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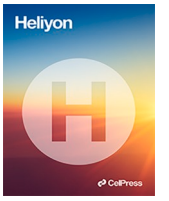

# Consortium of selected yeasts to produce healthy soy fermented beverage: Evaluation of microbial evolution, analytical, sensorial, and functional features

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ABSTRACT

Currently, an increasing number of intolerant and vegan consumers are driving the market towards plant-based milk alternatives. Here, selected probiotic yeasts, belonging to the *Candida zeylanoides*, *Kluyveromyces lactis* and *Debaryomyces hansenii* species, previously characterized for their aptitude to ferment animal milk, were tested in soy milk. Trials at different fermentation times with the developed yeast consortium (Yc) coinoculated with a lactic bacterium commercial strain were carried out.

Yc showed good fermentation performance, conferring distinctive analytical and aromatic properties to the resulted soy fermented beverage, a product similar to an industrial kefir. Analytical determinations did not show significant variations between the end of fermentation and cold storage (4 weeks at 4 °C), indicating full stability. Phenol amounts and antioxidant activity were significantly increased in soy fermented beverage fermented by Yc. All yeasts remained viable until the end of storage with a final concentration of approximately 8 Log CFU/ml, a value suitable for a probiotic commercial claim. Overall, the results suggest that Yc is a promising multistarter candidate for functional soy products.

## 1. Introduction

Kefir is an acidic fermented milk with high nutritional and therapeutic properties that is manufactured by bacteria and yeasts through lactic acid and alcoholic fermentation, respectively [1]. Among several health-promoting characteristics associated with kefir consumption, properties such as antioxidant, antifungal, blood pressure and hypocholesterolaemia regulation and bronchitis prevention have been demonstrated [2–4]. Sugar-fermenting microorganisms involved in domestic or industrial kefir production release lactic acid, alcohol, $CO_2$, β-complex vitamins, and other organic acids in the final product. In recent years, various types of kefirs have been marketed, mostly produced with the use of lactic acid bacteria or yeasts as probiotic microorganisms [5]. Although dairy products, including kefir, are excellent substrates for probiotics, there are some drawbacks related to milk composition, such as hypersensitivities like allergies or intolerance. There is a growing demand for vegetable foods, driven by ethical, ecological and health consumer demands. As a consequence, vegetarian product industries are growing approximately 10 % per year [6]. Moreover, many consumers do not drink dairy milk or derivatives for casein allergy, lactose intolerance or intestinal diseases related to irritable bowel syndrome (IBS).

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A valid alternative to dairy milk could be represented by vegetal beverages, and between them, soy milk reaches the first position. Traditionally, major worldwide soy consumers are identified in Asian populations, but in the last decade, the consumption of soy foods in Western countries has grown with the healthy lifestyle and beneficial perception of soy intake.

Although the sugar component is very high in vegetal milk, soy-based drinks contain functional ingredients such as high-quality protein, isoflavones, dietary fibre and some oligosaccharides with prebiotic properties [7,8]. A limit for the use of soy milk in food formulations is due to its unpleasant taste [9], commonly recognized as beany flavour linked to volatile compounds such as hexanol, 1-hexanol, 2-hexanol, 1-octene3-ol and pentanol. The presence of these off-flavours seems to be bypassed through the microbial fermentation that also increases the nutritional properties of soymilk beverages [10], which is why soy milk could represent a good candidate for vegetable-based, kefir-like fermented beverage.

Based on this evidence, soy milk was used for vegetable-based fermented beverage production in this study, with the general objective of obtaining a healthy vegetable drink by using a consortium of yeasts with probiotic characteristics, formerly tested in a previous work. For this, the fermentation was carried out by mimicking an industrial type of production, bypassing the traditional kefir grains, and inoculating a pool of selected yeast species, already studied with proven probiotic traits that were previously used in dairy milk fermentation [5,11]. Yc was employed in coculture with a commercial *Lactobacillus casei* strain. During the storage period, changes in microbial evolution, the main chemical compositions and sensory properties were evaluated with the aim of extending the knowledge concerning the microbial and physico-chemical diversity of kefir as a functional food influenced by multiple factors, such as matrix type, origin and type of fermenting microbial culture inoculated, fermentation and storage conditions.

The new functional nondairy beverage produced at the laboratory scale can meet the needs of some consumers, including vegans, vegetarians, and people with intolerance/allergy to dairy products, guaranteeing a functional beverage with a pleasant aromatic profile, high-value nutrient composition and the presence of probiotic microorganisms that remain alive until cold storage.

## 2. Materials and methods

### 2.1. Microorganisms: Bacteria and consortium of yeasts

The commercial probiotic bacterium *Lactobacillus casei* Shirota (LbS) was isolated from a Yakult beverage (Yakult Honsha Co., Ltd., Tokyo, Japan). Lbs was used in cofermentation with five different yeasts belonging to the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Ancona, Italy) previously isolated from artisan dairy environments [11]. The yeast consortium (Yc) was composed of two strains of *Debaryomyces hansenii* (36 and 78), *Candida zeylanoides* 13, *Kluyveromyces lactis* 80 and *Yarrowia lipolytica* 92, named Dh, Cz, Kl and Yl, respectively. Yeasts were previously characterized, and the probiotic features of each of them have been demonstrated. Moreover, Yc proposed here was previously tested by Agarbati et al. [5] for its fermentative aptitude to produce conventional kefir.

LbS was cultivated in MRS agar (LIOFILCHEM® S.r.l., Roseto degli Abruzzi, Teramo, Italy) at 37 °C under anaerobic conditions. Yeasts were cultivated in YPD agar (yeast extract, 10 g/l; dextrose, 20 g/l; peptone, 20 g/l; agar, 18 g/l) at 25 °C under aerobic conditions. All microorganisms were maintained at 4 °C for a short time and at – 80 °C for a long time by adding glycerol solution as a cryopreserant.

### 2.2. Viability of Yc tested in soy milk

The ability of Yc to survive in soy milk was carried out using a commercial product (Alpro soia, Alpro, Gand, Belgium), whose composition is reported in Table 1. Each yeast was precultured in YPD broth at 25 °C for 24 h, and then the biomass was recovered by centrifugation (4.000 rpm) and washed twice with 0.9 % NaCl saline solution. Yeasts were coinoculated in sterile glass jars containing 50 ml of soy milk following the setup concentration previously validated by Agarbati et al. [5]. Specifically, $5 \times 10^3$ cell/ml for both *D. hansenii* strains, $5 \times 10^2$ cell/ml for *C. zeylanoides* and *Y. lipolytica*, and $1 \times 10^3$ cell/ml for *K. lactis*. The glass jars were sealed with a sterile screw cap and incubated at 25 °C for 120 h. The test was carried out in triplicate. The ability of the yeasts to survive in soy milk was monitored daily until 120 h through viable cell counts using WL nutrient agar medium (LIOFILCHEM® S.r.l., Roseto degli Abruzzi,

**Table 1**
Main nutritional values of soy milk.

| Main nutritional values (100 ml of product) | |
|---|---|
| Energy (kcal) | 40 |
| Total fat (g) | 1.8 |
| Saturated fat (g) | 0.3 |
| Total carbohydrates (g) | 2.8 |
| Sugars (g) | 2.8 |
| Fibers (g) | 0.4 |
| Protein (g) | 3.0 |
| Salt (g) | 0.1 |
| Calcium (mg) | 160 |
| Vitamin D (μg) | 0.75 |
| Vitamin B2 (mg) | 0.21 |
| Vitamin B12 (μg) | 0.38 |

Teramo, Italy), which allowed the distinction between the species of yeast.

### 2.3. Set-up for soy beverage fermentation

Coculture of LbS and Yc was inoculated in 150 ml of soy milk in sterile glass jars, sealed with a sterile screw cap. LbS biomass was prepared in MRS broth at 37 °C for 48 h, recovered by centrifugation (14.000 rpm), washed twice with 0.9 % NaCl saline solution and inoculated at a $1 \times 10^8$ cell/ml final concentration. Yc was added following the yeast ratio described above, with the exception of *Y. lipolytica,* which did not survive in soy milk.

To produce distinct soy fermented beverage characterized by different aromatic traits, three fermentation sets were carried out. All trials started with a fermentation step carried out at 25 °C in which LbS was inoculated in pure culture and coinoculated with yeast consortium (Yc). Each trial differed in fermentation time: 24, 72 and 120 h, respectively. After this phase, a storage step at 4 °C for 4 weeks simulated the shelf life of the fresh product. All fermentations were carried out in triplicate. The resulting kefirs were analysed for microbial content, main analytical characters, fatty acid content, byproducts of fermentation and sensorial analyses.

### 2.4. Microbial monitoring

Each trial of soy fermented beverage was analysed for the viable microbial content at the end of the fermentation process and the end of the storage period (4 weeks). Viable cell counts were carried out by decimal serial dilutions and spread on MRS agar supplemented with 0.002 % cycloheximide to prevent yeast growth and on WL nutrient agar medium (LIOFILCHEM® S.r.l., Roseto degli Abruzzi, Teramo, Italy) supplemented with 0.005 % chloramphenicol to prevent LbS growth.

MRS agar was incubated at 37 °C for 5 days under anaerobic conditions, while WL nutrient agar was incubated at 25 °C for 3 days before microbial enumeration.

### 2.5. Main analytical characters of soy fermented beverage

All soy fermented beverages (24 h, 72 h, 120 h) were analysed for the main analytical characteristics, such as pH, acetic acid, lactic acid, ethanol, total phenolic compound content (TPCs), antioxidant activity, fatty acid profile (FA), total protein (TP) and total reducing sugars (TRS). All characteristics were evaluated at the end of the fermentation phase and after the storage period at 4 °C.

pH was determined using a pH-metre instrument (Meter S400, Mettler, Toledo). Acetic acid and ethanol were measured using the analytical method according to the Official European Union Methods (EEC, 2000), as described in Ref. [5]. Lactic acid was determined by titration with 0.1 N NaOH solution following the AOAC method (2002). TPCs, expressed as gallic acid equivalents, were determined spectrophotometrically using Folin-Ciocalteu reagent (Sigma–Aldrich, Saint Louis, USA) following the method described by Ref. [12]. The antioxidant activity of the kefir was evaluated using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma–Aldrich, Saint Louis, USA) following the procedure reported by Karaçalı et al. [8]. The results are reported as the percentage of DPPH scavenging, defined by solving the following equation: $[1\text{-}A_{517(sample)}/A_{517(blank)}] \times 100$ %. Soy milk was used as a blank. FA content was determined according to the ISO 16958 (ISO, 2015) gas chromatographic method with direct transesterification of the samples. Total proteins were determined following the procedure described by Bensadoun and Weinstein [13]. TRS was spectrophotometrically determined using dinitrosalicylic acid (DNS) as reported by Ref. [14].

### 2.6. Main byproducts of fermentation

All samples were analysed for the main byproducts of fermentation through the solid-phase microextraction (HS-SPME) gas chromatography method as described by Agarbati et al. [5]. The quantified compounds were ethyl acetate, n-propanol, acetaldehyde, isobutanol, acetoin, amyl alcohol and isoamyl alcohol. The quantification of each compound was obtained by comparison with external calibration curves and it was expressed as a relative percentage value considering the total peak area. The byproducts of fermentation were evaluated in soy fermented beverage samples after 4 weeks of storage.

### 2.7. Sensory analyses of soy fermented beverages

Sensory analyses of the soy fermented beverage were conducted by ten testers. They expressed an opinion regarding colour, texture, viscosity, acidity, bitterness, sweetness and overall appreciable sweetness of samples using a hedonic scale from 1 (dislike extremely) to 10 (like extremely) (Supplementary Fig. S1) [15]. The analyses were carried out at the end of the shelf life of the products. All testers expressed their informed consent regarding the sensory evaluation.

### 2.8. Statistical analyses

Experimental data regarding the main analytical characteristics and byproducts of fermentation were subjected to analysis of variance (ANOVA). The significant differences were determined using Duncan tests with associated $p$ *values* $< 0.05$. Furthermore, the mean values of the same data, normalized to eliminate the influence of hidden factors, were analysed by principal component analysis (PCA). Both statistical analyses were carried out using JMP® 11 statistical software (Statistical discovery from SAS, New York, NY, USA).

## 3. Results

### 3.1. Viability test

The ability of the yeasts to survive in soy milk during 120 h of fermentation is shown in Fig. 1. All yeast species belonging to Yc exhibited an increase of approximately two Log orders of magnitude in the first 24 h of fermentation, then each yeast showed a specific fermentation trend with increase in fermentation time. *D. hansenii* and *K. lactis* grew, reaching a concentration of approximately Log 6.5 CFU/ml at 120 h of fermentation, while *C. zeylanoides* decreased considerably (Log 2.2 CFU/ml, at 72 h) and remained alive. The only exception was observed for the yeast *Y. lipolytica*, which drastically decreased in the first 24 h and disappeared after 48 h of fermentation, highlighting its inability to survive in soy milk.

### 3.2. Microbial kinetics

The growth kinetics of LbS and yeasts in the trials at different times (24 h, 72 h, 120 h) are given in Fig. 2. LbS (dashed lines), inoculated at $10^8$ CFU/ml, increased by approximately one log order of magnitude in all fermentation trials at 25 °C and then remained almost constant during the 4 weeks of cold storage. The presence of yeasts did not modify the growth kinetics of LbS, which was comparable to that of the LbS pure culture in all fermentations.

Some differences were observed regarding the yeast behaviour in relation to the fermentation time (Fig. 2 a, b, c). In the trials fermented for 24 h (Fig. 2a), the three yeast species showed the same growth kinetic trend, reaching, at the end of the shelf life, a concentration of Log 7.5 CFU/ml for Dh and Kl and Log 6.5 CFU/ml for Cz. The two strains belonging to *D. hansenii* species 36 and 78, which were phenotypically indistinguishable, also showed similar fermentation kinetics in trials fermented for 72 h and 120 h (Fig. 2b and c). Otherwise, in both fermentations carried out at 72 h and 120 h, the Kl concentration remained almost at Log 4–5 CFU/ml until the end of cold storage. The yeast Cz showed a continuous decreasing trend up to 4 weeks of storage (Fig. 2b and c).

### 3.3. Main analytical characters of soy fermented beverages

The results of the chemical determinations of the soy fermented beverages fermented for 24, 72 and 120 h by LbS alone and LbS with yeast consortium (Yc) are shown in Table 2. Due to the non-survival of *Y. lipolitica*, the Yc will consist of all yeasts with exception of *Y. lipolitica*. For each trial, analytical results measured after the fermentation phase and after cold storage were compared.

As expected, Yc increased the acidity, and therefore, the pH of the cofermentation trials always showed a significant reduction in pH. The pH value decreased with increasing fermentation time for all trials, especially after 120 h. On the other hand, comparing the pH values between the end of the fermentation phase and cold storage, there were only slight reductions. Yc trials showed a significant increase in acetic and lactic acid. A stable level of these organic acids was detected between the end of fermentation and cold storage, with the only exception of the 120 h trials, where an increase in both acids after storage was shown.

The concentration of total residual sugars (TRS) was generally higher in the control trials (LbS pure cultures) than in the Yc theses. In all samples, TRS decreased with increasing fermentation time, especially in those carried out by Yc. The total radical scavenging activity (DPPH) and phenol content (TPC) of soy fermented beverage significantly increased in the Yc trials. Data trends seemed to be directly associated with the length of the fermentation, and higher values were recorded after 120 h of fermentation. A further increase in these analytical compounds was observed at the end of cold storage compared to the end of the fermentation phase.

The content of TP evaluated after each fermentation time revealed a slight reduction in the presence of Yc compared with pure LbS cultures. The analysis of total proteins (TP) in the Yc trials after cold storage always showed a drastic reduction compared to the residual TP assayed after fermentation times. After cold storage, the amount of residual protein in the trials fermented for both 72 h and 120 h was similar (approximately 10 g/l). This result indicated proteolytic activity by yeasts at low temperatures.

Ethanol was detected at a very low concentration of 0.05 % v/v in all trials and was not reported.

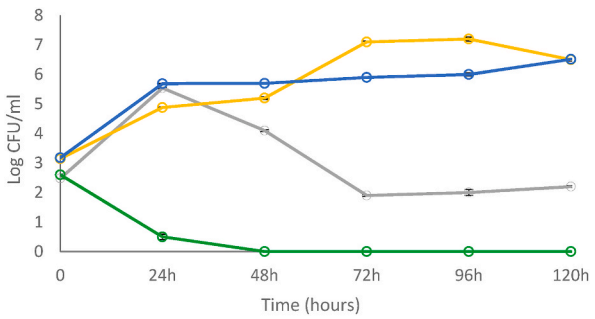
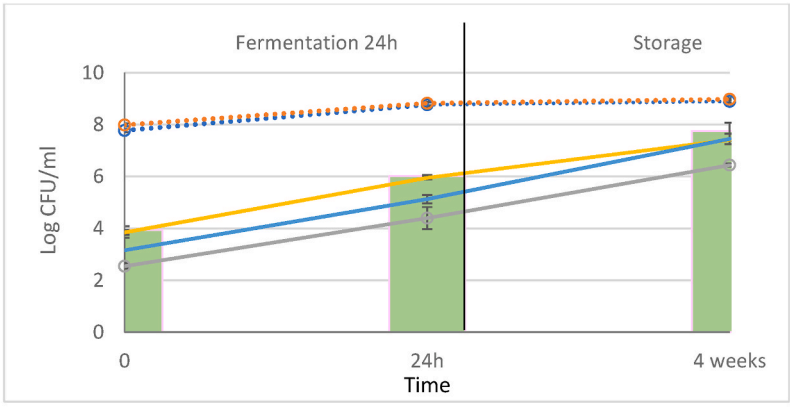
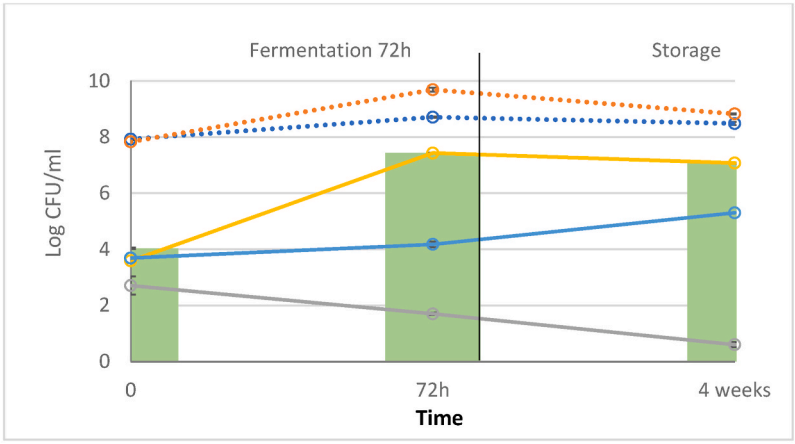


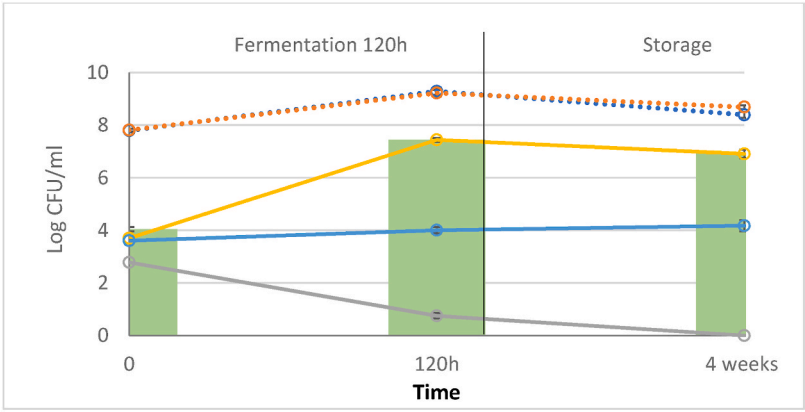

**Fig. 1.** Ability to survive in soy milk during 120 h of fermentation at 25 °C: (—○—) for *C. zeylanoides*; (—○—) for *D. hansenii* strains 36 and 78; (—○—) for *K. lactis*; (—○—) for *Y. lipolytica*. Data means ± standard deviations are represented as error bars.




(a)




(b)




(c)

**Fig. 2.** Growth kinetics of LbS and Yc detected at the end of fermentation and storage phases. LbS (dashed lines) in pure culture (····) and in coculture with Yc (····); (▬) for Yc; (—) for *C. zeylanoides*; (—) for *D. hansenii* strains 36 and 78; (—) for *K. lactis*. (a), (b), (c) represent trials with different fermentation times of 24, 72 and 120 h, respectively. Data means ± standard deviations are represented as error bars.

### *3.4. Main byproducts of fermentation*

The concentrations of the fermentation byproducts detected at the end of storage are reported in Table 3. Overall, soy fermented beverages fermented with Yc (without *Y. lipolitica*, not survived) showed significant differences compared with those inoculated with only LbS. In particular, acetaldehyde was not produced by the LbS trials, while Yc produced similar amounts at 24 and 72 h of fermentation (7–11%), while the amount was doubled after 120 h. Ethyl acetate showed relative percentages almost two times higher in Yc than in LbS in the 24 h fermentation trials. These differences were reduced with the increase in fermentation hours until being greater in the LbS than Yc in the 120-h thesis. Isobutanol was not produced by LbS at 24 and 72 h and increased in all Yc-fermented

**Table 2**
Main analytical characters of soy fermented beverages produced after different fermentation times (24, 72 and 120 h). Within each column are reported the data relatively to each soy fermented beverage at the end of the fermentation phase and after the cold storage at 4 °C. Data means ± standard deviations and values showing different superscript letters ($^{a,b,c,d,e,f,g,h,I,l}$) within each line are significantly different according to Duncan tests ($p < 0.05$).

| | 24 h-Fermentation | | Cold Storage | | 72 h-Fermentation | | Cold Storage | | 120 h-Fermentation | | Cold Storage | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LbS | Yc | LbS | Yc | LbS | Yc | LbS | Yc | LbS | Yc | LbS | Yc |
| pH | 6.60 ± 0.07$^{a}$ | 5.09 ± 0.01$^{d}$ | 6.37 ± 0.01$^{b}$ | 4.92 ± 0.13$^{e}$ | 6.39 ± 0.05$^{b}$ | 4.47 ± 0.06$^{f}$ | 5.55 ± 0.01$^{c}$ | 3.92 ± 0.02$^{h}$ | 4.36 ± 0.05$^{fg}$ | 4.00 ± 0.04$^{h}$ | 4.27 ± 0.00$^{g}$ | 3.71 ± 0.06$^{i}$ |
| Acetic acid (g/l) | 0.43 ± 0.09$^{e}$ | 0.44 ± 0.04$^{e}$ | 0.60 ± 0.03$^{cd}$ | 0.51 ± 0.08$^{de}$ | 0.40 ± 0.03$^{e}$ | 0.45 ± 0.00$^{e}$ | 0.42 ± 0.01$^{e}$ | 0.62 ± 0.06$^{c}$ | 0.66 ± 0.01$^{c}$ | 0.79 ± 0.03$^{b}$ | 0.78 ± 0.03$^{b}$ | 0.91 ± 0.04$^{a}$ |
| Lactic acid (%) | 0.25 ± 0.03$^{ef}$ | 0.23 ± 0.01$^{ef}$ | 0.29 ± 0.03$^{e}$ | 0.27 ± 0.03$^{ef}$ | 0.23 ± 0.01$^{ef}$ | 0.16 ± 0.01$^{f}$ | 0.27 ± 0.00$^{ef}$ | 0.25 ± 0.03$^{ef}$ | 0.54 ± 0.05$^{d}$ | 0.27 ± 0.00$^{e}$ | 0.712 ± 0.03$^{c}$ | 2.00 ± 0.14$^{a}$ |
| TRS (g/l) | 21.87 ± 0.67$^{a}$ | 9.66 ± 0.39$^{f}$ | 18.76 ± 0.30$^{b}$ | 7.50 ± 0.28$^{gh}$ | 17.10 ± 0.66$^{c}$ | 8.28 ± 0.02$^{g}$ | 15.17 ± 0.19$^{d}$ | 6.69 ± 0.13$^{hi}$ | 15.76 ± 0.34$^{d}$ | 5.93 ± 0.08$^{i}$ | 11.85 ± 0.51$^{e}$ | 3.30 ± 0.14$^{i}$ |
| DPPH (%) | 28.66 ± 1.80$^{de}$ | 31.85 ± 0.29$^{de}$ | 29.62 ± 0.84$^{de}$ | 34.71 ± 0.58$^{cd}$ | 26.62 ± 0.45$^{ef}$ | 35.03 ± 0.39$^{bcd}$ | 22.17 ± 0.84$^{f}$ | 39.39 ± 0.90$^{bc}$ | 26.94 ± 0.77$^{ef}$ | 41.08 ± 0.32$^{b}$ | 29.17 ± 0.06$^{de}$ | 46.94 ± 0.13$^{a}$ |
| TPCs (g/l) | 0.48 ± 0.04$^{de}$ | 0.52 ± 0.01$^{cd}$ | 0.46 ± 0.05$^{de}$ | 0.58 ± 0.02$^{c}$ | 0.43 ± 0.07$^{de}$ | 0.68 ± 0.02$^{b}$ | 0.40 ± 0.04$^{e}$ | 0.76 ± 0.07$^{b}$ | 0.47 ± 0.06$^{de}$ | 0.86 ± 0.02$^{a}$ | 0.47 ± 0.01$^{de}$ | 0.91 ± 0.00$^{a}$ |
| TP (g/l) | 25.80 ± 0.48$^{a}$ | 24.54 ± 0.26$^{ab}$ | 25.14 ± 0.38$^{a}$ | 16.01 ± 0.36$^{d}$ | 23.82 ± 0.23$^{bc}$ | 21.46 ± 0.66$^{bc}$ | 23.91 ± 0.23$^{bc}$ | 10.06 ± 0.02$^{e}$ | 22.58 ± 0.50$^{c}$ | 17.42 ± 0.20$^{d}$ | 23.05 ± 0.63$^{abc}$ | 10.66 ± 0.23$^{e}$ |

**Table 3**
Main byproducts of fermentation evaluated at the final step of storage (4 weeks) of each trial (24 h-Fermentation, 72 h-Fermentation, 120 h-Fermentation). Data means ± standard deviations and values showing different superscript letters ($^{a,b,c,d}$) within each line are significantly different according to Duncan tests ($p < 0.05$).

| Main by-products (relative %) | 24 h-Fermentation | | 72 h-Fermentation | | 120 h-Fermentation | |
|---|---|---|---|---|---|---|
| | LbS | Yc | LbS | Yc | LbS | Yc |
| Acetaldehyde | 0.00 ± 0.00$^{d}$ | 7.51 ± 0.16$^{c}$ | 0.00 ± 0.00$^{d}$ | 11.26 ± 0.08$^{b}$ | 0.00 ± 0.00$^{d}$ | 24.27 ± 0.07$^{a}$ |
| Ethyl acetate | 49.08 ± 0.35$^{bc}$ | 75.33 ± 0.79$^{a}$ | 37.16 ± 0.63$^{c}$ | 50.77 ± 0.06$^{b}$ | 68.03 ± 1.15$^{ab}$ | 54.32 ± 0.20$^{b}$ |
| n-propanol | 36.99 ± 0.35$^{b}$ | 4.08 ± 0.28$^{d}$ | 49.12 ± 1.35$^{a}$ | 21.52 ± 0.14$^{c}$ | 23.91 ± 1.29$^{c}$ | 6.66 ± 0.69$^{d}$ |
| Isobutanol | 0.00 ± 0.00$^{c}$ | 2.19 ± 0.18$^{b}$ | 0.00 ± 0.00$^{c}$ | 4.30 ± 0.28$^{a}$ | 2.38 ± 0.19$^{b}$ | 4.65 ± 0.37$^{a}$ |
| Amyl alcohol | 0.43 ± 0.00$^{d}$ | 5.96 ± 0.24$^{b}$ | 2.94 ± 0.14$^{c}$ | 7.63 ± 0.08$^{a}$ | 0.00 ± 0.00$^{d}$ | 5.71 ± 0.36$^{b}$ |
| Isoamyl alcohol | 8.85 ± 0.67$^{a}$ | 1.44 ± 0.10$^{c}$ | 7.40 ± 0.04$^{a}$ | 0.00 ± 0.00$^{d}$ | 3.85 ± 0.24$^{b}$ | 2.55 ± 0.18$^{bc}$ |

trials. Amyl alcohol was produced at significantly higher amounts by Yc than LbS. Isoamyl alcohol was produced mostly by LbS with a behaviour that decreases with increasing fermentation time. The same alcohol was produced in variable amount in Yc theses (<3%), regardless of the fermentation time. Acetoin was not detected in all trials and thus was not reported.

The overall contribution of Yc to the final product in relation to the different fermentation times compared to the LbS pure culture

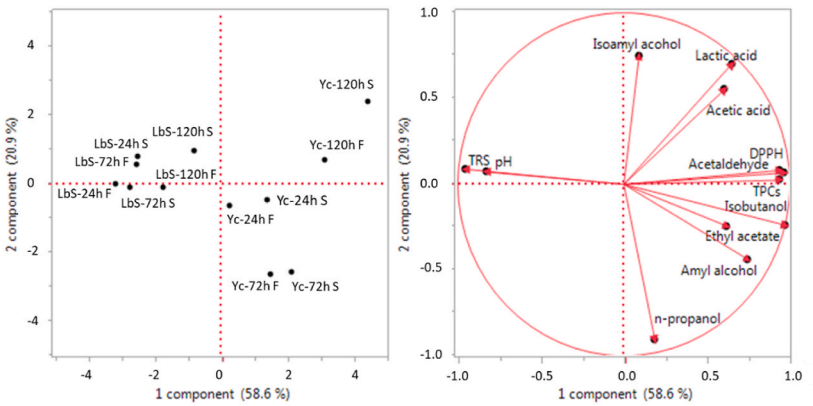



**Fig. 3.** Principal component analysis (PCA) of the main analytical characteristics and byproducts of fermentation of all samples. Samples are reported as LbS (pure bacterial culture) and Yc (yeast consortium in cofermentation with bacteria) associated with fermentation time (24 h, 72 h, 120 h) and the letters S or F to indicate samples at the end of fermentation and cold storage, respectively.

and the stability of each soy fermented beverage from the end fermentation until the end of the storage period was analysed by principal component analysis (PCA), and the results are reported in Fig. 3. The results highlighted the greater complexity of Yc soy fermented beverage, independent of the fermentation time, due to yeast metabolism during the fermentation processes compared with the fermentation carried out by LbS. Indeed, all soy fermented beverages fermented by Yc were grouped on the right side of the graph, while all LbS kefirs were grouped on the left side of the graph (PC1 58.6 %). Furthermore, all LbS kefirs were grouped close together, showing little difference in relation to the duration of fermentation, and differed mainly from Yc kefirs in pH values and TRS content. Regarding Yc soy fermented beverage, they showed greater differences in relation to the fermentation time. Indeed, Yc soy fermented beverage fermented at 120 h were grouped in the upper right quadrant, while those fermented at 24 h and 72 h were grouped in the lower right quadrant (PC2 20,9 %). As expected, the Yc soy fermented beverage fermented for 120 h differed mainly in the content of organic acids.

Regarding the influence of the storage period, the same inoculated trials at the end of fermentation (F) and storage (S) were grouped close together, underlining the stability of the product until the presumed expiration date.

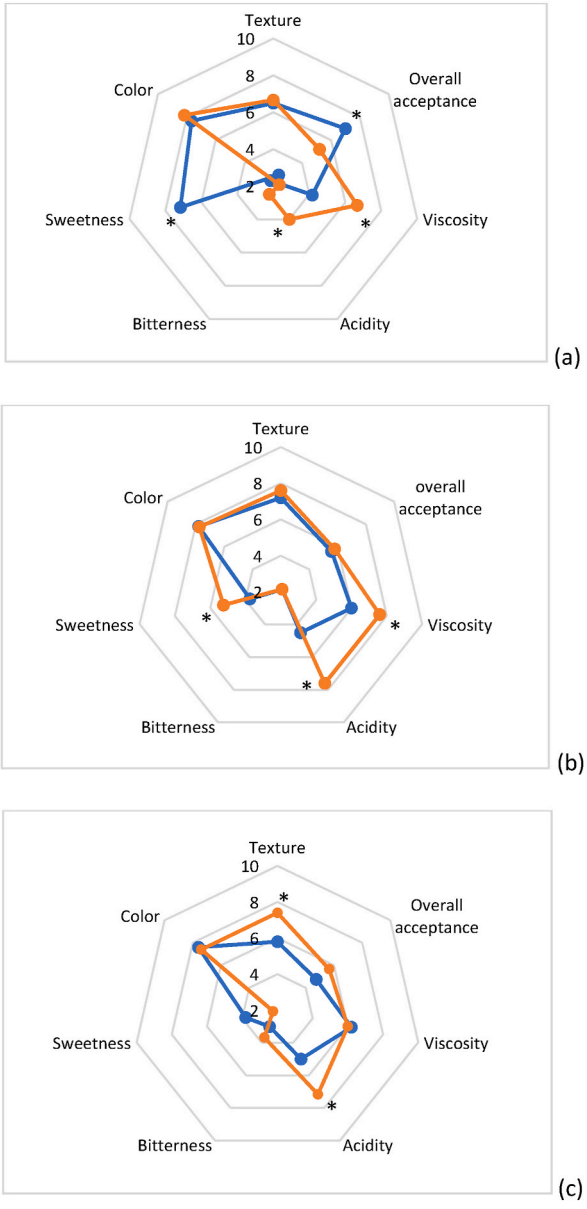



**Fig. 4.** Sensory analysis of soy kefirs-like. (a), (b), (c) Graphs represent the results of the testing of soy fermented beverage fermented for 24 h, 72 h and 120 h, respectively. (—●—) for LbS; (—●—) for Yc. * = Significant differences between trials.

### 3.5. Sensorial analysis

The organoleptic evaluation of all soy fermented beverages is reported in Fig. 4. In general, the kefirs-like prepared with soy milk were positively evaluated by all testers. Indeed, the data comparing the overall acceptance properties highlighted that kefirs-like from Yc were always preferred, obtaining higher acceptability values than the others. The only exception was represented by LbS fermented for 24 h (Fig. 4a), which reached a higher score, probably in association with a higher sweetness note. The soy fermented beverage samples fermented for 72 h and 120 h (Fig. 4b and c) by Yc reached a higher acidity score, supported by lower pH values than those fermented for 24 h. Nevertheless, the overall acceptance of these soy fermented beverage samples was comparable to or greater than that of the pure LbS culture.

Moreover, the soy fermented beverage fermentation for 72 h by Yc (Fig. 4b) also achieved higher scores for viscosity and sweetness than the pure LbS culture. After a comparative analysis of the other descriptors, greater suitability of the soy fermented beverage fermented by Yc emerged, which probably confers greater complexity to the finished product. Conversely, LbS trials show good texture, good colour and medium viscosity.

## 4. Discussion and conclusion

Good flavour is the most important attribute of a food product, determining the possibility of success in the market [16]. In this study, a well-established Yc, previously tested during conventional milk fermentation [5], was tested in soy milk.

Within the current market, which is increasingly rich in foods with potential benefits for consumer health, nondairy-based analogues and all soy products represent an important segment. Among analogues of milk and fermented milk products, soy products are widely perceived as healthier, less caloric, and suitable for vegan diets or intolerant consumers. However, disagreeable bean flavour remains one of the most important aspects of soy beverages and foods that reduces consumer acceptability. Moreover, its content in raffinose and stachyose, which produce flatulence, represents another limitation [17,18]. In contrast, the related soy milk fermented products generally showed improved flavour and texture, as well as enhanced beneficial health properties [19]. On the other hand, several studies have shown that soy products, particularly soy yogurt, may be good vehicles for probiotic bacteria [20,21]. Similarly, in this work, the positive effect on the aroma and taste of the fermentation carried out by Yc consortium (without *Y. lipolitica*, not survived) was revealed in the final soy fermented beverage. The viability of all probiotic yeasts that composed the consortium until 4 weeks of soy fermented beverage storage at 4 °C further strengthens the functional effect of this fermented product. Effectively, the maintenance of viable yeasts even during cold storage guarantees an even minimal metabolic activity which is essential for the maturation of the product in terms of production of secondary compounds. Moreover, in accordance with data published by Yang et al. [22], the overall results demonstrated that the soy fermented beverage ecosystem remained quite stable since Yc (yeast consortium), and LbS did not show negative interactions between them. Specifically, the proliferation and metabolic activities of inoculated microorganisms that slowly continue during cold storage may contribute to peculiar sensory characteristics such as sweetness, acidic taste and refreshing flavour.

In this regard, there are various studies on the potential positive effect of probiotic bacteria used to produce soy yogurt. Champagne and coworkers [23] demonstrated that fermented soy milk obtained with the presence of *Bacillus natto*, *Bacillus subtilis* and *Bifidobacterium* spp. Represents a good source of bioactive peptides, such as anti-ACE, antioxidative and immunomodulatory properties. Moreover, Bedani et al. [24] recently focused on the anticancer properties of bioactive compounds of soy fermented beverage, such as polysaccharides and peptides. Bau et al. [25] demonstrated the functionality of soy products supplemented with soy fibre and fermented with probiotic *Lactococcus lactis*.

On the other hand, works regarding the potential role of probiotic yeasts in soy-based fermented products are lacking in the literature. The addition of probiotic Yc in cofermentation with recognized commercial probiotic bacteria positively influenced the analytical characteristics of soy fermented beverage. Indeed, the presence of yeasts increased the functional properties of this fermented product, improving the antioxidant activity and phenolic compound content, both commonly known for their benefits to human health [26]. Moreover, the absence of ethanol guarantees the possibility of using the claim “functional” to this soy fermented beverage [27].

The fermentative activity of yeasts contributes to reducing the residual sugars in the final products, making it suitable for consumers who must/want to follow a low-sugar diet. Indeed, the soy milk present in the market is generally enriched with sugar to improve consumer pleasantness; otherwise, it is hardly appreciated in taste due to the presence of off-odours [28,29].

In recent decades, it has become well known that soy foods contain an array of biologically active compounds called phytochemicals that may confer important health benefits [30]. In particular, well-characterized soy proteins are considered a rich source of isoflavone compounds that possess antihemolytic, antioxidative, antifungal, oestrogenic and antitumour activities. In fermented soy foods, isoflavones are in the aglycone form that is most biologically active [31]. On the other hand, isoflavones are the source of compounds involved in undesirable tastes, such as bitter and astringent. The fermentation process represents a key phase to convert thorough microbial metabolism, off-odours in desirable molecules, reduce unpleasant taste and maintain the functional role of this protein class [8]. Effectively, after fermentation, the texture and taste improved, diminishing the bitterness, astringency, and vegetable flavour, such as in tofu preparation [32].

Based on these statements, this work showed the potential of a fermented product, combining the benefits of fermentation byproducts of yeasts and their probiotic power [5]. This evidence is appreciated by the panel experts who positively evaluated the soy fermented beverage product. Furthermore, the aforementioned characteristics remained unchanged. Among the three types of soy fermented beverages fermented also by Yc, those fermented for 72 h were the most appreciated, particularly for their acidity balanced

by sweetness. This fermentation time could represent a valid compromise between the rapidity of the process required for industrial production and the time necessary for yeasts to release metabolites such as organic acids, ethanol, and aromatic compounds [22], as typically happens in artisanal production.

In conclusion, this work demonstrated the synergistic action of a probiotic consortium (LAB and yeasts) in the production of soy-based fermented beverage. This consortium enhanced the final products in different ways: (i) yeast and LAB remained viable until the presumed expiration date at a concentration suitable to satisfy the probiotic claim; (ii) Yc increased the functional properties of the soy fermented beverage through the release of bioactive metabolites; and (iii) Yc reduced unpleasant flavour (linked to soybean milk) and released desirable compounds through the fermentation process, increasing the aroma complexity of the final product.

This work had the main purpose of evaluating the fermentative efficacy and the functional contribution of a consortium of probiotic yeasts, previously tested in the production of a traditional animal milk-based kefir. Here, the effectiveness of the consortium was also demonstrated in a vegetable matrix such as soy milk with the aim to enter the market, in the future, after appropriate validation studies, a little-known product such as soy kefir. Indeed, although in recent years various types of fermented yoghurt starting from soybean milk have begun to appear on the market, no soy kefir is currently present.

For these reasons, Yc could be proposed as a functional multistarter able to obtain soy fermented beverage in a standardized and controlled process. Further research will be needed to determine if Yc can be applied under pilot and/or industrial scales to place appreciable and reproducible products on the market.

## Ethics statement

The experiments were conducted according to established ethical guidelines. All voluntary participants in the sensory panel gave written consent to take part in the experiment. They were previously informed of the purpose and the procedures of the study, ensuring their confidentiality of the data. Participants were in good health and had no known allergy to the components tested.

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## Data availability statement

Data included in article/supplementary material/referenced in article.

## CRediT authorship contribution statement

**Alice Agarbati:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Maurizio Ciani:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Laura Canonico:** Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing. **Francesca Comitini:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

## 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.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20979.

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