroides* K09**	2	>16	<2	**128**	32	0.125	<0,125	8	4
***Leuconostoc pseudomesenteroides* K05**	2	>16	4	**64**	64	0.0625	**2**	2	4
Cut-off values for strain *L. paracasei* LMG12586	0.5-2	*n.r.*	1-4	16-64	8-32	0.062-0.25	0.062-0.25	1-4	4-8
***Lacticaseibacillus paracasei* LMG12586**	1	>16	4	64	32	0-125	<0,125	2	4

Legends

Fig. 1. Fermentation of the commercial soy drink with kefir grains. **A**, Kefir grains used for the inoculation of the soy drink. **B**, Soy drink before inoculation. **C**, Fermented soy drink obtained after two weeks of daily subculturing. In panel C, the absence of grains and the homogenous creamy texture of the fermented product are evident.

Fig. 2. Analysis of soy drink texture. Histograms represent mean ± standard deviation of five replicates. Samples indicated with a different letter are significantly different ($P<0.05$) according to unpaired Student's t test. Unferm., unfermented soy drink. Mix, soy milk fermented with the four bacterial strains inoculated together. C.S.Y., commercial yogurt-like soy product (containing *Streptococcus thermophilus*).

Fig. 3. Sugars detected and quantified by UPLC-MS/MS in soy drinks before and after fermentation. Numbers above bars refer to the g of sugar per liter of soy drink. Data show results from one out of at least three independent experiments. Unferm., unfermented soy drink. Mix, soy drink fermented with the four bacterial strains inoculated together. *S.t.*, *Streptococcus thermophilus* SY isolated from a commercial yogurt-like soy product.

Fig. 4. Isoflavones in soy drinks. **A**, isoflavones detected by UPLC-MS/MS in unfermented soy drink. Numbers above bars refer to the mg of molecule per liter of soy drink. **B**, relative conversion of flavonoid glycosides in aglycones (calculated as molarity) by bacterial fermentation (24 h at 30 °C). Unferm., unfermented soy drink.

785 Mix, soy milk fermented with the four bacterial strains inoculated together. *S.t.*,
786 *Streptococcus thermophilus* SY isolated from a commercial yogurt-like soy product.

787 **Fig. 5**. Estrogenic activity of the soy drink under study before and after the
788 fermentation with lactic acid bacteria. Results are from three independent experiments
789 conducted in triplicate. FOI, fold of induction. ZEN, zearalenone; Mix, soy drink
790 fermented by the combination of the four strains isolated from kefir. Unferm.,
791 unfermented soy drink. *S.t.*, *Streptococcus thermophilus* SY isolated from a
792 commercial yogurt-like soy product. Statistics is according to two-way unpaired
793 Student's t test. Different letters (a-c) significant differences ($p<0.05$). Asterisks
794 indicate significant difference from control (i.e., unfermented soy drink); **, $p<0.01$; *,
795 $p<0.05$.

e 1

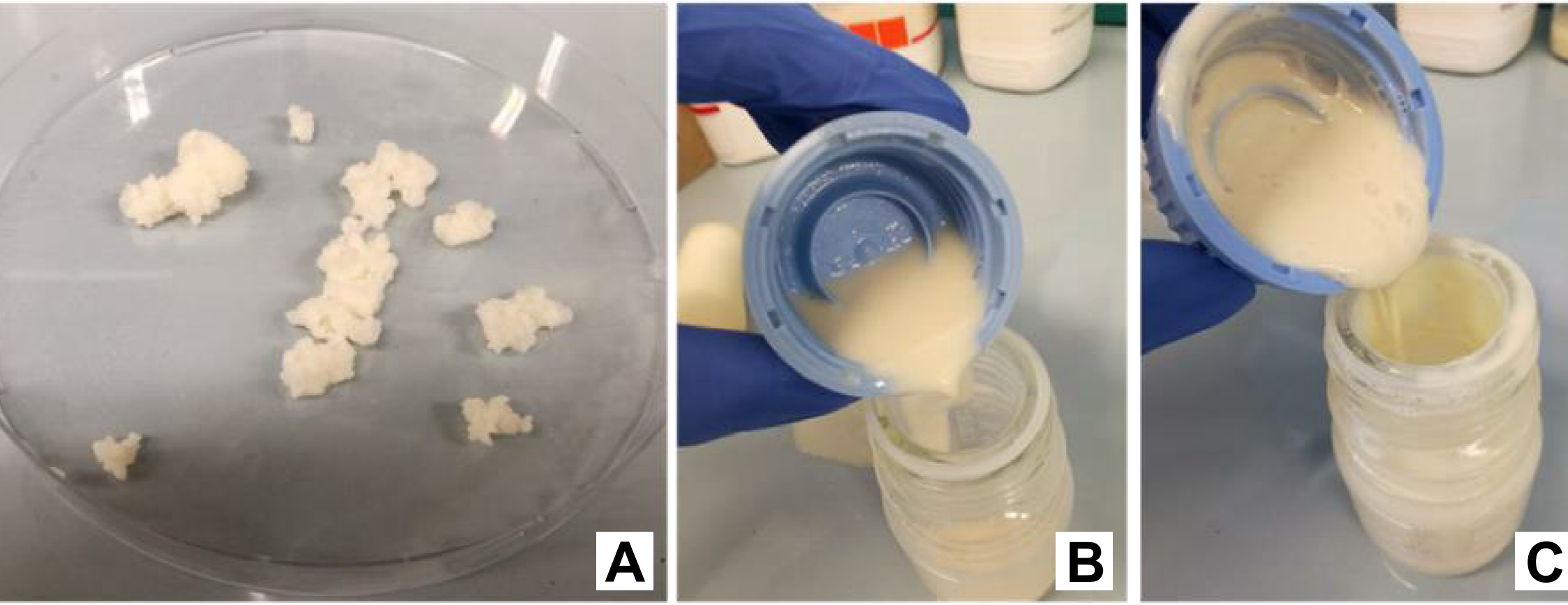
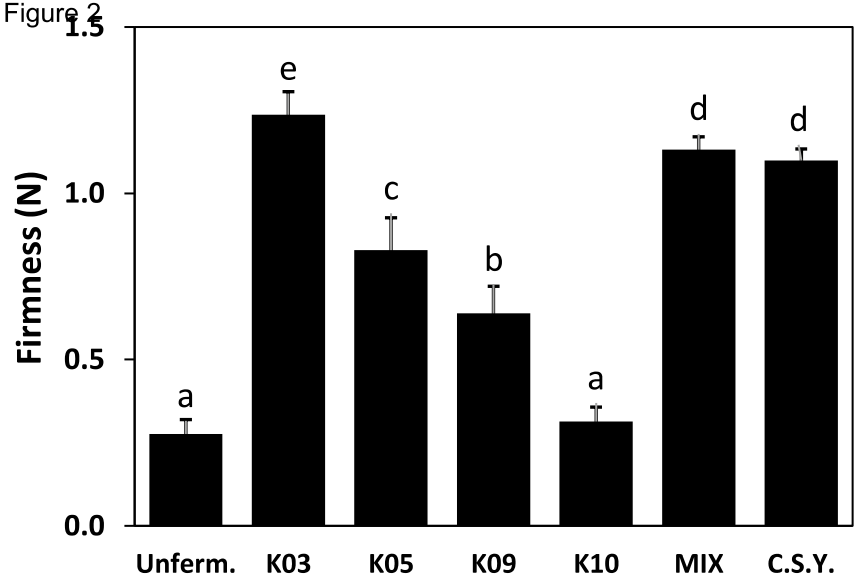


Figure 2


Firmness (N)
1.5
1.0
0.5
0.0
a
e
c
b
a
d
d
Unferm.
K03
K05
K09
K10
MIX
C.S.Y.

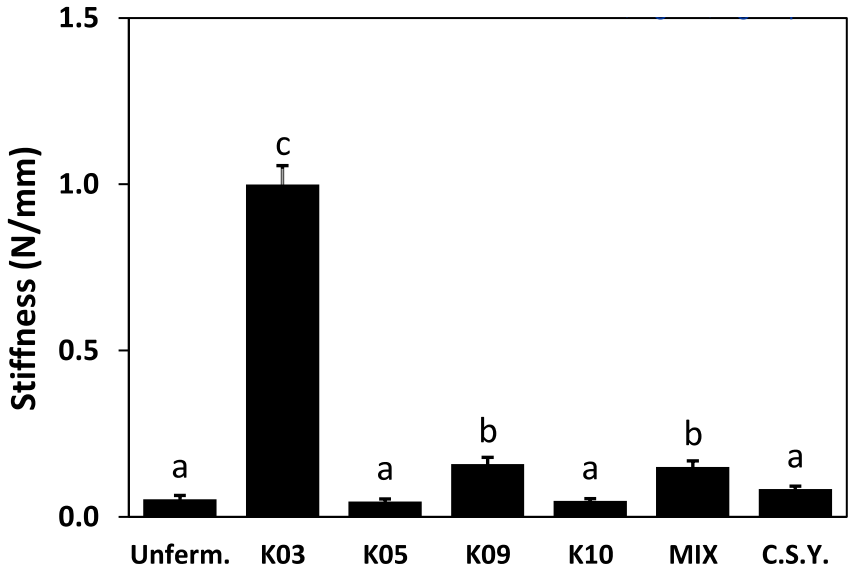

Stiffness (N/mm)
1.5
1.0
0.5
0.0
a
c
a
b
a
b
a
Unferm.
K03
K05
K09
K10
MIX
C.S.Y.

Figure 3

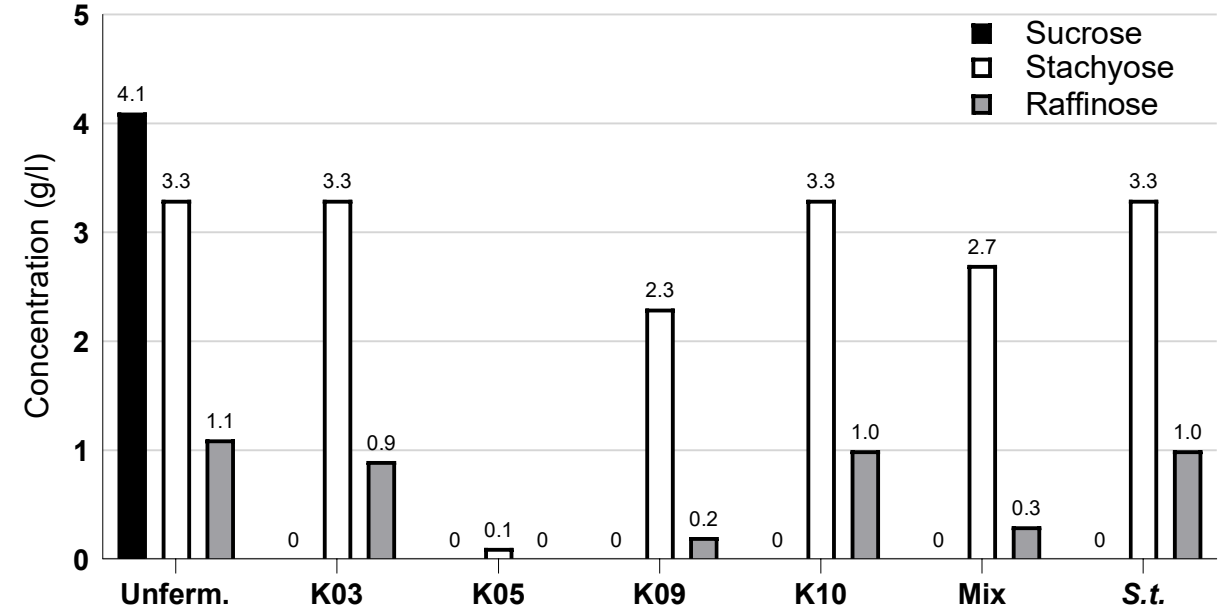

Sucrose
Stachyose
Raffinose
Concentration (g/l)
5
4
3
2
1
0
4.1
3.3
1.1
0
3.3
0.9
0
0.1
0
0
2.3
0.2
0
3.3
1.0
0
2.7
0.3
0
3.3
1.0
Unferm.
K03
K05
K09
K10
Mix
S.t.

Figure 4

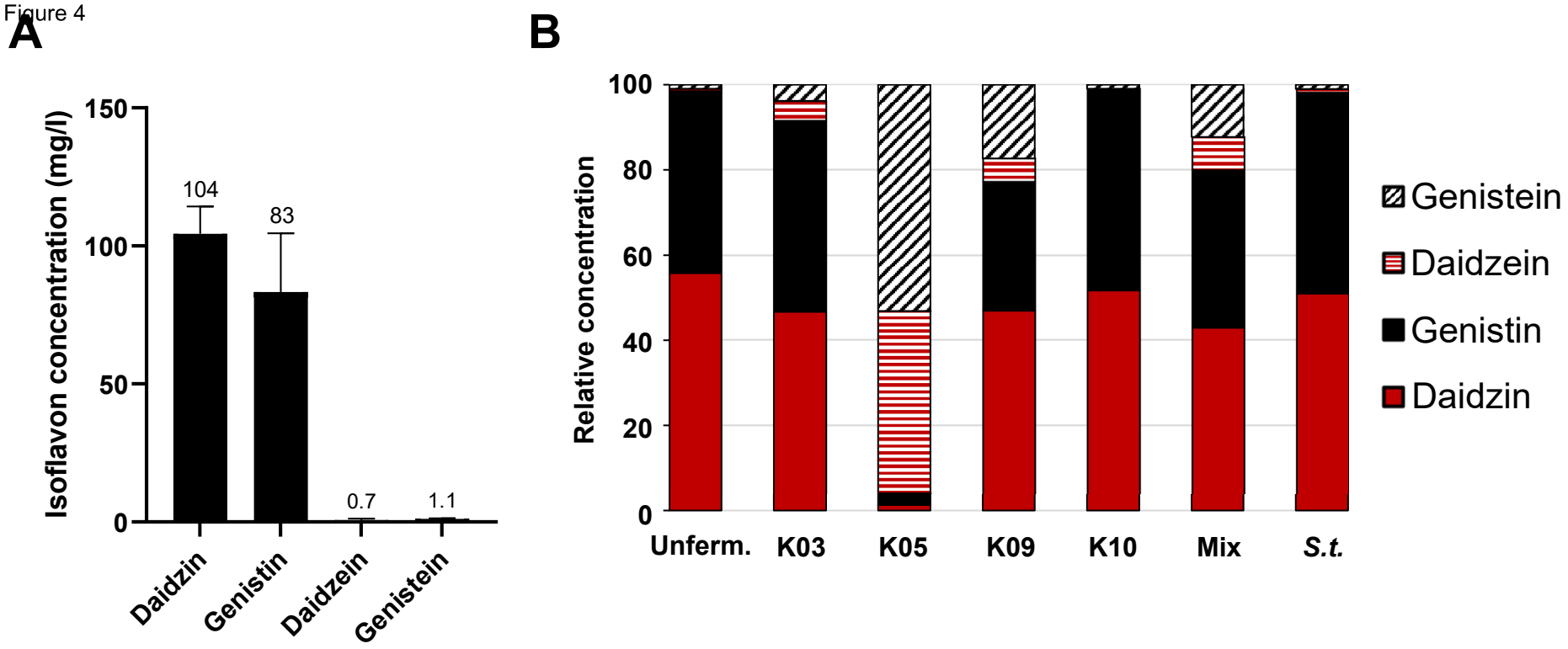
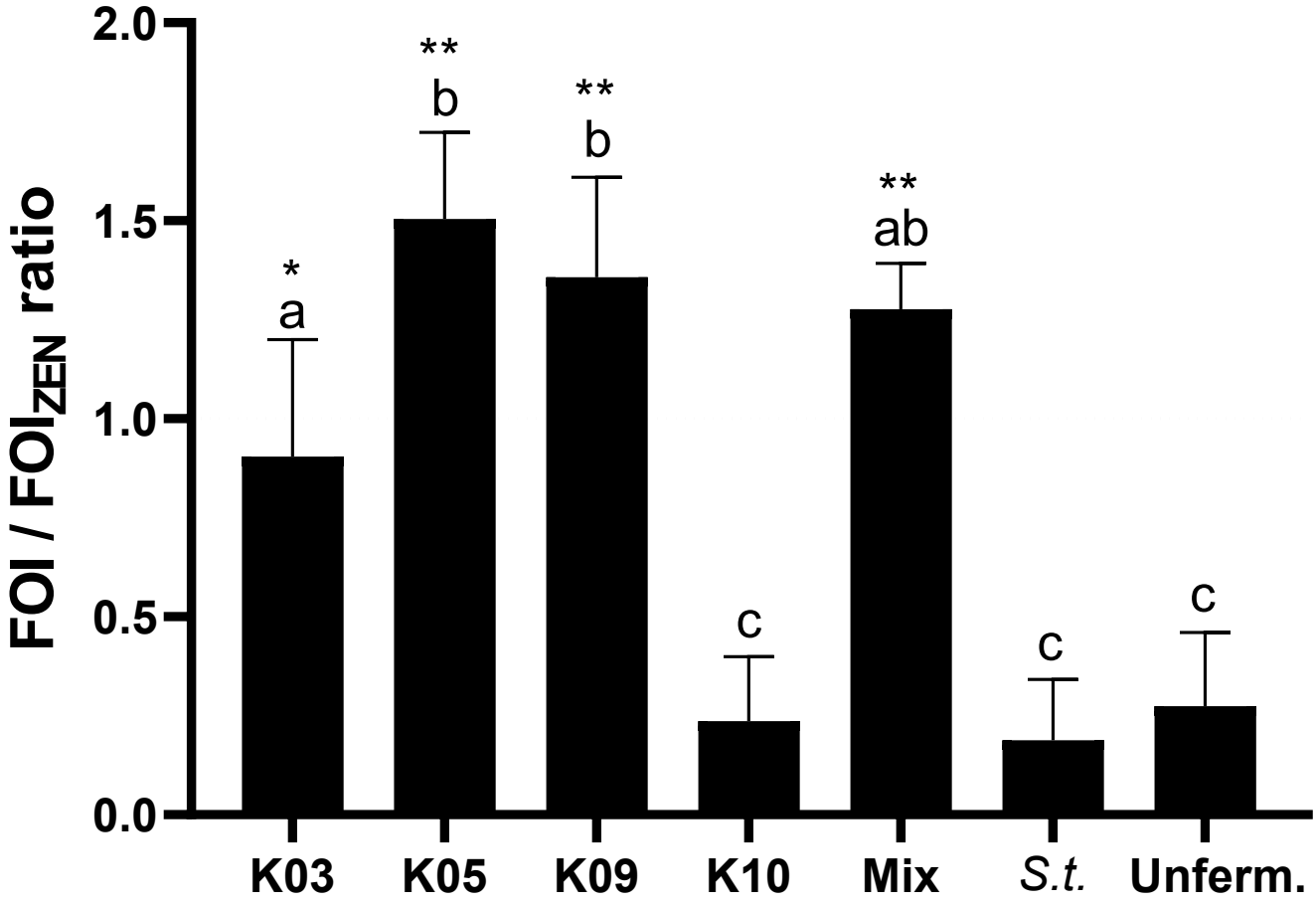
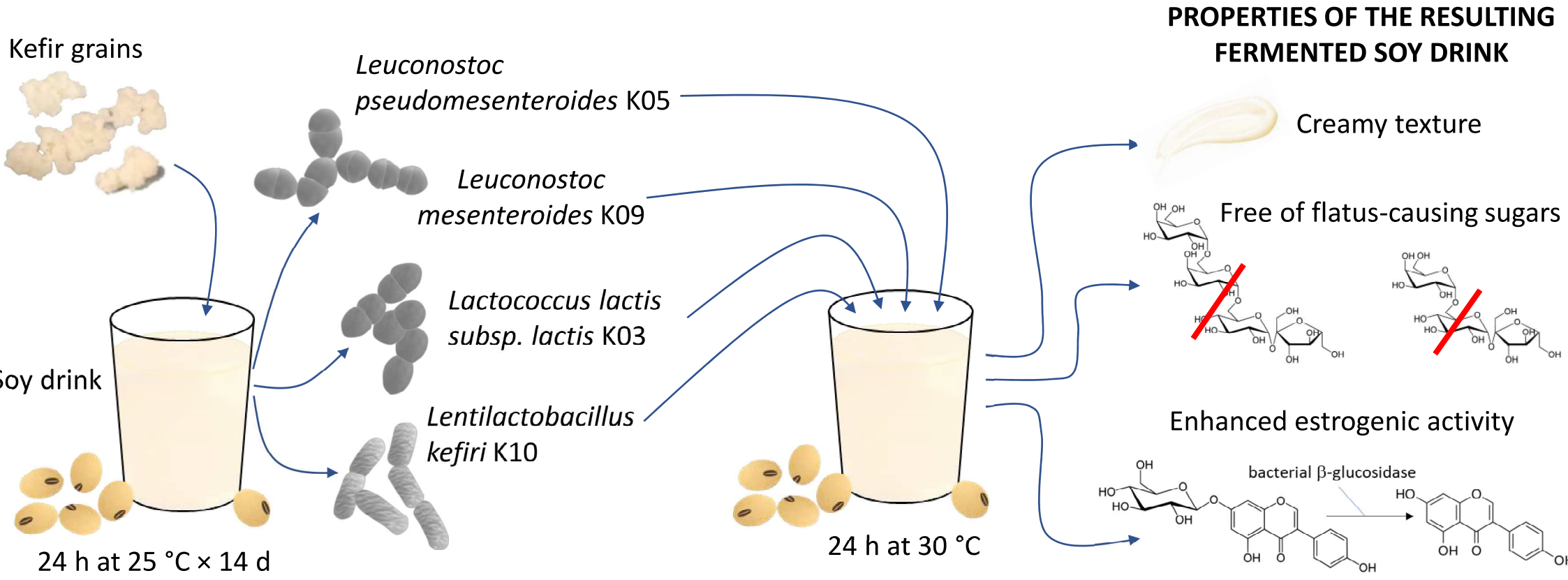

A
Isoflavon concentration (mg/l)
150
100
50
0
104
83
0.7
1.1
Daidzin
Genistin
Daidzein
Genistein
B
Relative concentration
100
80
60
40
20
0
Unferm.
K03
K05
K09
K10
Mix
S.t.
Genistein
Daidzein
Genistin
Daidzin

Figure 5


FOI / FOIZEN ratio
2.0
1.5
1.0
0.5
0.0
*
a
**
b
**
b
**
ab
c
c
c
K03
K05
K09
K10
Mix
S.t.
Unferm.


Kefir grains
Leuconostoc pseudomesenteroides K05
Leuconostoc mesenteroides K09
Lactococcus lactis subsp. lactis K03
Lentilactobacillus kefiri K10
Soy drink
24 h at 25 °C × 14 d
24 h at 30 °C
PROPERTIES OF THE RESULTING FERMENTED SOY DRINK
Creamy texture
Free of flatus-causing sugars
Enhanced estrogenic activity
bacterial β-glucosidase