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.

Manuscript

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

4

5 **Running Title: Kefir LABs to ferment soy drink**

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19

20 **Abstract**

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

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

43 **1. Introduction**

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

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

65 A category of food-associated molecules that attracted great attention for its
66 impact on human health are phytoestrogens (PEs), bioactive compounds naturally
67 present in several vegetal foods that are structurally and/or functionally similar to
68 mammalian estrogens (Patisaul & Jefferson, 2010). These compounds have been
69 studied for more than 40 years for their potential effects in numerous hormone-
70 associated conditions such as breast cancer (Cohen, Zhao, Pittman, & Scimeca, 2000;
71 Martin, Horwitz, Ryan, & McGuire, 1978), prostate cancer (Adlercreutz et al., 1995),
72 cardiovascular disorders (Baum et al., 1998; Potter et al., 1998), and menopausal
73 symptoms (Jayachandran & Xu, 2019; Kurzer, 2000; Potter et al., 1998). A rich source
74 of PEs are plants belonging to the *Fabaceae* family, including soy, green peas, and red
75 clover, which contain isoflavones, a subclass of flavonoids that are among the first PEs
76 discovered (Rossiter & Beck, 1966). Isoflavones are naturally present in plants in the
77 form of β-glycosides, acetyl glycosides and malonyl glycosides, which are much less
78 estrogenic than their respective aglycones (Křížová, Dadáková, Kašparovská, &
79 Kašparovský, 2019; Landete et al., 2016). Once ingested, isoflavones glycosides can
80 be hydrolyzed by β-glycosidases and further modified by intestinal bacteria, producing
81 PE molecules such as equol, dihydrodaidzein and o-desmethylangolensin (Lampe,
82 2009). The deglycosylation of isoflavones can also occur in food through the
83 fermentation of starter microorganisms possessing the β-glucosidase activity such as
84 lactic acid bacteria (e.g., *Lacticaseibacillus casei*; (Matsuda et al., 1994)) or
85 filamentous fungi like *Rhizopus oryzae* and *Mucor racemosus*, which are used to
86 produce the soybean fermented product tempeh (He & Chen, 2013; Nakajima, Nozaki,
87 Ishihara, Ishikawa, & Tsuji, 2005).

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

93

94 **2. Materials and methods**

95 *2.1. Adaptation of kefir microbial consortium to soy drink*

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

104 *2.2. Isolation and identification of microbial strains in the fermented soy drink*

105 Aliquots of the fermented product obtained after 2 weeks of subculturing were sown by
106 spread-plating on two agar culture media: (i) deMan Rogosa Sharpe (MRS; Difco
107 Laboratories Inc., Detroit, MI, USA) at pH 5.5, and (ii) M17 (Difco) supplemented
108 with 1% (w/v) glucose and 1% lactose (w/v) (Sigma-Aldrich S.r.l., Milano, Italy).
109 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

132 prepared as indicated above. Then, cell viability was assessed by flow cytometry in
133 accordance with the ISO 19344 protocol. In brief, the fluorescent dyes SYTO24™
134 (Thermo Fisher Scientific Inc., Monza, Italy) and propidium iodide (Sigma-Aldrich)
135 were added to a diluted cell suspension in saline solution (0.9% NaCl) at a final
136 concentration of 0.1 μM and 0.2 μM, respectively. The samples were then incubated at
137 37 °C for 15 min in the dark before analysis by flow cytometer (BD Accuri™ C6 Plus
138 Flow Cytometer, BD Biosciences, Milan, Italy). Subsequently, bacterial cells were
139 recovered by centrifugation from the broth culture, washed once with saline, and
140 diluted in saline to 1×10^8 Active Fluorescent Unit (AFU)/ml. Then, each strain was
141 individually inoculated at a concentration of 1×10^6 AFU/ml in the commercial soy
142 drink. An inoculum composed of an equal part of the four strains at a final
143 concentration of 10^6 AFU/ml (2.5×10^5 AFU/ml for each strain) was also used to
144 ferment the soy drink. In addition, a strain of *Streptococcus thermophilus* was isolated
145 from a commercial soy-based yogurt-like product prepared with the same quantity of
146 decorticated soybeans (7%) (strain SY). This strain was cultivated in M17 medium +
147 2% (w/v) sucrose and then inoculated in soy drink as per the other strains. For
148 subsequent experiments, each combination was cultivated in triplicate at 30 °C for 24
149 h.

150 *2.4. Viable bacterial count of fermented soy products*

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

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

158 *2.5. Analysis of texture of the fermented soy products*

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

171 *2.6. Antibiotic resistance profiles*

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

176 ampicillin (from 0.5 to 16 μg/ml), vancomycin (1-32 μg/ml), gentamicin (8-256
177 μg/ml), kanamycin (from 16 to 512 μg/ml), streptomycin (from 8 to 256 μg/ml),
178 erythromycin (from 0.25 to 8 μg/ml), clindamycin (from 0.25 to 8 μg/ml), tetracycline
179 (from 1 to 32 μg/ml) and chloramphenicol (from 2 to 64 μg/ml). All antibiotics were
180 purchased from Sigma-Aldrich. The MICs were determined by micro-dilution method,
181 using a media made up of ISO sensitest broth (Oxoid, Fisher Scientific Italia, Rodano,
182 Italy) 90% (w/w) and MRS (Difco) 10% (w/w) (ISO-MRS), and MIC tests were
183 performed in 384-well plates, filled with an automatic liquid handling system
184 (EpMotion, Eppendorf, Milan, Italy) to a final volume of 80 μl. Each strain was
185 exposed, in duplicate, to each antimicrobial concentration, starting from overnight
186 cultures in ISO-MRS. For each strain, a positive control (inoculated medium without
187 antibiotic) and a negative control (medium without inoculum) were included. Bacterial
188 cells were precultured in ISO-MRS, quantified by flow cytometry and inoculated at a
189 concentration of 1×10^5 AFU/ml. *Lacticaseibacillus paracasei* LMG12586 was used as
190 reference strain according to ISO10932. The 384-well plates were incubated 48 h at 30
191 °C for the four isolated strains and 37 °C in anaerobiosis for the reference strain, and
192 the cell density evaluated by $O.D._{600nm}$ measurement using a spectrophotometer
193 (MicroWave RS2, Biotek, USA) and the Gene5 software (Biotek, USA). The MIC was
194 determined as the lowest antibiotic concentration that inhibited bacterial growth and
195 the results were interpreted according to the EFSA Guidance on the assessment of
196 bacterial antimicrobial susceptibility (EFSA, 2012).

197 *2.7. Genome sequencing, annotation, and comparative analysis*

198 The draft genome of the four bacterial strains isolated from the fermented soy infusion
199 was determined using an Illumina Hiseq 2500 system with paired-end and shotgun
200 libraries. From each strain, we obtained reads length of 151 nucleotides for both R1
201 and R2. The number of high-quality paired-end reads (quality Phred score > 30)
202 obtained per strain was: K03 = 5'949'531; K05 = 6'532'359; K09 = 5'331'816; K10 =
203 6'158'418. The SPAdes version 3.14.1 (Bankevich et al., 2012) algorithm was used for
204 assembling reads into contigs and then in scaffolds. The success of the assembly was
205 tested with Bandage version 0.8 (Wick, Schultz, Zobel, & Holt, 2015). General
206 information on the obtained draft genomes is shown in **Supplementary Table S1**.
207 Draft genome annotation was carried out by means of the automated pipeline RAST
208 (Rapid Annotations using Subsystems Technology (Aziz et al., 2008). Putative
209 antibiotic resistance genes were searched using two different tools: (i) the antimicrobial
210 resistance gene detection tools of AMRFinderPlus (Feldgarden et al., 2021) and (ii) the
211 Resistance Gene Identifier (RGI) on Comprehensive Antibiotic Resistance Database
212 (CARD; updated April 2022; (Alcock et al., 2020)). Concerning RGI, “Perfect” and
213 “Strict” algorithms were used to detect perfect match and previously unknown AMR
214 genes variants, respectively. The “Strict” algorithm uses detection models with
215 CARD's curated similarity cut-offs to ensure the detected variant is likely a functional
216 AMR gene. Sequencing data were deposited in the European Nucleotide Archive of the
217 European Bioinformatics Institute under the accession code PRJEB52922.

218 *2.8. Sugar and isoflavone determination in soy drink*

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

233 Sugar analysis was performed by an UHPLC mod. Flexa (Thermo) coupled to a High-
234 Resolution MS Spectrometry model Exactive (Thermo) equipped with an ESI
235 interface operating in negative mode. A 1.7 µm BEH Amide column (150x2.1 mm,
236 Waters, Milford, MA, USA) was used in isocratic mode for the separation at a
237 flow/rate of 0.2 ml/min. The eluent was 0.02% NH_4OH in acetonitrile: 0.02% NH_4OH
238 in water (65:35, v/v). The column and the sample were maintained at 35 and 20°C,
239 respectively. The Mass conditions were the following: spray voltage -3 kV, sheath gas
240 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

286 **3. Results**

287 *3.1. Subculturing of kefir grains in soy drink and microbial composition of the*

288 *resulting fermented product*

289 Artisanal kefir grains were inoculated into a commercial soy drink and propagated

290 through a daily subculturing (1:50 inoculum) for 2 weeks. After a few days, kefir

291 grains were no longer visible, and after 2 weeks a homogeneous creamy product was

292 obtained (**Fig. 1**).

293 The microscope examination with methylene blue staining of the fermented soy

294 drink obtained after two weeks of subculturing revealed the exclusive presence of

295 bacterial cells, whereas cells/structures ascribable to fungi were not observed

296 (**Supplementary Fig. S2**). Most of the bacterial cells had a coccoid morphology;

297 nonetheless, we also found rod-shaped bacteria, which were rarely observed in

298 aggregates of a few tens of cells (Supplementary Fig. S2C).

299 Dilution plating of the fermented product on gyBHI revealed a viable microbial

300 count of 2×10^9 CFUs per ml. Similar microbial cell count was calculated when the

301 MRS and gM17 media were used. On the differential medium HHD, four different

302 types of colonies were observed (**Supplemetary Fig. S2D**), accounting collectively for

303 a viable count not dissimilar from that calculated with the other agar media.

304 Several colonies, representative of the four morphologies observed on HHD agar,

305 were isolated and characterized by BOX-PCR genetic fingerprinting. The isolates were

306 clustered into four genotypic groups (**Supplementary Fig. S3**), which matched with

307 the colony morphologies observed on HHD agar. Two representative isolates for each

308 group were chosen to perform the taxonomic assignment by sequencing of the 16S

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

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

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

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

320 *3.2. Characterization of soy drink fermented with the selected bacterial strains*

321 The four selected strains were cultivated singularly or in combination in the
322 commercial soy drink for 24 h at the optimal temperature of the isolated bacteria, i.e.,
323 30 °C. Viable count and pH of the resulting fermented products are reported in **Table**
324 **1**. Viable count on HHD agar plates revealed that strain *L. kefiri* K10 has a limited
325 ability to grow in the soy infusion compared to the other strains, either as inoculated
326 alone or in combination. Accordingly, strain K10 only marginally reduced the pH
327 (from 7.3 to 6.5), whereas the other strains acidified the soy drink to pH < 5 (Table 1).

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

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

340 The same fermented samples were also used to define the sugars utilized by
341 bacterial cells during the fermentation. The main sugar in the unfermented soy drink
342 was sucrose, at a concentration of 4.1 g/l. In addition, stachyose and raffinose were
343 detected at a concentration of 3.3 and 1.1 g/l, respectively. On the contrary, verbascose,
344 a penta-saccharide commonly found in soy (Ibrahim, 2018), was not detected. After
345 fermentation with all strains, sucrose was not detected anymore, suggesting that it was
346 completely utilized in the bacterial metabolism (**Fig. 3**). Only strain *L.*
347 *pseudomesenteroides* K05 consumed all stachyose and raffinose. A partial reduction of
348 these two sugars was also observed after fermentation with *L. mesenteroides* K09 and
349 the mix of bacterial strains. On the contrary, On the contrary, stachyose and raffinose
350 were only marginally affected after fermentation with *L. lactis* K03, *L. kefiri* K10 and
351 *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

396 tract, counteracting the detrimental consequences of bacterial deprivation that occurs
397 in the environment and food of industrialized societies (Allaerts & Chang, 2017;
398 Şanlier et al., 2019). In Western countries, most of the fermented products available on
399 the market are dairy (milk-based) foods. Nonetheless, the animal rights aware choice of
400 a vegan lifestyle, intolerances/allergies to milk-based products, and the general
401 perception on the sustainability of dairy farming are increasingly orienting consumers
402 towards plant-based dairy alternatives. One well-known and widely diffused example
403 of plant-based dairy alternative is represented by yogurt-like fermented soy infusions
404 (soy yogurt), which is conventionally produced at the industrial level through the direct
405 fermentation of a soy infusion with the conventional dairy starter *Streptococcus*
406 *thermophilus*.
407 In this context, we carried out this study to generate a novel vegan (non-animal)
408 fermented product that could enhance the health-promoting properties of soy by means
409 of unconventional microorganisms (*i.e.*, different from yogurt starters or commonly
410 used probiotics), which can provide additional functionalities to the product. To this
411 aim, we used milk kefir grains as the initial source of microorganisms, because this
412 fermented product possesses a complex consortium of microorganisms including lactic
413 acid bacteria, yeasts, and acetic bacteria (Garofalo et al., 2015; Prado et al., 2015) with
414 demonstrated health-promoting properties (Hertzler & Clancy, 2003; Jeong et al.,
415 2017; Merenstein, Foster, & D'Amico, 2009; Silva, Rodrigues, Filho, & Lima, 2009;
416 Turan, Dedeli, Bor, & İlter, 2014; Yılmaz, Dolar, & Özpınar, 2019).
417 The subculturing of kefir grains in a commercial soy drink generated a fermented
418 product with a creamy texture and induced the selection of four bacterial strains

419 belonging to four species of lactic acid bacteria reported to be common members of the
420 kefir microbiota (Korsak et al., 2015; Kotova, Cherdyntseva, & Netrusov, 2016; Leite
421 et al., 2012). The absence of a strain capable to produce the kefiran exopolysaccharide
422 such as *Lactobacillus kefiranofaciens* among our isolates could explain the absence of
423 granules in the fermented soy drink (Wang, Ahmed, Feng, Li, & Song, 2008).
424 All four isolated bacterial species, *viz. Lactococcus lactis*, *Lentilactobacillus*
425 *kefiri*, *Leuconostoc mesenteroides*, and *Leuconostoc pseudomesenteroides*, possess the
426 "qualified presumption of safety" (QPS) status, which allows the deliberate
427 introduction of these microorganisms into the food chain in the European Union
428 (EFSA, 2007, 2020). In addition, these LAB species are listed in the "*Inventory of*
429 *microbial food cultures with safety demonstration in fermented food products*" of the
430 International Diary Federation (IDF; Bulletin N° 514/2022). The safety of the isolated
431 bacteria was also evaluated at strain level by the characterization of their antibiotic
432 resistance profile. MIC analysis revealed a reduced susceptibility to one or two
433 antibiotics for each strain, in particular for aminoglycoside antibiotics: kanamycin for
434 *Leuconostoc* strains, and streptomycin for the *L. lactis* strain. The profiles of
435 kanamycin sensitivity in *Leuconostoc* spp. were shown to vary largely among strains
436 (Adimpong, Nielsen, Sorensen, Derkx, & Jespersen, 2012; Florez et al., 2016), and the
437 reduced susceptibility to streptomycin was reported in several *L. lactis* strains
438 (Toomey, Bolton, & Fanning, 2010). However, specific genes supporting these
439 phenotypes have not been reported for these bacteria (Salvetti, Campedelli, Larini,
440 Conedera, & Torriani, 2021) and have not been found for the strains here investigated
441 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

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

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

476 Isoflavones are phytoestrogens with a structure resembling that of the human
477 female hormone 17-β-estradiol. After binding to the estrogen receptors, isoflavones
478 can exert a potent estrogenic activity that can provide many health benefits related to
479 breast and prostate cancer prevention, postmenopausal symptoms, osteoporosis, and
480 cardiovascular diseases (Alshehri et al., 2021; Boutas, Kontogeorgi, Dimitrakakis, &
481 Kalantaridou, 2022; Chen & Chen, 2021; Vitale, Piazza, Melilli, Drago, & Salomone,
482 2013). Isoflavones are present in natural sources primarily as glucose-conjugates.
483 According to literature, here we found that the main isoflavones in the investigated soy
484 drink were the glucose-conjugated forms daidzin and genistin, whereas the
485 corresponding aglycons daidzein and genistein were detected at 100 times lower
486 concentration. However, glycosylated isoflavones are difficult to absorb by the
487 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

511 **5. Conclusion**

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

521

522 **Authorship Contribution Statement**

523 SG conceived and supervised the study. ADV performed the experiments of kefir
524 bacteria selection by soy drink, LAB isolation and taxonomic identification with the
525 assistance of VT, and GM. CG performed UPLC-MS/MS experiments for the
526 quantification of sugars and isoflavones in soy drink. AL and CA set up and carried out
527 the analysis of texture of the fermented soy products. SA carried out the antibiotic
528 resistance experiments with the contribution of VT. GG performed the experiment of
529 genome sequencing, annotation, and comparative analysis with the contribution of
530 ADV and GM. MK, GM, ADV and RD set up and performed the experiments with the
531 estrogenic reporter system. ADV and GM contributed to the realization of all
532 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 $p<0.05$.

Figure 1

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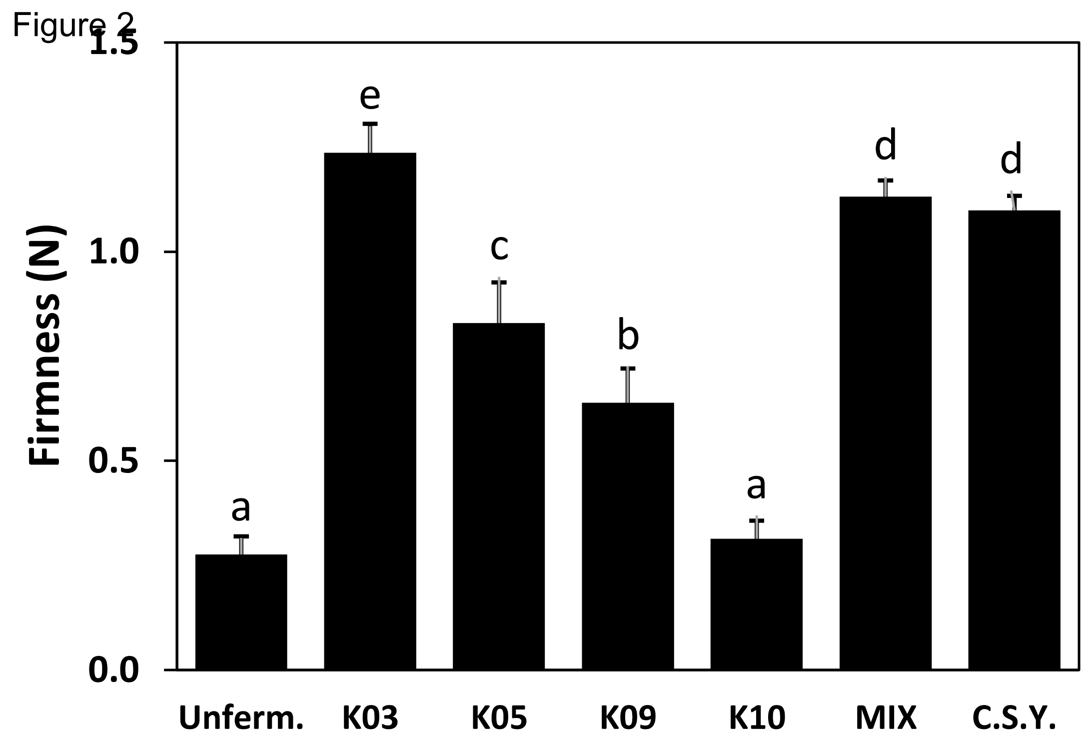
Figure 2



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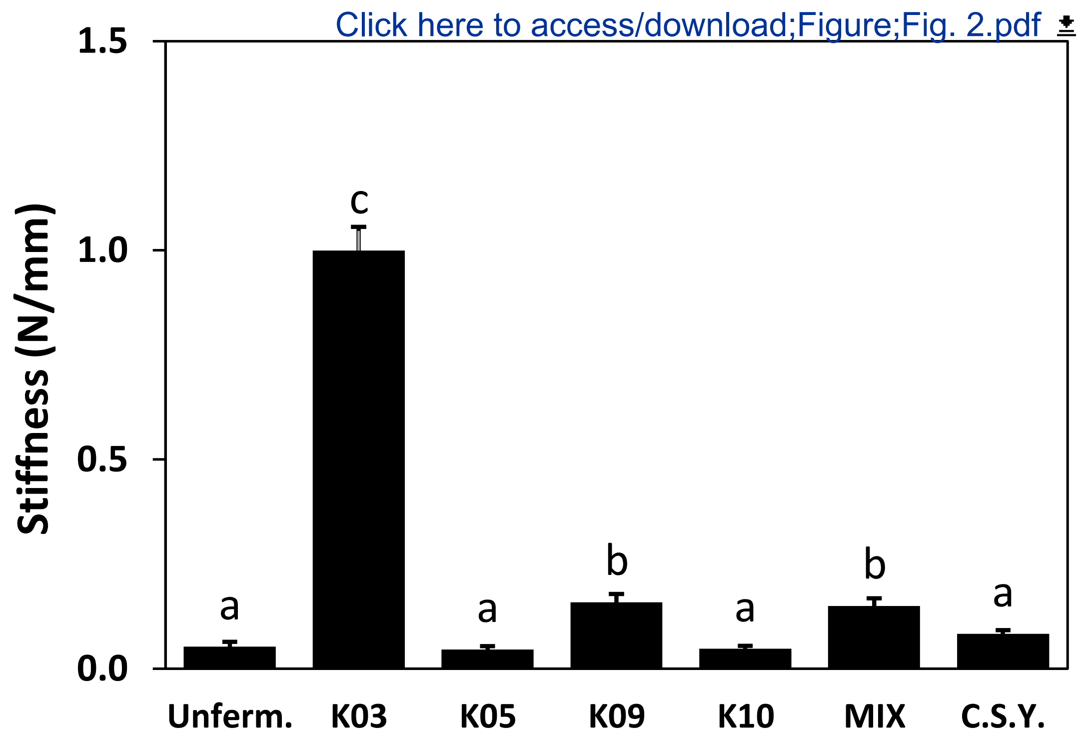

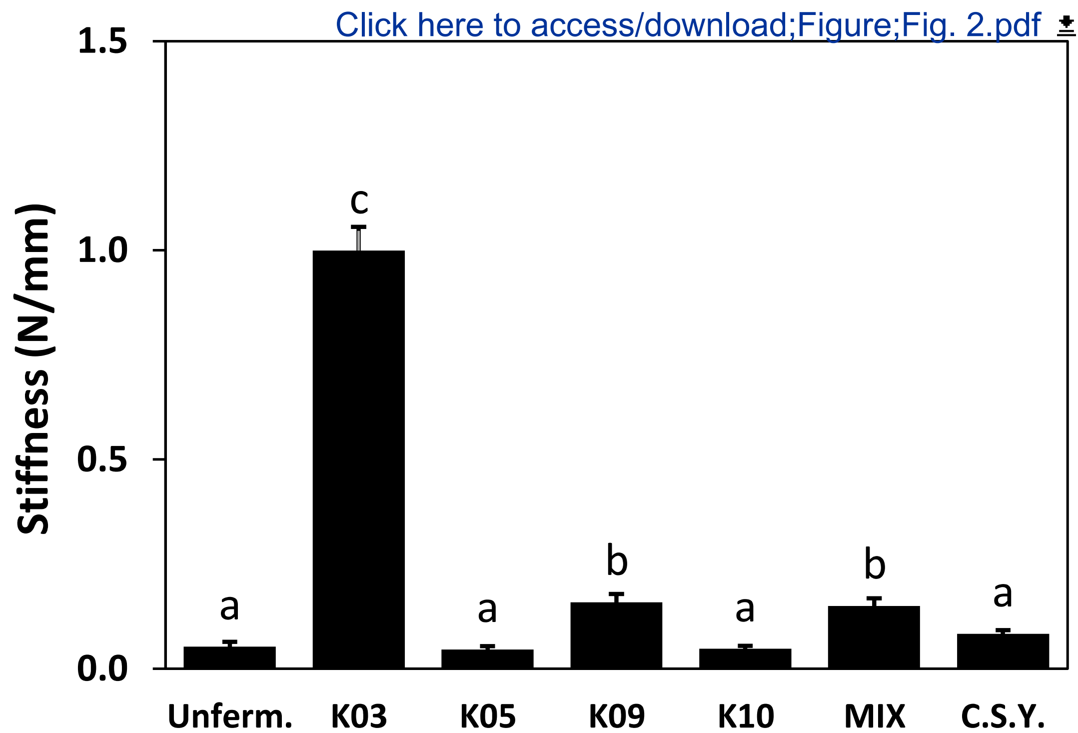

Figure 3

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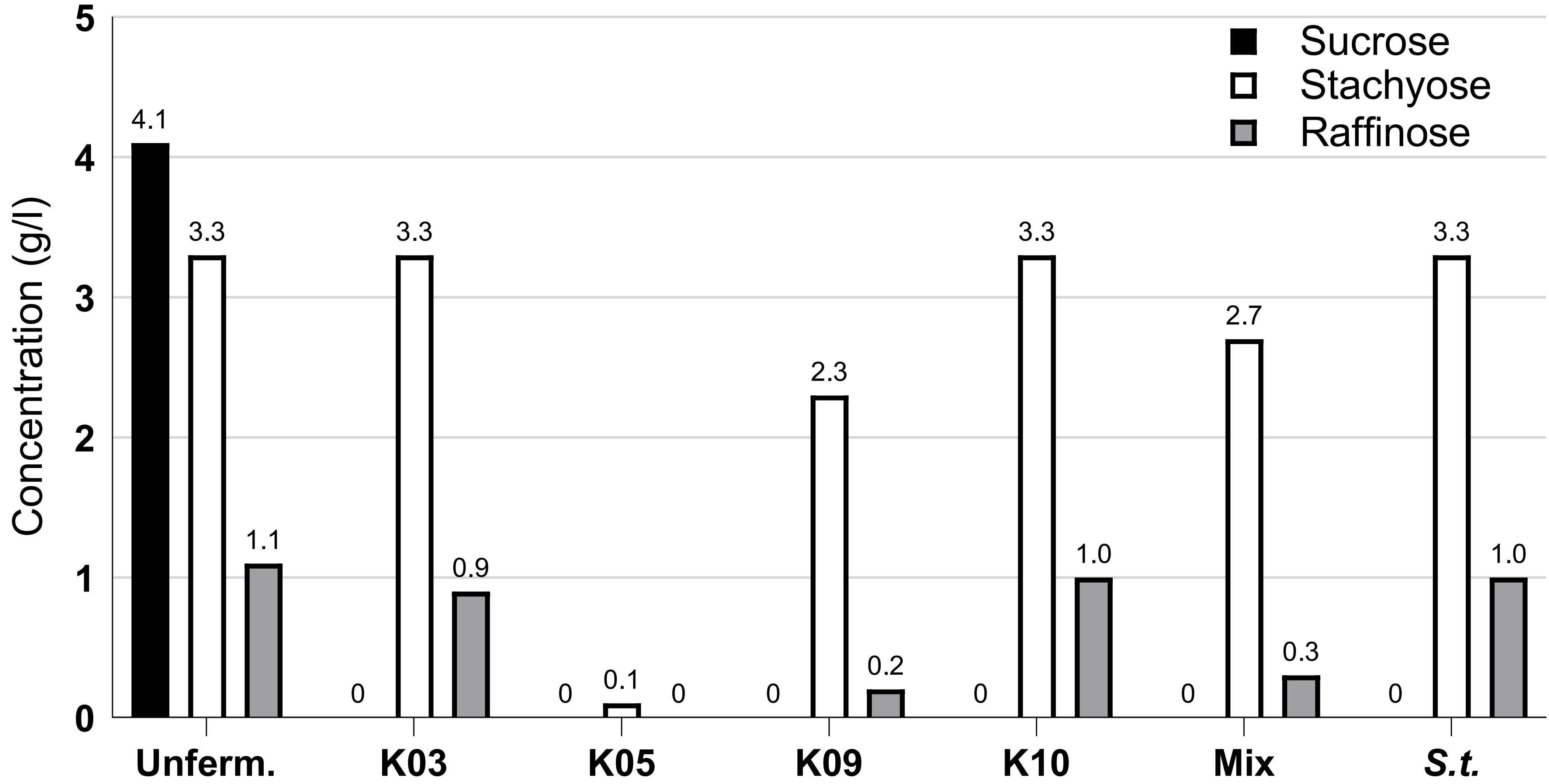

Figure 4

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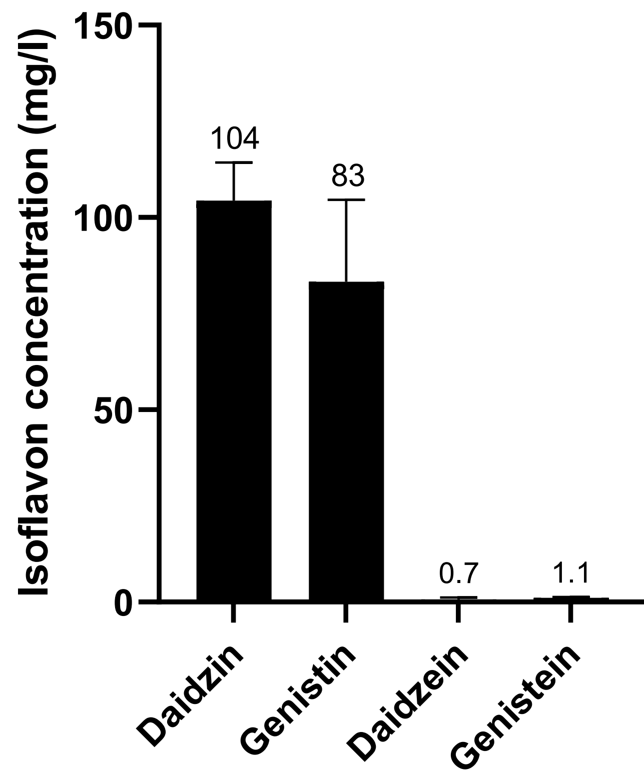

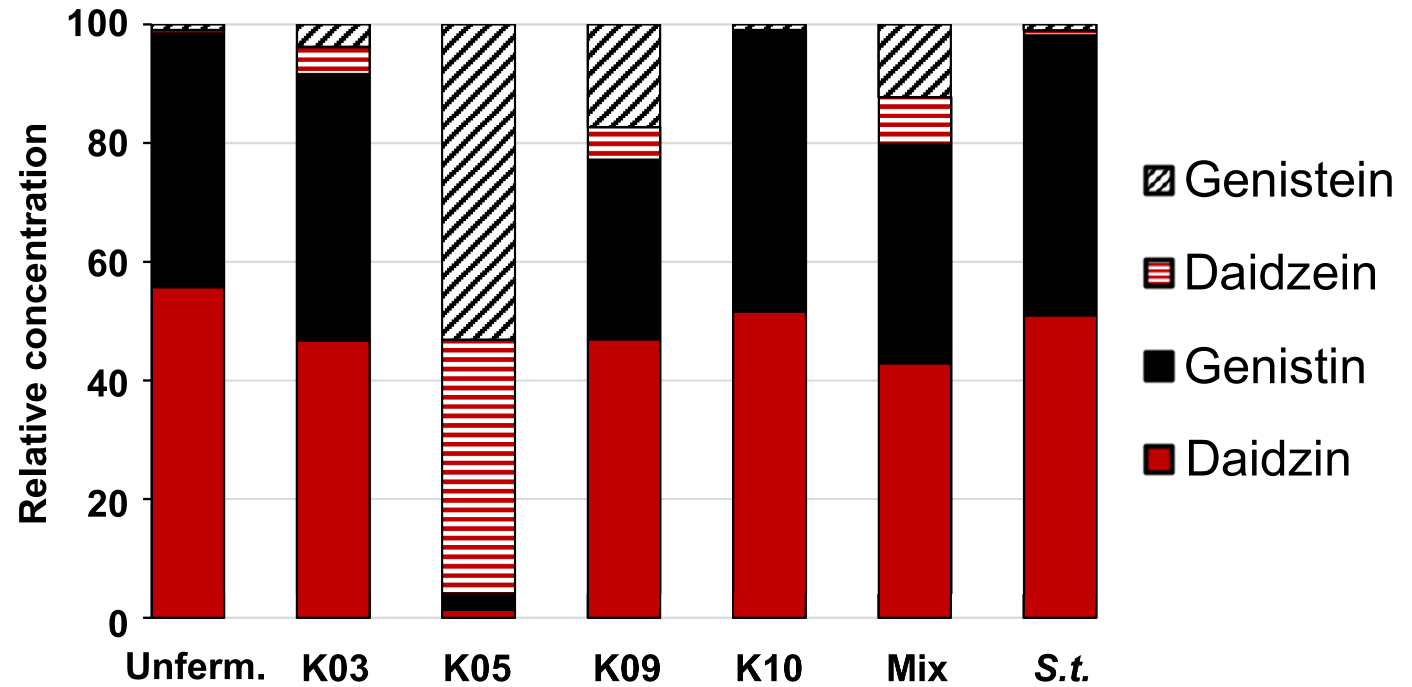

Figure 5

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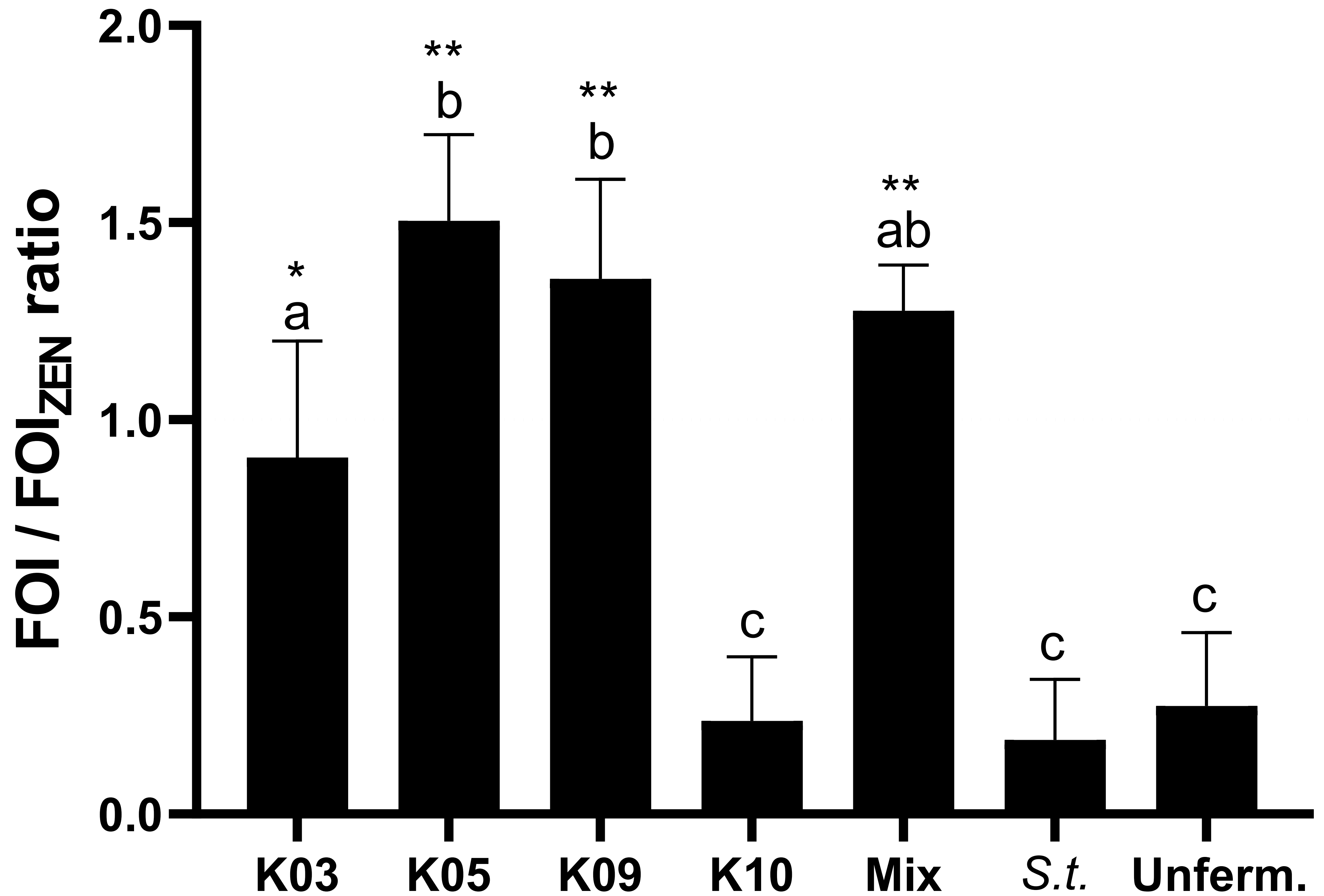

Graphical Abstract