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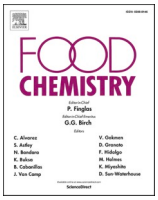

Effect of commercial yeasts (*Saccharomyces cerevisiae*) on fermentation metabolites, phenolic compounds, and bioaccessibility of Brazilian fermented oranges

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ABSTRACT

Brazil is the largest producer of oranges worldwide, as well as one of the largest producers of orange juice. Alcoholic fermented beverages have been considered a marketable alternative for oranges. In this study, four *S. cerevisiae* commercial yeasts were evaluated for metabolites generated during orange juice (cv. 'Pêra D9') fermentation. Alcohols, sugars, and organic acids were evaluated by HPLC-DAD-RID during fermentation, and phenolic compounds were analyzed in fermented orange. Orange juice and fermented oranges were also subjected to digestion simulations. The yeasts presented an adequate fermentation activity, based on sugar consumption, and high ethanol (>10.5%) and glycerol (4.8–5.5 g/L) contents. The yeast strains T-58 and US-05 produced high levels of lactic acid. Phenolic compounds and antioxidant activity did not differ amongst yeasts, presenting hesperidin levels between 115 and 127 mg/L, respectively. The fermented orange showed a >70% bioaccessibility, compared to juice, especially for catechin, epigallocatechin-gallate, procyanidin-B2, rutin, and procyanidin-B1.

1. Introduction

Citrus fruits, such as oranges (*Citrus sinensis* L.), are among the most widely cultivated worldwide. In 2020, Brazil produced 16.5 million tons of oranges. The FAO estimates that > 67% of the world's production of orange juice (FAO, 2020; USDA, 2021) stems from Brazil, meaning that Brazil is the largest orange juice producer in the world. The production is concentrated in the states of São Paulo and Minas Gerais, but other states in Brazil have invested in the production of oranges including the states of Paraná (Costa, Neves & Telles, 2020), Bahia, and Sergipe. Recently, the lower-middle San Francisco Valley, in the Brazilian semi-arid region, has cultivated several new Brazilian citrus cultivars, such as cv. 'Pêra D9', 'Pêra D12', 'Pêra C21', 'Natal 112', 'Valencia Tuxpan', 'Baianinha', and 'Bahia' (Coelho et al., 2021).

Oranges are mostly processed to produce whole and concentrated juices. However, recent market surveys suggest that alcoholic beverages such as fermented orange may be a viable alternative for the market (Kelebek et al., 2009; Lee et al., 2013; Wu et al., 2017). Oranges have several bioactive phenolic compounds such as hesperidin, naringin, naringenin, quercetin, rutin, and gallic, caffeic, *p*-coumaric and chlorogenic acids, which are associated with high antioxidant activity, and provide health benefits when consumed (Wang, Chuang & Ku, 2007; Coelho et al., 2021; Noori et al., 2022). The quantification of the chemical compounds of juice from the new Brazilian orange cultivars, planted in the lower-middle San Francisco Valley in northeastern Brazil, has revealed high concentrations of sugars (approximately 90 g/L), low acidity (<10 g/L), high phenolic content (e.g., hesperidin), and high antioxidant activity (Coelho et al., 2021). These characteristics are ideal

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for the production of fermented beverages, such as fermented orange.

Consumption of fermented orange has been reported to reduce risk factors associated with cardiovascular diseases in healthy mice. The main benefits are associated with phenolic compounds and to its moderate alcohol content (Escudero-López et al., 2015). However, to ensure that the beneficial effects can be maximized, phenolic compounds must be bioaccessibile. This means that after ingestion, the compounds must be stable to be able to achieve systemic circulation, having the ability to pass through the intestinal barrier (intestinal absorption) (Lingua, Wunderlin & Baroni, 2018). In the study by Stinco et al. (2020) the main bioaccessible phenolic compound in orange juice was hesperidin, however the simulation of digestion was performed up to the intestinal phase. According to Macêdo et al. (2023), the fermentation of Brazilian fruits by non-*Saccharomyces* yeasts has strongly increased the bioaccessibility of phenolics in the simulation of *in vitro* digestion.

The composition and quality of fermented orange have been previously reported in the literature. Such studies have investigated the chemical composition in the 'Kozan' cultivar; fermented beverages obtained by spontaneous fermentation (Kelebek et al., 2009); the influence of *S. cerevisiae* strains on the volatiles profile of fermented orange (Lee et al., 2013); evaluation of different processing protocols on final quality (Schvab et al., 2015); and the use of pectinases to change the final methanol content of the fermented beverage (Wu et al., 2017). The effect of different *Saccharomyces cerevisiae* strains on the bioactive phenolic compounds content, antioxidant capacity and bioaccessibility of fermented orange has not been extensively studied before. To address this gap in the literature, we aimed to evaluate the differences in the influence of phenolic bioactive compounds content, antioxidant capacity, and chemical composition of Brazilian fermented orange cv. 'Pêra D9', using four different commercial strains of *S. cerevisiae*. In this study, fermented oranges were produced and the bioaccessibility of phenolic compounds using a digestion model with intestinal barrier simulation was evaluated.

2. Materials and methods

2.1. Fruit harvesting, juice extraction and preparation

Brazilian oranges cv. 'Pêra D9' (*Citrus sinensis*) were obtained from Embrapa semi-arid station, located in the lower-middle San Francisco Valley in Petrolina, Pernambuco, Brazil (9°23′54″ S, 40°30′02″ W). The fruits were harvested during the commercial maturation stage, based on the size and color of the peel. Juice was obtained from 100 kg of oranges: the fruits were cut in half and squeezed using an electric juicer model E-10 Turbo 250 W (Mondial®, Brazil), avoiding rupturing the orange albedo, to prevent bitterness. To the juice, 50 ppm of sulfite, and 6 mL/100 L pectinase Endozym Pectofruit PR (AEB Biochemistry, Brazil), was added and the mixture was allowed to incubate at room temperature for 60 min. to breakdown pectin. Bentonite (0.4 g/L of the juice) was later added and the mixture refrigerated at 5 °C for 48 hrs. Sucrose was added to the supernatant to achieve 18 °Brix, and a bio-activator (Nutrozim®, Ever, Italy) was added at a dose of 200 mg/L to improve the fermentation matrix.

2.2. Evaluation of commercial S. cerevisiae strains in orange juice fermentation and chemical composition of the fermented product

During fermentation, four yeast strains were tested; two *S. cerevisiae* ale strains, traditionally used in beer manufacturing: SafAle™ US-05 (yeast with neutral fermentation profile) and SafAle™ T-58 (yeast selected for its strong fermentation character, and production of fruity esters) (Fermentis, Lesaffre, France); and two *S. cerevisiae* var. *bayanus* used in wine manufacturing: Red Star - Premier Classique (Montrachet) and Red Star - Premier Cuvée (Lesaffre, France). The juice (400 mL in 500 mL flasks) was inoculated individually with 0.55 g/L of yeasts US-05 and T-58, and closed with airlocks, according to manufacturer recommendations. For Premier Classique (Pclass) and Premier Cuvée (Pcuvée), 0.2 g/L of yeasts were used for inoculation. All closed flasks were incubated at 18 ± 0.5 °C in a biological oxygen demand - BOD incubator (Caltech, Recife, Brazil). For each yeast tested, fermentation experiments were carried out in triplicate. The state of alcohol fermentation was monitored daily by measuring concentrations of glucose, fructose, sucrose, ethanol, glycerol, and methanol using HPLC-RID (Refractive Index Detector) methodology described by Viana et al. (2021). At the end of fermentation, analyses of phenolic compounds and organic acids were performed in HPLC-DAD (Diode Array Detector), in addition to antioxidant capacity.

2.3. Bioaccessibility evaluation

Three 10 L fermentation bottles (triplicate) containing the orange juice (8 L) were inoculated with Premiere Classique (Pclass) yeast (200 mg/L). Alcohol fermentation was carried out at 18 °C. After fermentation, clarification was performed by adding 50 g /100 L silica sol, and the fermented oranges were refrigerated at 5 °C for 7 days. Later, free sulfite was adjusted to 40 mg/L and the fermented orange was bottled in 750 mL Bordeaux bottles. Bottles were sealed with an agglomerated natural cork and after 30 days of storage, the *in vitro* gastrointestinal digestion simulation was performed.

2.4. Wine quality analysis

The orange juice and the fermented oranges were evaluated for density, free sulfite, pH (PHS-3B digital bench potentiometer (Tecnal, Brazil)), °Brix (HI 96,801 digital refractometer (Hanna, United States)), and titratable acidity (expressed as g/L of citric acid), following the procedures of the International Organization of Vine and Wine (OIV, 2011).

2.5. Antioxidant capacity

The antioxidant capacity (AOX) of the samples was evaluated by using the free radical scavenging methods $ABTS^{\bullet+}$ (Re et al., 1999) and $DPPH^{\bullet}$ (Kim, Guo & Packer, 2002). Trolox was used as the standard to construct a calibration curve and the results were expressed as Trolox equivalent per liter (mmol TE/L). The orange juice and fermented oranges were centrifuged at 3000 *g* for 5 min. The absorbance (734 nm) readings were carried out in a spectrophotometer UV–vis model UV 2000A (Instrutherm, Brazil).

$DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) scavenging activity was measured spectrophotometrically at $\lambda = 517$ nm. The analysis was performed by mixing a 100 μL sample with 2.9 mL $DPPH^{\bullet}$ radical ethanolic solution (100 μM), followed by incubation in the dark for 30 min. $DPPH^{\bullet}$ solution was diluted with ethanol to achieve an absorbance value of 0.950 ± 0.050 at 517 nm.

In the $ABTS^{\bullet+}$ method, the antioxidant activity was determined by the decay rate of the absorbance ($\lambda = 754$ nm) of the $ABTS^{\bullet+}$ radical, which was produced by the reaction between 5 mL of $ABTS^{\bullet+}$ 7 mM and 5 mL of potassium persulfate 2.45 mM. The mixture was kept in the dark for 16 h prior to analysis. Later, the $ABTS^{\bullet+}$ solution was diluted with ethanol to adjust the initial absorbance to 0.700 ± 0.050 at 734 nm. 30 μL of the sample was added to 3.0 mL of the $ABTS^{\bullet+}$ solution and the readings were performed immediately and after 6 min of reaction.

The FRAP method was performed according to the methodology recommended by Rufino et al. (2006), with some modifications. Briefly, the FRAP reagent was prepared by mixing 25 mL of acetate buffer solution (300 mM; pH 3.6), 2.5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL of $FeCl_3$ aqueous solution (20 mM). 90 μL of the fermented beverage and 270 μL of deionized water were added to 2.7 mL of the FRAP reagent, followed by incubation at 37 °C for 30 min. Absorbance was measured at 595 nm. The results obtained were compared with a standard ferrous sulfate curve (100 – 2000 μmol/L) and

expressed in mmol of Fe^{2+} per liter of the sample.

2.6. Sugars, organic acids, alcohols, and phenolic compounds by HPLC-DAD-RID

High-performance liquid chromatography (HPLC) analyses were performed using an Agilent 1260 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a quaternary pump (model G1311C), vacuum degasser, thermostatic column compartment (model G1316A), automatic sampler (model G1329B), diode array detector (DAD; model G1315D) and refractive index detector (RID; Model G1362A).

Sugar consumption and alcohol production during fermentation were determined by HPLC-RID, using the methodology described by Viana et al. (2021). The juice/fermented beverage, previously diluted in ultrapure water, was filtered through a 0.45 μm filter and injected. The separation was obtained in an Agilent Hi-Plex H column (300 × 7.7 mm, 8.0 μm), protected by a PL Hi-Plex H guard column (5 × 3 mm) (Agilent Technologies, Santa Clara, CA, USA). The column temperature was maintained at 50 °C. The mobile phase was H_2SO_4 4 mmol/L. The HPLC external standards used were glucose, fructose, sucrose, ethanol, glycerol, and methanol. All calibration curves presented $R^2 > 0.996$.

Phenolic compounds determination followed the methodology described by Padilha (2017), with some modifications detailed by Dutra et al. (2018). Briefly, the compounds were separated using the Zorbax Eclipse Plus RP-C_{18} column (100 × 4.6 mm, 3.5 μm) and the pre-column Zorbax C_{18} (12.6 × 4.6 mm, 5 μm). The run time lasted 33 min., using the following gradient: 0–5 min: 5% B; 5–14 min: 23% B; 14–30 min: 50% B; 30–33 min: 80% B. Oven temperature was set at 35 °C and flow rate was 0.8 mL.min^{-1}. The mobile phases consisted of a 0.1 M phosphoric acid solution with pH = 2.0 (A) and methanol acidified with 0.5% phosphoric acid (B). Phenolic compounds were detected at 220 nm for (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, (-)-epicatechin gallate, procyanidin B1, and procyanidin B2; 280 nm for gallic and syringic acids, hesperidin, *cis*-resveratrol and naringenin; 320 nm for caftaric acid, caffeic acid, chlorogenic acid, p-coumaric acid, and *trans*-resveratrol; 360 nm for quercetin 3- glucoside, rutin, and kaempferol. Data collection and processing were performed using OpenLAB CDS ChemStation Edition (Agilent Technologies, Santa Clara - USA). The external standards of phenolic compounds were used for calibration curves, and all analytical curves presented $R^2 > 0.995$.

Simultaneous determination of organic acids and sugars was done in a HPLC-DAD/RID system. The analytical procedure followed the methodology validated by Coelho et al. (2018). The determination of tartaric, malic, lactic, succinic, formic, citric, and acetic acids was performed at 210 nm using DAD. Sucrose, glucose, fructose and rhamnose were analyzed using RID. The injection volume of the sample was 10 μL, with a flow rate of 0.7 mL min^{-1}. The mobile phase used was H_2SO_4 4 mmol/L.

2.7. Simulation of in vitro digestion with passage through the intestinal barrier

To evaluate *in vitro* gastrointestinal digestion of orange juice and fermented product, the INFOGEST protocol (Minekus et al., 2014) was used supplementing the simulation of passage through the intestinal barrier with the protocol described by Carneiro et al. (2022). Digestion simulation was performed in triplicate. For mimicry, a solution containing 5 mL of juice/fermented product, 3.5 mL of simulated salivary fluid (SSF), 0.5 mL of α-amylase (1500 U/mL), 25 μL of $CaCl_2$ 0.3 M, and 975 μL of ultrapure water was prepared (oral phase). The oral phase solution pH was adjusted to 7.0 and the mixture was incubated at 37 ± 1 °C for 2 min at 90 rpm. Soon after, the oral phase was mixed with 7.5 mL of simulated gastric fluid (SGF), 1.6 mL pepsin (25 000 U/mL), 5 μL of $CaCl_2$ 0.3 M, 200 μL of HCl 1.0 M, and 0.695 μL of ultrapure water. The pH of gastric phase solution was adjusted to 3.0. The mixture was incubated at 37 ± 1 °C for 2 h at 90 rpm.

The simulation of the intestinal phase was performed by mixing 20 mL of gastric chyme with 11 mL of simulated intestinal fluid (SIF), 5 mL of pancreatin (800 U/mL), 1 mL of bile salts (25 mg of bile/mL of sample), 40 μL of $CaCl_2$ 0.3 M, 150 μL of NaOH 1.0 M and 1.31 mL of ultrapure water. The pH was adjusted to 7.0 with NaOH 1.0 M, and the mixture was maintained at 37 ± 1 °C for 2 h at 90 rpm. This mixture was transferred to a 12 kDa dialysis bag (Sigma-Aldrich, USA), to simulate the passive absorption of phenolic compounds by the intestine membrane. The fully filled bags, without air bubbles, were immersed in 0.1 M $NaHCO_3$, and incubated in the dark at 37 °C for 2 h at 90 rpm. The non-dialysable fraction remaining inside the bag was separated and stored, representing the material that remained in the gastrointestinal tract. The fraction that permeated the membrane (dialysate) was separated, being the fraction available for absorption via the circulatory system by passive diffusion. The bioaccessibility of phenolic compounds was determined by Eq. (1):

$$\text{Bioaccessibility}(\%) = \frac{Dialyzedfraction}{Non-dialyzedfraction(juiceorfermented)} \times 100$$

2.8. Statistical analysis

The data obtained from chemical analyses were subjected to analysis of variance (one-way ANOVA), and mean values were compared by the Tukey test at 5% probability, using SPSS version 20.0 for Windows (SPSS, Chicago, IL, USA). Principal Component Analysis (PCA) and dendrogram analysis were performed using Past 4.03 (USA). The PCA was processed in a 5-line (4 yeast strains + orange juice) and 32-column (phenolic compounds, sugars, organic acids, and antioxidant capacity) array.

3. Results and discussion

3.1. Sugar consumption and alcohol production during fermentation

Fig. 1 shows the kinetics of sugar consumption and alcohol production during the fermentation of orange juice by the studied yeast strains. The juice initially had concentrations of 75, 66, and 31 g/L of sucrose, glucose, and fructose, respectively, totaling 172 g/L of fermentable sugars. One day after inoculation of starter yeasts, sucrose, glucose, and fructose consumption began, and ethanol was produced by all strains (0.42 to 2.05 g/L), except for US-05 which started producing ethanol after day 3. Sucrose was consumed at higher rates by US-05 and T-58 yeast strains compared to the Pclass and Pcuvée yeasts, however, at day 6, the sucrose content was almost completely depleted. With respect to glucose and fructose, Pclass and Pcuvée yeasts consumed the sugars at a higher rate than US-05 and T-58 yeasts.

Sucrose is consumed first as per the figures due to extracellular enzymes breaking down the sucrose, however, yeasts preferentially consume monosaccharides rather than disaccharides and oligosaccharides. Thus, the higher consumption rate for glucose was observed; besides the *S. cerevisiae* is also fructophilic (Chan et al., 2019), this explains why there was a longer time for consumption of fructose in the orange juice (9 days). The US-05 yeast had a significantly lower sugar consumption rate ($p < 0.05$) compared to other yeasts, consuming glucose and fructose on day 12 of fermentation. The consumption of sugars by yeasts resulted in increased ethanol and glycerol contents, where Pclass, Pcuvée, and T-58 yeasts achieved the maximum values (84.5–89.3 g/L ethanol and 4.8–5.5 g/L glycerol, respectively) on the seventh day of fermentation.

The US-05 strain had a low fermentation rate ($p < 0.05$), achieving the maximum ethanol (85.9 g/L) and glycerol (5.06 g/L) content on day 12. Even with a lower viable cell count (**Figure S2**). Wine yeasts had higher fermentation rates than beer yeasts.

Viana et al. (2021) evaluated beer fermentation processes of

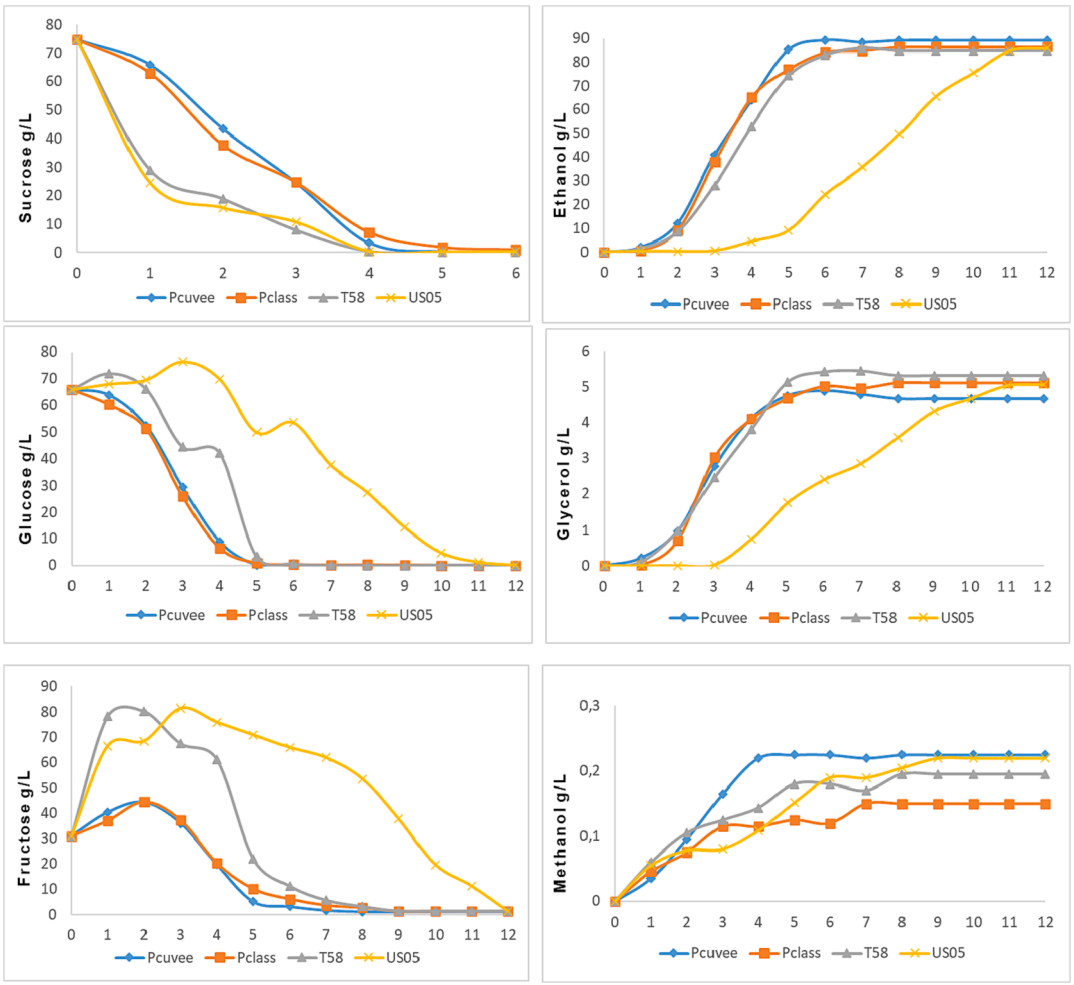

Fig. 1. Sugar consumption and alcohol production during alcoholic fermentation of orange juice by *S. cerevisiae*. Data are expressed as mean values of triplicates. × axis = day.

commercial strains of *S. cerevisiae* and also observed that the US-05 strain had a lower fermentation rate, however, it did produce higher concentrations of ethanol than other strains, besides having higher sugar consumption rates. Glycerol production by yeasts is dependent on the specificity of each strain, and strains with higher glycerol yield have been sought due to the sensorial characteristics they provide in alcoholic beverages (Zhao, Procopio & Becker, 2015). Based on the results from our study, at the end of the alcohol fermentation process, similar glycerol production was observed from the use of four tested commercial yeasts.

The concentration of ethanol produced by yeasts during fermentation depends on several factors including the amount of sugar available, temperature, availability of oxygen and micronutrients, and the metabolism of the strain. *S. cerevisiae* yeasts which have several ways of converting the energy obtained from sugars into biomass – this can decrease the formation of ethanol (Canonico et al., 2019). However, it was observed that all studied yeast strains produced high ethanol yield ($\geq$10.5% v/v) from 172 g/L of fermentable sugars, and high viable cells count during fermentation ($>10^7$ CFU/mL).

The non-*Saccharomyces* yeasts completed the fermentation process between 7 and 10 days (25 °C) and produced a lower ethanol concentration compared to *S. cerevisiae* which fermented the juice (150 g/L of sugars) in 4 days. However, Hu et al. (2018) reported that non-*Saccharomyces* yeasts such as *H. uvarum*, *H. opuntiae*, *H. occidentalis*, *P. kudriavzevii,* and *T. delbrueckii* improved the flavor of fermented orange beverages.

An orange juice (cv. 'Ponkan') containing 24% sugar was fermented with several *S. cerevisiae* strains, isolated from the oranges, and compared with the commercial wine *S. cerevisiae* HF-08. This yielded an ethanol concentration varying between 9.43 and 11.86% at various fermentation times; the HF-08 strain was shown to have the best performance and highest ethanol concentration yield (Lee et al., 2013). In the present study, the orange (cv. 'Pêra D9′) juice standardized in 18 °Brix produced > 10.5% of ethanol in the fermented product for all the yeast strains studied, confirming the high efficiency of generating ethanol by used yeasts.

One of the concerns of fermenting pectin-rich fruits, such as orange, is the formation of methanol, which occurs during hydrolysis of the methyl ester groups of pectin by oenological enzymes with pectinesterase activity. Methanol is a toxic alcohol that causes damage to the central nervous system, and its content in fermented fruit beverages should be monitored (Wu et al., 2017).

In our study, small concentrations of methanol (0.035 to 0.06 mg/L) were produced from the second day after inoculation of starter yeasts, reaching maximal values of 0.15 and 0.225 mg/L for Pclass and Pcuvée yeasts, respectively, at the end of fermentation (Fig. 1). These concentrations of methanol are within the acceptable values established by the Brazilian legislation for fermented fruit products, which allows a maximal concentration of 400 mg/L. Our results show that all evaluated yeast strains presented adequate fermentative behavior.

Based on the metabolites generated during the fermentation process, the four *S. cerevisiae* yeast strains evaluated showed that they had different fermentative behaviors, although all of them produced high concentrations of ethanol, similar concentrations of glycerol, and

methanol (within the limit established by Brazilian legislation). Thus, it can be concluded that these four strains are adequate in producing fermented beverages from oranges.

3.2. Sugars, organic acids, alcohols, and polyphenols in fermented orange

The values of metabolites associated with the fermentation process of orange juice are shown in Table 1. All yeast strains consumed the sucrose, glucose, and fructose, but there were residual amounts of each, particularly fructose, varying from 1.13 to 3.22 g/L for Pcuvée and T-58 yeasts, respectively. It is known that even with the complete fermentation of orange juice, residual fermentable and non-fermentable sugars do still remain (Viana et al., 2021). Regarding the total sugars quantified by HPLC system, all fermented beverages had values $\leq$ 3.68 g/L, which according to Brazilian legislation characterizes fermented oranges as "semi dry". The "dry" classification is applied to sugar values $\leq$ 3.0 g/L (Brasil, 2019) and only the wine yeasts Pclass and Pcuvée produced "dry" beverages.

Regarding the alcohol concentration in this study, the highest average value for ethanol was obtained with the wine yeast Pclass (88.47 g/L), differing significantly ($p < 0.05$) from the others, which presented values ranging from 84.88 to 86.94 g/L (10.6 to 10.9% v/v). Glycerol concentrations varied from 4.67 to 5.32 g/L for Pcuvée and T-58 yeasts, respectively. Glycerol is an alcohol that positively affects the mouthfeel and taste of alcoholic beverages, and its content can be increased by optimizing the fermentation process or by using genetically modified yeasts, reaching values up to 14 g/L in strains selected for this purpose. Nevertheless, flavor profile is unique for each beverage. The sweetness threshold of glycerol in wine is of 5.2 g/L (Zhao, Procopio & Becker, 2015). All yeasts produced ~ 0.23 g/L of methanol, not differing statistically ($p > 0.05$) from each other.

Amongst the quantified organic acids, only lactic acid differed significantly ($p < 0.05$) depending on the yeast used. The fermented product obtained with the T-58 and US-05 yeasts had the highest malic acid content (0.31 and 0.23 g/L, respectively). The most abundant organic acid present in the fermented orange was citric acid, ranging from 5.88 to 6.42 g/L, however, there were no significant differences ($p < 0.05$) among the different fermented products. Kelebek et al. (2009) reported the citric and malic acids values in a fermented orange (cv. 'Kozan') beverage, being 6.03 and 0.3 g/L, respectively. These values are similar to those found in the current study.

Acetic acid is a compound responsible for "*off-flavor*" in beverages, however, it was not detected in our study, or its concentration was well below the detection limit (0.07 mg/L). The metabolism of *S. cerevisiae* is related to specific genes for producing organic acids, which can influence the pH of beverage (Yoshida & Yokoyama, 2012). Yeasts used in brewing processes are usually selected for their high lactic acid production capacity (Viana et al., 2021), which may explain the higher levels of lactic acid in the fermented oranges obtained with US-05 and T-58 yeasts.

A total of 19 compounds classified as flavanols, phenolic acids, and flavanones were quantified by RP-HPLC/DAD system (Table 1). For the evaluated phenolic compounds, there was no significant difference ($p < 0.05$) among the fermented beverages with the different *S. cerevisiae* strains. Chemical differences in total phenolics quantified were observed only when comparing the orange juice (230 mg/L) and the fermented product (154–165 mg/L). The decrease of phenolic compounds in the fermented oranges may be associated with the precipitation and adsorption of compounds during clarification treatments, fact normally observed in wine making (Ghanem et al., 2017).

Amongst the quantified phenolic compounds, the most abundant was hesperidin, with concentrations ranging from 115.4 to 180.2 mg/L in orange juice. The fermented orange obtained from the yeasts Pclass, Pcuvée, T-58, and US-05 had lower hesperidin values (115.4 to 126.7 mg/L) compared to orange juice and did not differ significantly ($p < 0.05$). The presence of *trans*-caftaric acid (7.5–8 mg/L), chlorogenic acid

Table 1
The chemical profile of juice and fermented beverages from orange (cv. 'Pêra D9') produced using commercial *S. cerevisiae*.

Variables	Orange juice	Fermented beverages with yeasts			
		P.Cuvée	*P.Class*	*US05*	*T58*
pH	3.77 ± 0.01ab	3.72 ± 0.01d	3.69 ± 0.01e	3.74 ± 0.01bc	3.79 ± 0.01[a]
°Brix	18 ± 0.2	–	–	–	–
Titratable acidity g/L	7.54 ± 0.6b	7.47 ± 0.8a	8.58 ± 0.4a	8.64 ± 0.3a	8.76 ± 0.2a
SO_2 free (mg/L)	36 ± 1	38 ± 1	39 ± 1	36 ± 1	38 ± 1
Density g/cm^3	1.069 ± 0.002a	0.993 ± 0.001b	0.993 ± 0.001b	0.993 ± 0.001b	0.993 ± 0.001b
Sugars g/L					
Sucrose	75.39 ± 1.11a	0.1 ± 0b	0.35 ± 0.04b	0.37 ± 0.05b	0.13 ± 0.05b
Glucose	66.3 ± 3.31a	ND	0.07 ± 0.07b	1.02 ± 0.34b	0.13 ± 0.01b
Fructose	30.81 ± 1.19a	1.13 ± 0.09c	2.64 ± 0.63bc	2.29 ± 0.05bc	3.22 ± 0.76b
∑ quantified sugars	172.5	1.23	3.0	3.68	3.48
Alcohols g/L					
Ethanol	ND	86.94 ± 1.81b	88.47 ± 3.39a	85.92 ± 0,33b	84.88 ± 1.9b
Glycerol	ND	4.67 ± 0.08c	5.12 ± 0.05b	5.06 ± 0.08b	5.32 ± 0.01a
Methanol	ND	0.24 ± 0.02a	0.26 ± 0.01a	0.23 ± 0.01a	0.21 ± 0.05[a]
∑ quantified alcohols	–	91.85	93.95	91.17	90.41
Organic acids g/L					
Citric	5.67 ± 0.22a	6.05 ± 0.77a	6.42 ± 0.4a	5.88 ± 0.41a	5.93 ± 0.4a
Lactic	0.08 ± 0.01c	0.09 ± 0.01c	0.03 ± 0.02d	0.23 ± 0.01b	0.31 ± 0.01b
Acetic	ND	ND	ND	ND	ND
Succinic	0.73 ± 0.21a	0.68 ± 0.13a	0.78 ± 0.1a	0.73 ± 0.14a	0.88 ± 0.08a
Malic	0.88 ± 0.07a	0.85 ± 0.025a	0.96 ± 0.04a	0.92 ± 0.04	0.84a ± 0.03
∑ quantified acids	7.36	7.67	8.18	7.76	7.96
Phenolic compounds mg/L					
Flavanols					
Catechin	3.53 ± 0.01a	0.79 ± 0.02b	0.86 ± 0.03b	0.78 ± 0.05b	0.83 ± 0.02b
Epicatechin	0.73 ± 0.25a	0.53 ± 0.05b	0.54 ± 0.09b	0.51 ± 0.04b	0.46 ± 0.05b
Epicatechin gallate	8.85 ± 1.6a	7.5 ± 1.0a	8.33 ± 1.2a	6.9 ± 0.2a	7.45 ± 0.7a
Epigallocatechin gallate	2.48 ± 0.87a	2.91 ± 0.03a	3.04 ± 0.25a	3.09 ± 0.1a	2.78 ± 0.16a
Procyanidin A2	1.39 ± 0.04a	1.27 ± 0.15a	1.53 ± 0.02a	1.77 ± 0.16a	1.37 ± 0.01[a]
Procyanidin B1	2.04 ± 0.02c	2.55 ± 0.04b	2.63 ± 0.29b	2.4 ± 0.06bc	3.5 ± 0.13a
Procyanidin B2	1.21 ± 0.04a	0.88 ± 0.01b	0.89 ± 0.08b	0.90 ± 0.04b	0.84 ± 0.03b
Flavonols					
Myricetin	0.17 ± 0.12a	0.08 ± 0.03a	0.09 ± 0.02a	0.20 ± 0.10a	0.1 ± 0.03a
Quercetin-3-glucoside	0.41 ± 0.05a	0.27 ± 0.01b	0.27 ± 0.01b	0.26 ± 0.01b	0.24 ± 0.01b
Rutin	0.60 ± 0.35a	0.87 ± 0.05b	0.89 ± 0.11b	0.88 ± 0.08b	0.81 ± 0.1b
Kaempferol-3-glucoside	0.71 ± 0.46a	0.79 ± 0.12a	0.76 ± 0.21a	0.69 ± 0.17a	0.68 ± 0.16a
Isorhamnetin	0.15 ± 0.06a	0.41 ± 0.03b	0.44 ± 0.08b	0.47 ± 0.06b	0.41 ± 0.04b
Phenolic acids					
trans-caftaric acid	9.98 ± 0.97a	7.77 ± 0.06b	7.99 ± 0.58b	8.033 ± 0.16b	7.47 ± 0.1b
Chlorogenic acid	8.21 ± 1.64a	7.01 ± 1.71a	6.95 ± 1.22a	7.33 ± 1.44a	6.77 ± 1.15a
Caffeic acid					

(*continued on next page*)

Table 1 (*continued*)

Variables	Orange juice	Fermented beverages with yeasts			
		P.Cuvée	*P.Class*	*US05*	*T58*
	0.42 ± 0.09a	0.56 ± 0.04a	0.57 ± 0.09a	0.73 ± 0.1a	0.63 ± 0.05[a]
p-Coumaric acid	1.39 ± 0.07a	0.17 ± 0.01 cd	0.09 ± 0.02d	0.87 ± 0.45bc	0.48 ± 0.02c
Syringic acid	0.1 ± 0.02a	0.06 ± 0.01ab	0.07 ± 0.03ab	0.06 ± 0.02ab	0.05 ± 0.01b
Flavanones					
Hesperidin	180.2 ± 14.63	126.74 ± 16.6	124.68 ± 10.5	115.5 ± 24.85	115.37 ± 18
Naringenin	5.86 ± 0.46a	4.27 ± 0.37b	4.23 ± 0.13b	4.01 ± 0.52b	3.89 ± 0.38b
∑ quantified phenolics	230.17	165.47	164.87	156	154.17

Legend: Different letters in the same row indicate significant difference ($p < 0.05$); Results expressed as mean ± standard deviation (n = 3). Evaluation on wort (day 0) and final orange wine. ND = not detected or < LOD.

(6.8 to 7.3 mg/L), epicatechin gallate (6.9 to 7.5 mg/L), and naringenin (3.9 to 4.3 mg/L), were among the main phenolic compounds in this study.

Previous studies that characterized orange juices also showed that hesperidin was the most abundant phenolic compound in orange juice (Coelho et al., 2021; Stinco et al., 2020), and in the juice of the cv. 'Pêra D9', an average value of 101 mg/L is mentioned from the fruit obtained from the lower-middle San Francisco valley, Brazil (Coelho et al., 2021).

Guo et al. (2020) demonstrated the high absorption capacities of hesperidin and naringenin, the main phenolics of orange in the gastrointestinal protection of rats. Their study shows that the fermented orange, given its high hesperidin values should be a focus for future study so as to better understand the protective functional properties.

Of the total phenolic compounds quantified, the orange juice had higher values (230 mg/L) when compared to the fermented product obtained with the yeasts Pclass (165 mg/L), Pcuvée (165.4 mg/L), T-58 (154 mg/L) and US-05 (156 mg/L). For several orange cultivars planted in the lower-middle San Francisco valley, the total phenolic compounds quantified by HPLC system for all juices varied from 65 to 220 mg/L, with emphasis on cv. 'Bahia', 'Cara-cara', and 'Pêra' (Coelho et al., 2021). This showed that to obtain a fermented beverage with high bioactive content, the choice of the cultivar should be a primary consideration.

There were lower concentrations of phenolic compounds in fermented orange (cv. 'Kozan') beverages, compared to the juice. This was also observed by Kelebek et al. (2009), who showed that hesperidin was also the main phenolic compound present in the orange juice (171.2 mg/L) and in the fermented orange beverage (90.7 mg/L). The presence of the flavanones narirutin (21.7 mg/L), apigenin (16 mg/L), ferulic acid (9.9 mg/L), and chlorogenic acid (4.7 mg/L) was also highlighted, corroborating the results in this study. Other phenolic compounds were shown to decrease in the fermented orange compared to juice, also corroborating our results.

3.3. Antioxidant capacity

The antioxidant capacity of the orange juice and fermented oranges is shown in **Supplementary Figure S1.** The antioxidant capacity (AOX) as measured by free radicals scavenging (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) and ferric reducing antioxidant power (FRAP), did not differ significantly ($p < 0.05$) between the yeasts studied. The mean values obtained for the ABTS$^{\bullet+}$ analyses ranged from 2.62 to 3.06 mmol TE/L. For the DPPH$^{\bullet}$ method, the values ranged from 2.17 to 3.31 mmol TE/L. For the FRAP method, the mean values ranged from 6.5 to 8.14 mmol Fe^{2+}/L. The antioxidant capacity of the orange juice was preserved in the fermented oranges, which is a positive factor of fermented orange beverages.

3.4. Differentiation of yeasts by PCA chemometric analyses

Yeasts were separated by their metabolite profile from each of the fermented orange beverages by principal component analysis (PCA) (Fig. 2). PCA represented 82.2% of the variance of the experiment. CP1 > 0 separated the orange juice from fermented oranges mainly due to its high sugar content, absence of alcohols, and higher levels of phenolic compounds, as discussed in sections 5.1 and 5.2. The differentiation of yeasts was calculated by PC2, where beer yeasts (US-05 and T-58) were grouped in the positive part, associated with the highest levels of lactic acid, *p*-coumaric acid, and myricetin. PC2 < 0 (negative part) grouped the wine yeasts Pclass and Pcuvée and were associated with higher levels of kaempferol, citric acid, and lower lactic acid levels. The main factor responsible for the separation of the two groups of yeasts was the higher concentrations of lactic acid produced by the Pclass and Pcuvée beer yeasts.

The differentiation of yeasts by PCA is more powerful than that obtained by statistics such as the Tukey test and Pearson correlations (Lima et al., 2022), and in this study we found this to be true. Although all the yeasts studied had adequate fermentative behavior to produce fermented oranges, differences in lactic acid and non-evaluated compounds such as volatile compounds may lead to sensory differentiation of the wine. Future studies should also investigate the role that other metabolites play in fermentation of oranges.

3.5. Simulation of digestion and bioaccessibility of phenolic compounds

A small proportion of both the orange juice and the fermented product obtained from the Pcuvée yeast was used for *in vitro* digestion experiments to simulate the passage of compounds through the intestinal barrier. These results for bioaccessibility of phenolic compounds are presented in Tables 2 and 3. The non-dialyzed fraction corresponded to compounds that remained in the intestine until elimination, and the dialyzed fraction represented the fraction that permeated the intestinal barrier and hence would be available in the bloodstream, i.e., bioaccessibility.

For orange juice (Table 1), most of the quantified compounds remained intact until the intestinal phase, however, only catechin (1315%), epigallocatechin gallate (71%), and procyanidin B2 (188%) from the juice remained bioaccessible at values > 70%. Catechin was extremely high and remained highly bioaccessible. The main phenolic compound present in the juice was hesperidin (138 mg/L); this was preserved in the stomach and intestine, and ~ 30% of this was bioaccessible. Although not totally bioaccessible, hesperidin was the main phenolic compound that permeated the intestinal barrier (44.8 mg/L).

There are few studies that have evaluated the bioaccessibility of phenolic compounds in orange juice, however Stinco et al. (2020) showed that the bioaccessibility of flavonoids and hesperidin presented values between 16 and 20% in processed orange juice, however in this study, the simulation of digestion was performed up to the intestinal phase.

In this study, the presence of hesperidin in the stomach, gastric and intestinal fractions is a positive aspect of the juice and fermented orange product. According to Guo et al. (2020) this compound, and its derivatives, are absorbed by the duodenum and ileum. Also, hesperidin deglycosylation metabolites can be absorbed by the cecum, and phenolic acids can be absorbed by the ileum, cecum, and colon. These results may contribute to the understanding of the intestinal bioactivities of hesperidin.

There was a higher proportion of bioaccessible phenolic compounds in fermented orange beverages than in the orange juice (Table 3). Catechin, epigallocatechin gallate, and procyanidin B2 were also bioavailable at values>70%, especially catechin (2825%) and procyanidin B2 (247%). However, compounds such as rutin (1655%) and procyanidin B1 (69%) also showed good bioaccessibility in the fermented products, but were not bioavailable in the juice. Hesperidin, the

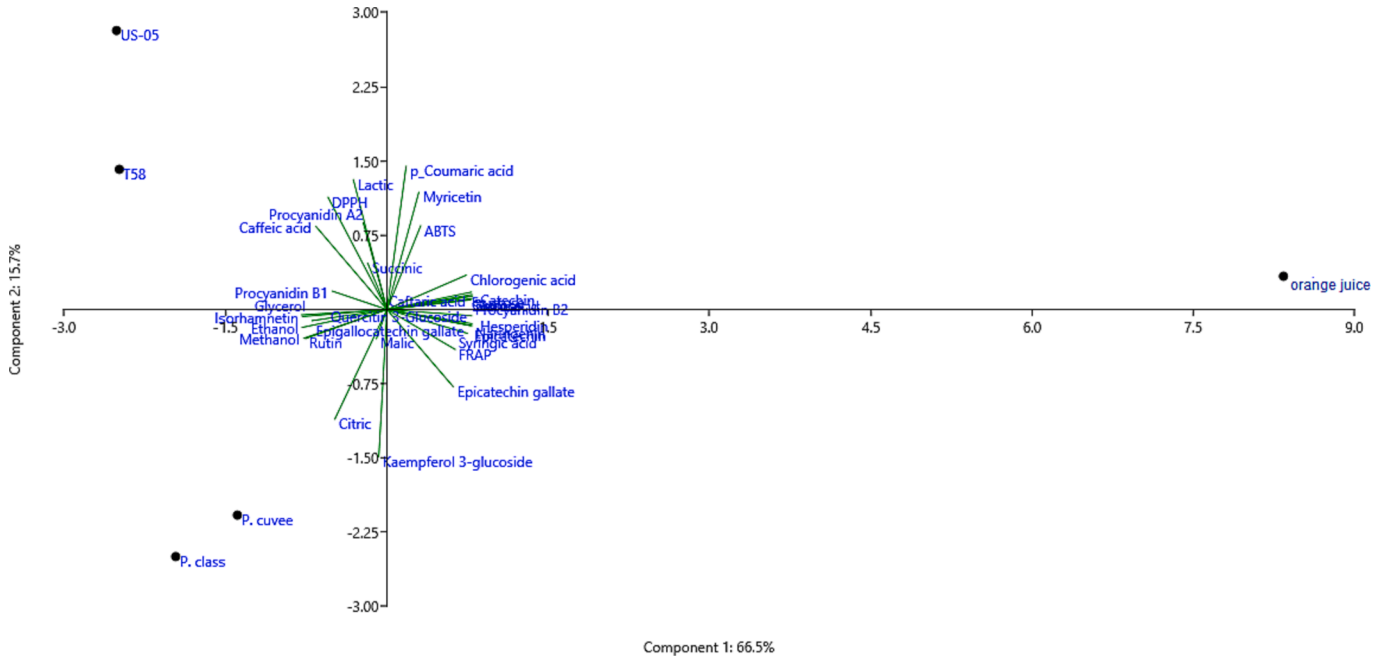

Fig. 2. Principal component analysis (PCA) of the chemical profile of fermented orange beverage produced by different *S. cerevisiae* strains.

Table 2
Individual polyphenolic compounds concentration (mg/L) in orange juice cv. 'Pêra D9' and at different stages of GI digestion.

Phenolic compounds mg/L	Juice	Stomach	Nondialysable	Dialysable	Bioaccessibility (%)
Flavanols					
Catechin	2.16 ± 1.11	5.54 ± 0.21	37.44 ± 0.10	28.48 ± 0.10	1315.5 ± 196.1
Epicatechin	0.63 ± 0.05	0.22 ± 0.02	0.08 ± 0.10	ND	–
Epicatechin gallate	4.25 ± 3.47	ND	ND	ND	–
Epigallocatechin gallate	2.25 ± 0.52	1.12 ± 0.09	1.04 ± 0.10	1.60 ± 0.10	71.1 ± 18.20
Procyanidin A2	1.31 ± 0.03	ND	ND	ND	–
Procyanidin B1	2.30 ± 0.24	1.94 ± 0.11	ND	ND	–
Procyanidin B2	1.02 ± 0.11	2.82 ± 0.11	4.00 ± 0.10	1.92 ± 0.10	188.2 ± 21.56
Σ Flavanols	13.92	11.64	42.56	32	
Flavonols					
Quercitin 3-glucoside	0.36 ± 0.08	0.52 ± 0.09	ND	ND	–
Rutin	0.77 ± 0.11	0.40 ± 0.004	0.56 ± 0.10	ND	–
Kaempferol 3-glucoside	0.79 ± 0.10	0.20 ± 0.08	0.73 ± 1.11	ND	–
Myricetin	0.02 ± 0.01	ND	ND	ND	–
Σ Flavonols	1.94	1.12	1.29	–	–
Stilbenes					
Cis-resveratrol	ND	ND	ND	ND	–
Trans-resveratrol	ND	ND	ND	ND	–
Σ Stilbenes	–	–	–		–
Flavanones					
Hesperidin	138.14 ± 22.42	122 ± 6.00	85.91 ± 0.10	44.80 ± 7.10	33.43 ± 5.51
Isohametin	0.29 ± 0.11	ND	ND	ND	–
Naringenin	4.65 ± 0.61	3.84 ± 0.19	2.56 ± 0.10	ND	–
Σ Flavanones	143.08	5.06	88.47	44.8	
Phenolic acids					
Galic acid	ND	ND	ND	ND	–
Syringic acid	0.09 ± 0.01	ND	ND	ND	–
Caftaric acid	8.35 ± 0.52	4.86 ± 0.08	1.12 ± 0.10	ND	–
Chlorogenic acid	5.93 ± 0.51	ND	ND	ND	–
Cafeic acid	1.35 ± 0.61	1.26 ± 0.08	0.52 ± 0.10	ND	–
p-Coumaric acid	0.49 ± 0.25	ND	ND	ND	–
Σ phenolic acids	16.21	6.12	1.64	–	–

Data represent the mean values for each sample ± standard deviation ($n = 3$).

main phenolic compound in the fermented beverage, had a bioaccessibility of 43.7%, which was greater than in juice. This suggests that orange juice fermentation has increased the bioaccessibility of phenolic compounds.

The increased bioaccessibility of hesperidin is a strong factor that shows that the fermented orange has the potential to be a strong candidate which can be used in pharmaceuticals, functional foods, dietary supplements, and nutraceuticals (Pandey & Khan, 2021). Noori et al. (2022) also reported that hesperidin has anti-cancer properties in an *in vitro* study. Therefore, increasing the bioaccessibility of hesperidin in our fermented orange is another positive outcome from this study.

Studies evaluating the influence of fermentation on phenolic

Table 3
Individual polyphenolic compounds concentration (mg/L) in fermented orange cv. 'Pêra D9' beverages at different stages of GI digestion.

Phenolic compounds mg/L	Orange wine	Stomach	Nondialysable	Dialysable	Bioaccessibility (%)
Flavanols					
Catechin	0.79 ± 0.01	3.54 ± 0.11	20.20 ± 5.97	22.32 ± 2.02	2825.3 ± 227.2
Epicatechin	0.53 ± 0.02	0.24 ± 0.02	ND	ND	–
Epicatechin gallate	4.24 ± 3.46	ND	ND	ND	–
Epigallocatechin gallate	2.91 ± 0.02	0.78 ± 0.14	1.20 ± 0.06	2.16 ± 0.03	74.4 ± 1.6
Procyanidin A2	1.27 ± 0.01	ND	ND	ND	–
Procyanidin B1	2.55 ± 0.03	2.64 ± 0.06	1.48 ± 0.35	1.76 ± 0.10	68.9 ± 1
Procyanidin B2	0.87 ± 0.01	3.40 ± 0.03	2.08 ± 0.65	2.16 ± 0.06	246.8 ± 6.3
Σ Flavanols	13.16	10.6	24.96	28.4	215.8
Flavonols					
Quercitin 3-glucoside	0.27 ± 0.01	ND	ND	ND	–
Rutin	0.87 ± 0.03	0.64 ± 0.06	0.44 ± 0.13	14.4 ± 0.65	1655.2 ± 12.9
Kaempferol 3-glucoside	0.78 ± 0.10	0.26 ± 0.01	0.46 ± 025	ND	–
Myricetin	ND	ND	ND	ND	–
Σ Flavonols	1.92	0.9	0.9	14.4	750
Stilbenes					
Cis-resveratrol	ND	ND	ND	ND	–
Trans-resveratrol	ND	ND	ND	ND	–
Σ Stilbenes	–	–	–	–	–
Flavanones					
Hesperidin	126.73 ± 13.10	128.94 ± 6.12	56. 00 ± 16.26	55.36 ± 0.65	43.7 ± 4.1
Isohametin	0.405 ± 0.02	ND	ND	ND	–
Naringenin	4.26 ± 0.29	4.14 ± 0.24	ND	ND	–
Σ Flavanones	131.4	133.08	56	55.36	42.9
Phenolic *acids*					
Galic acid	ND	ND	ND	ND	–
Syringic acid	0.05 ± 0.01	ND	ND	ND	–
Caftaric acid	7.76 ± 0.04	3.94 ± 0.21	2.28 ± 0.55	ND	–
Chlorogenic acid	7.01 ± 1.71	ND	ND	ND	–
Cafeic acid	0.55 ± 0.03	0.62 ± 0.01	0.23 ± 0.19	ND	–
p-Coumaric acid	0.17 ± 0.01	ND	ND	ND	–
Σ phenolic acids	15.54	4.56	2.51	–	–

Data represent the mean values for each sample ± standard deviation ($n = 3$).

bioaccessibility in oranges are scarce. However, Lingua et al. (2018) evaluated the bioaccessibility of phenolic compounds in grape juice and its subsequent wine (cv. 'Syrah'); they reported that as beverages progress through each stage of digestion, several compounds are degraded by the extreme pH conditions in the gastrointestinal tract. They showed that catechin was the most bioavailable phenolic compound in grape juice (88%), and fertaric acid was most bioavailable in wine (68%). In a recent study by Macêdo et al. (2023), the fermentation of umbu-cajá and soursop pulps by non-*Saccharomyces* yeasts increased the bioaccessibility of some phenolics, reaching values of 4800% (procyanidin B2), 703% (gallic acid), 2540% (quercetin), 791% (syringic acid), 122% (epicatechin), 120% (myricetin), 364% (epigallocatechin gallate), 563% (*p*-coumaric acid), corroborating with the present study.

The results obtained in the present study provide evidence that the fermented fruits by *S. cerevisiae* yeasts can be a potential technology to obtain the products with more significant amounts of bioaccessible phenolic compounds.

4. Conclusions

In this work, a fermented orange beverage was produced which met the criteria for commercialization, according to Brazilian legislation. It has been shown that the four tested yeast strains had adequate fermentation profiles according to the consumption of sugars and all fermented orange beverages achieved a high production of ethanol and glycerol, and acceptable low concentrations of methanol. The beer yeasts T-58 and US-05 produced higher concentrations of lactic acid, and the strains studied did not differ significantly ($p < 0.05$) in the production of each quantified phenolic compound. Fermented orange beverage had a higher bioaccessibility of several compounds compared to orange juice, especially catechin, epigallocatechin-gallate, procyanidin B2, rutin, and procyanidin B1, all with bioaccessibility of > 70%. Fermentation of orange juice significantly increased the bioaccessibility of phenolic compounds. Based on the results of the present study, it can be concluded that all yeasts evaluated are suitable for the production of fermented orange.

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CRediT authorship contribution statement

Suzana Maria Andrade Barreto: Investigation, Data curation, Writing – original draft. **Ana Beatriz Martins da Silva:** Formal analysis, Investigation. **Maria da Conceição Prudêncio Dutra:** Formal analysis. **Debora Costa Bastos:** Resources, Visualization. **Ana Júlia de Brito Araújo Carvalho:** Visualization, Formal analysis. **Arão Cardoso Viana:** Formal analysis, Visualization. **Narendra Narain:** Conceptualization, Visualization, Writing – review & editing. **Marcos dos Santos Lima:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.135121.

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