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Development of fermented beverage with citrus fruit extract using probiotics: impact on antioxidant activity and in vitro digestibility

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Abstract

Redhyang *(Citrus hybrid 'Kanpei'*,CHK) is a subtropical citrus species introduced in Korea due to climate change. To enhance the nutritional value and usability of CHK as a processed food product, CHK extract was fermented with four types of commercial starters (YoFlex Harmony 1.0 (YFH), ABY-3 (ABY), YC-X11 (YXC), and YC-180 (YC)), and their antioxidant activities and changes in chemical properties during fermentation were investigated. The consumer acceptance of probiotic beverages containing fermented CHK extracts and their viability and antioxidant activity through in vitro digestion were also elucidated. The enumeration of lactic acid bacteria (LAB) in all samples after fermentation was above 7.60 log colony-forming units (CFU)/mL, with YC exhibiting the highest number after 24 h. Fermented CHK extracts containing higher levels of organic acids, total polyphenols, and flavonoids tended to exhibit higher antioxidant activities. YFH, ABY, and YC showed maximum antioxidant activity at 24 h, whereas YXC showed differences in the types of LAB at 12 h. After in vitro digestion, YXC showed higher antioxidant activity and LAB viability than the control. This result indicates that CHK extract fermented with YXC can increase antioxidant activity, bioactive ingredients, and sensory preference and positively impact the production of probiotic beverages.

Keywords *Citrus fruit*, *Redhyang*, Lactic acid bacteria, Fermentation, Antioxidant activity, Probiotic beverage

Introduction

The subtropical climate is expanding due to continued global warming, which has enabled the cultivation of subtropical crops that were previously not produced in South Korea [1] and the total cultivated area of subtropical crops has increased from 33.9 ha in 2010 to 171.3 ha in 2020 [2]. Currently, there are about 20 subtropical crops have been selected for cultivation in Korea. Among them, 5 types of fruit trees (mango, passion fruit, dragon fruit, olive, and papaya) and 8 types of vegetables (okra, leek, bitter gourd, turmeric, sugar beet, jicama, and artichoke) had been developed with cultivation technology and distributed to farms [2, 3]. The demand for subtropical crops is expected to increase in the future due to changes in consumer preferences, and multicultural families as well as climate change [2]. Subtropical fruits are nutritionally excellent and contain various useful ingredients, but their rarity in Korea leads to high prices. To prepare for this, it is necessary to increase the utilization of subtropical crops by improving their quality in terms of safety and freshness, as well as exploring new functions and efficacy.

Citrus is an important fruit crop worldwide and is recognized as a rich source of useful phytochemicals, including bioactive polyphenolics, flavonoids, and dietary fiber [4]. Phytochemicals ingested through citrus fruits or their products used for them are known to have various biological functions such as antioxidant, anti-inflammatory, anti-carcinogenic, and anti-aging effects on human health [5, 6]. Furthermore, citrus fruits and

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their byproducts have a wide range of technological potential that can be used not only in the food industry but also in the cosmetics and pharmaceutical industries [6]. In Korea, among the various citrus fruits consumed, 'Setoka' (Citrus hybrid 'Setoka'), 'Kanpei' (C. hybrid 'Kanpei'), 'Ehime kashi No. 28' (C. hybrid 'Ehime kasha 28 gou'), and 'Shiranuhi' (C. hybrid 'Shiranuhi') are increasingly common [7]; domestic cultivation of these subtropical crops means that they are sold without heat treatment or freezing and offer the advantages of excellent freshness, taste, and quality over imported crops [2]. Despite their nutritional benefits, domestic subtropical crops are often subjected to physical damage during harvesting, sorting, and transportation, and their high moisture content can lead to mold growth, resulting in a very short shelf life. Therefore, to solve this problem and expand the utilization of subtropical crops, innovative food-processing technologies must be developed.

Fermentation is a technology that is widely used to improve the shelf life, nutritional quality, and taste of food while removing undesirable compounds [8]. Lactic acid bacteria (LAB) are used in food production for preservation, flavor enhancement, and consumption of probiotics. Probiotics provide various health benefits, including the treatment and regulation of diseases and the detoxification of chemical food contaminants [9]. In addition, they effectively prevent foreign contaminants from colonizing the intestine and alleviate toxicity [10]. Fermentation with LAB is a promising alternative food-processing technology that increases the value of food by producing nutrients and functional ingredients while also improving storage capacity through the biosynthesis of antibacterial substances [10, 11]. Recently, subtropical crops have been proposed as good media for the growth of LAB because of their vitamin and antioxidant benefits, in addition to nutritional components, such as carbohydrates, dietary fibers, and minerals. However, few studies have been conducted on LAB fermentation in *Citrus hybrid Kanpei* (CHK), characterized by low pH and the absence of lactose.

Therefore, the aim of this study was to enhance the utilization of CHK in processed foods through lactic acid bacteria (LAB) fermentation. Fermentation conditions were established using commercial starters, and the effects of LAB starter types on CHK fermentation were investigated in terms of chemical properties, antioxidant activity, and LAB viability. Additionally, a fermented beverage was prepared using LAB-fermented CHK extract, and sensory evaluation along with an in vitro digestion assessment was conducted to estimate the CHK-fermented beverage.

Materials and methods

Preparation of lactic acid bacteria (LAB)-fermented *Citrus hybrid Kanpei* (CHK) extracts

Domestic CHK was purchased from a farm on Jeju Island in February 2021. CHK was washed, peeled, and squeezed into juice using a juicer (H-AA-DBF17B; Hurom Co., Seoul, Korea). The CHK extract was heated at 70 °C for 10 min for pasteurization and cooled in a − 40 °C deep freezer for further analysis. The pH of the original CHK extract (before fermentation) was approximately 4.0, with an acidity of 0.5 and a brix of 15. Based on preliminary experiments, 10% skimmed milk powder and 0.1% of four kinds of LAB starters (YoFlex Harmony 1.0 (YFH), ABY-3 (ABY), YC-X11 (YXC), and YC-180 (YC), Chr Hansen, Hønsholm, Denmark) were added in CHK extract. The mixtures were fermented under anaerobic conditions at 37 °C for 48 h to produce LAB-fermented CHK extract (Fig. 1). Samples were collected at 0, 12, 24, and 48 h of fermentation. Table 1 shows the characteristics of LAB starters.

Microbiological evaluation of LAB-fermented CHK extracts

The enumeration of LAB on fermented CHK extract was performed by serially diluting the samples in phosphate buffered saline and plating them onto De Man, Rogosa, and Sharpe (MRS) agar plates. The plates were then incubated at 37 °C for 24–48 h and the resulting colonies were

Table 1 Types of microorganisms in lactic acid bacteria starter

	ST[1)]	LB	L.lac	L.fer	BB-12	LA-5
YFH[2)]	O	O		O		
ABY	O	O			O	O
YXC	O	O				
YC	O	O	O			

[1)] ST: *Staphylococcus thermophilus,* LB: *Lactobacillus delbrueckii. Bulgaricus,* L.lac: *Lactococcus lactis,* L. fer: *Lactobacillus fermentum,* BB-12: *Bifidobacterium animalis spp. Lactis*

LA-5: *Lactobacillus acidophilus*

[2)] YFH: YoFlex Harmony 1.0, ABY: ABY-3, YXC: YC-X11, YC: YC-180

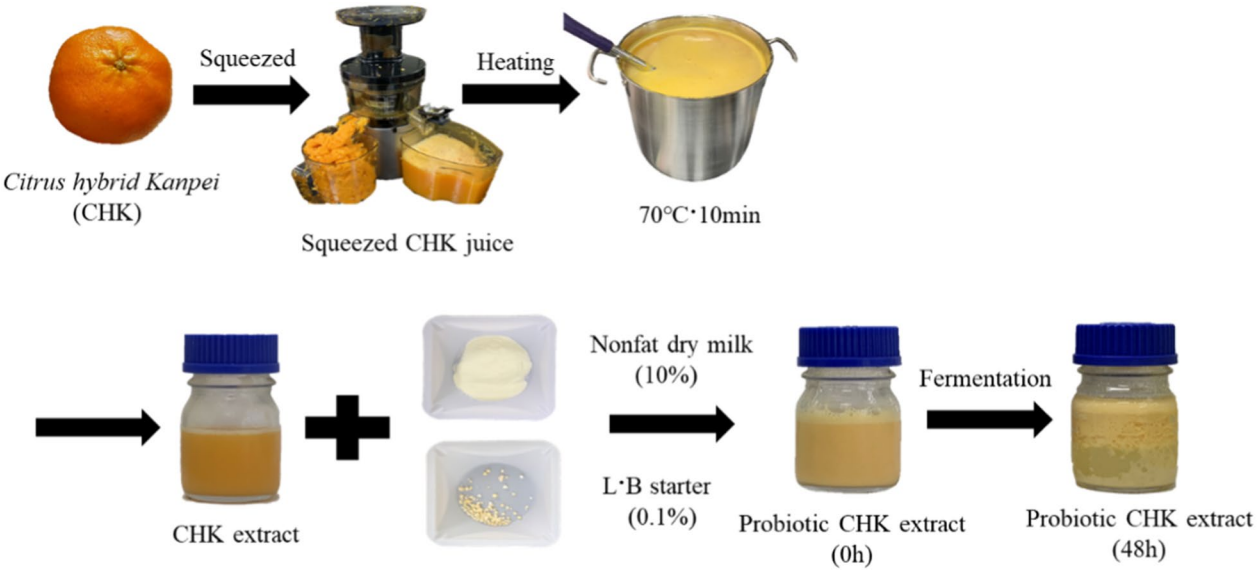

Fig. 1 Preparation of fermented *Citrus hybrid kanpei* (CHK) extracts by lactic acid bacteria

counted. The number of colony-forming units (CFU/mL) was determined by multiplying the colony count with the appropriate dilution factor.

pH, acidity, brix, and color of LAB-fermented CHK extracts

The pH of the LAB-fermented CHK extracts was measured during fermentation using a pH meter (SI Analytics GmbH D-55122, Mainz, Germany). Titratable acidity, expressed as percent acetic acid, was determined by titrating fermentation extract samples with 0.1 N NaOH to pH 8.3. Brix was measured using a Brixmeter (SCM-1000, HmDigital, Seoul, Korea), and color was measured using a colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan). All experiments were conducted in triplicate and the results are expressed as mean ± standard deviation.

Organic acid content

The organic acid content of the LAB-fermented CHK extract was analyzed by High Performance Liquid Chromatography (HPLC) (Shimadzu Co., Kyoto, Japan). The sample was centrifuged (3000 rpm, 15 min), and the resulting supernatant was treated with Sep-Pak C18 and filtered through a 0.45 μm membrane filter. Standards including oxalic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid (Sigma Chemical Co., St. Louis, USA), and ascorbic acid were used to quantify the organic acids. The HPLC analysis conditions for organic acid analysis were as followed: Column, Prevail 5 Organic Acid (250×4.6 mm); mobile phase, 25 mM KH_2PO_4 (pH 2.5); column temperature, 40 °C; flow rate, 1.0 mL/min; detector, 440 nm.

Free sugar content

The free sugar content of the LAB-fermented CHK extract was determined by HPLC analysis. LAB-fermented CHK extract (0.5 g) was mixed with 10 mL of distilled water and placed in a water bath for 30 min at 85 °C. The mixture was then centrifuged at 3000 rpm for 10 min, and the collected supernatant was treated with Sep-Pak C18 and passed through a 0.45 μm membrane filter. Subsequently, 20 μL of the filtrate was analyzed by HPLC (Shimadzu Co.) which was equipped with a pump system and a UV/Vis detector (SPD-20A) monitored at 210 nm. Free sugars were simultaneously analyzed on a carbohydrate column (250×4.6 mm, Alltech Co., Lexington, KY, USA) under the following analytical conditions: flow rate, 1.0 mL/min; mobile phase, acetonitrile:water at 75:25 (v/v).

Total polyphenol content

The total polyphenol content of LAB-fermented CHK extracts was determined using the method described by Hashemi and Hosseini [12]. Specifically, 0.1 mL of LAB-fermented CHK extract was mixed with 2 mL 2% Na2CO3 and allowed to react for 3 min. Then, 0.1 mL of 50% Folin–Ciocalteu reagent was added, and the reaction mixture was allowed to stand in the dark for 30 min. The absorbance was measured at 750 nm using a spectrophotometer, and a calibration curve was prepared using gallic

acid (Sigma Chemical Co.). Total polyphenol (mg/mL) of the LAB-fermented CHK extract was calculated based on the calibration curve.

Total flavonoid content

To quantify the total flavonoid content (TFC) of LAB-fermented CHK extracts, a modified method sourced from Baba and Malik [1] was used. Briefly, 250 µL of sample extract was thoroughly mixed with 1 mL of distilled water and 75 µL of 5% $NaNO_2$. Then, 150 µL of 10% $AlCl_3H_2O$ was added and the reaction was stopped after 6 min by adding 500 µL of 1 N NaOH. The absorbance of the resulting solution was measured immediately at 510 nm using a UV spectrophotometer. Quercetin (Sigma Chemical Co.) was used as a standard, and the TFC was expressed as milligrams of quercetin equivalent per 100 g of sample (mg QE/100 g).

2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The antioxidant activity of LAB-fermented CHK extracts against the stable free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) was determined spectrophotometrically. The radical scavenging activity of the extract was measured using a slightly modified version of the method described by Seephonkai, Samchai [2]. CHK-fermented extracts were dissolved in ethanol and mixed with 0.2 mM DPPH solution. The mixture was then incubated in the dark for 30 min at 25 °C and the absorbance of the sample was measured at 517 nm using a spectrophotometer (C40; Implen, München, Germany). Radical scavenging activity was calculated using the following formula:

$$\text{Scavenging effect\%} = A_{\text{Control517nm}} - A_{\text{Sample517nm}} / A_{\text{Control517nm}} \times 100.$$

2′-Amino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging activity

To induce the 2,2′-amino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of LAB-fermented CHK extracts, a solution containing 7 mM ABTS reagent and 2.4 mM potassium persulfate was prepared (1:1 ratio) and allowed to react for 12–16 h in the dark at 25 °C. The resulting solution was diluted with distilled water to achieve an absorbance value of 0.70 ± 0.05 at 732 nm. Subsequently, 0.4 mL of LAB-fermented CHK extracts were mixed with 3.6 mL of the prepared ABTS solution, and the mixture was allowed to react in the dark for 30 min. The absorbance was measured at 732 nm and a control measurement was performed using distilled water instead of the sample. ABTS radical scavenging activity was calculated using the following formula:

$$\text{Scavenging effect\%} = A_{\text{Control732nm}} - A_{\text{Sample732nm}} / A_{\text{Control732nm}} \times 100.$$

Superoxide Dismutase (SOD)-like activity

Superoxide Dismutase (SOD) is an important antioxidant enzyme that catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen, thereby protecting cells from oxidative damage. The SOD-like activity of LAB-fermented CHK extracts was measured using the method described by Kim, Jeon [3]. 0.2 mL of LAB-fermented CHK extracts were mixed with 0.2 mL of 7.2 mM pyrogallol and 2.6 mL of Tris–HCl buffer solution (50 mM Tris + 10 mM EDTA, pH 8.5). The mixture was incubated at 25 °C for 10 min. HCl (0.1 mL, 1 M) was added to stop the reaction and the absorbance of the mixture was measured at 420 nm. SOD activity was determined by calculating the rate of decrease in absorbance at the addition and non-addition ports of the sample solution and was expressed as a percentage.

$$\text{SOD activity \%} = A_{\text{Blank420nm}} - A_{\text{Sample420nm}} / A_{\text{Blank732nm}} \times 100.$$

Preparation of fermented beverage with LAB-fermented CHK extracts

LAB-fermented CHK extracts at 24 h were selected for the production of fermented beverage since that was the duration with the highest number of LAB. The mixture ratio was determined based on the results of a preliminary study. LAB-fermented CHK extracts (25 mL) were mixed with 35 mL of purified water, 10 mL of CHK extract (not fermented), 2 g of citric acid, 15 g of oligosaccharide, and 5 g of fructose and stored in a refrigerator.

Consumer acceptance of fermented beverage

The fermented beverage samples were served cold in glasses denoted with random numbers to a panel of 40 students from Suncheon National University. Consumer acceptance was assessed using a 9-point hedonic scale for appearance, color, flavor, sweetness, sourness, swallowing, and overall acceptability, with ratings ranging from 9 (extremely favorable) to 1 (extremely unfavorable), following approval by the Suncheon National University Institutional Review Board (IRB approval number:1040173-202111-HR-040-02).

In vitro digestibility of fermented beverage

The simulated in vitro digestibility of the four types of fermented beverage was evaluated using a slightly modified method described by Ryan, Hutchings [4]. Gastric juices were prepared by suspending pepsin (3 g/L) in a 0.5% NaCl solution, and the pH was adjusted to 3.0 using HCl. Intestinal juices were prepared by suspending pancreatin (1 g/L, Sigma Chemical Co.) in a 0.5% NaCl solution with bovine bile salts (3 g/L, Sigma Chemical Co.), and the pH was adjusted to 7.0 using 0.1 N NaOH. One milliliter of the sample was mixed with 9 mL of gastric or intestinal juice. Gastric juice samples were incubated at 37 °C for a total of 60 min, and 1 mL was collected at 0, 1, and 60 min. Intestinal juice samples were incubated at 37 °C for a total of 120 min, and 1 mL was collected at 0, 1, and 120 min. The collected samples were streaked onto MRS agar plates to enumerate the probiotic cells, and the DPPH radical-scavenging ability of each collected sample was measured at 517 nm using a spectrophotometer.

Statistical analysis

All experiments were performed in triplicates, and the results were presented as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) in the SPSS 26 statistical software (IBM Corp., Armonk, NY, USA), and the 95% confidence level was determined using Duncan's multiple range test.

Results and discussion

Changes in LAB viability during CHK fermentation

The cell counts LAB in CHK extracts during fermentation are presented in Fig. 2A. The number of LAB at the initial stage of fermentation was 4.56–7.66 log CFU/mL, and after 24 h of fermentation, all samples maintained a count of 7.63 log CFU/mL or higher. Among the samples, YFH, YXC, and YC exhibited the highest numbers of LAB after 24 h of fermentation, with YC showing the greatest growth at 9.40 log CFU/mL. The decrease in LAB count after 24 h of fermentation was consistent with a previous study that showed that fermented kiwifruit juice exhibited a rapid increase in LAB count after 24 h of fermentation, followed by a decrease [5]. This decrease is attributed to the low pH produced during LAB fermentation, which inhibits microbial growth [6]. However, ABY showed a maximum LAB count of 8.10 log CFU/mL after 48 h of fermentation. This difference between the samples may be attributed to the variation in the LAB starter complexity, which consists of various LAB [7].

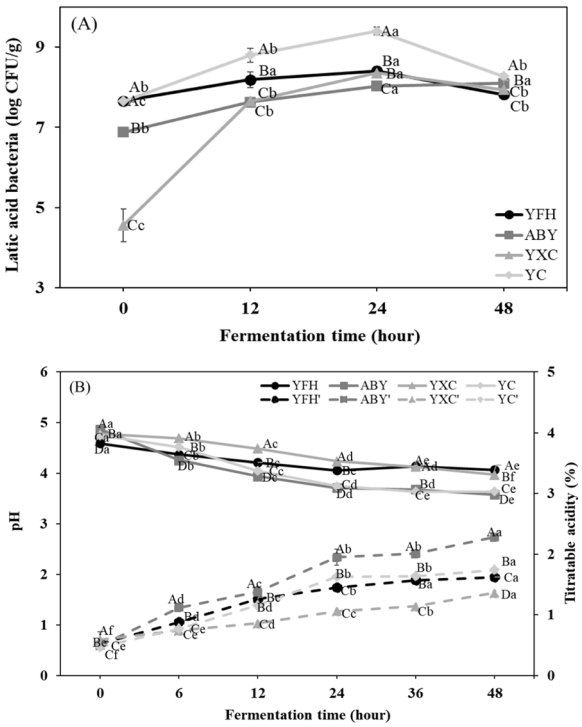

Fig. 2 Changes in in the viability of lactic acid bacteria (LAB) (**A**) and pH (solid lines) & titratable acidity (dotted lines) (**B**) of *Citrus hybrid kanpei* (CHK) extracts during fermentation. Values followed by different small letters (a–e) and by different capital letters (A–D) are significantly different at $p < 0.05$ according to the fermentation time and the groups, respectively

Changes in pH, acidity, brix, and color of CHK extracts during fermentation

To elucidate the variation in the growth of LAB based on starter type, it is essential to examine the changes in pH and acidity of the CHK extracts. The pH and titratable acidity were measured by collecting samples at 0 h, 12 h, 24 h, and 48 h. The pH exhibited a declining trend over time in all four (YFH, ABY, YXC, YC) samples, while the titratable acidity showed an increasing trend (Fig. 2B). The initial fermentation pH ranged from 4.59–4.87 and decreased to 3.58–3.97 after 48 h. These results are consistent with the pH results for LAB-fermented apple juice [8]. The pH varied depending on the type of LAB starter, with ABY and YC exhibiting the most rapid changes at 0.36% and 0.26%, respectively. Acidity also showed the most significant change in the early stages of fermentation, with the ABY starter exhibiting the largest change from 0.49% to a final value of 2.28%. A rapid decrease in pH at the start of fermentation is crucial to ensure the quality of the final product because a rapid increase in acidity minimizes the influence of spoilage bacteria [9].

The brix values of the CHK-fermented extracts are presented in Table 2 and were observed to be in the range of

Table 2 Changes in brix and Hunter's color values of *Citrus hybrid kanpei* (CHK) extracts during fermentation

		0 h	12 h	24 h	36 h	48 h
Brix	YFH	21.20 ± 0.00^{Bb}	20.00 ± 0.00^{Cd}	20.93 ± 0.12^{Ac}	19.80 ± 0.00^{Ce}	19.00 ± 0.00^{Df}
	ABY	21.07 ± 0.12^{Ba}	20.00 ± 0.00^{Cc}	19.00 ± 0.00^{Cd}	20.20 ± 0.00^{ABb}	20.00 ± 0.00^{Bc}
	YXC	22.00 ± 0.00^{Aa}	21.20 ± 0.00^{Ab}	21.07 ± 0.12^{Ab}	20.07 ± 0.12^{Bd}	20.27 ± 0.12^{Ac}
	YC	21.07 ± 0.12^{Ba}	20.27 ± 0.12^{Bb}	19.80 ± 0.00^{Bc}	20.27 ± 0.12^{Ab}	19.53 ± 0.12^{Cd}
L	YFH	66.36 ± 1.75^{Ba}	66.18 ± 0.93^{Cab}	65.06 ± 1.68^{Bb}	65.01 ± 1.15^{Cb}	63.79 ± 2.24^{Ac}
	ABY	67.28 ± 0.81^{Aa}	66.85 ± 0.30^{Ba}	66.86 ± 0.47^{Aa}	64.46 ± 0.61^{Cb}	64.94 ± 0.00^{Ab}
	YXC	65.91 ± 0.47^{Bc}	67.48 ± 0.24^{Aa}	66.82 ± 0.69^{Ab}	66.59 ± 0.18^{Ab}	65.42 ± 0.43^{Aa}
	YC	66.03 ± 0.71^{Bb}	66.69 ± 0.45^{Ba}	66.94 ± 0.31^{Aa}	65.83 ± 0.61^{Bb}	64.52 ± 0.54^{Ac}
a	YFH	-2.80 ± 0.04^{Bd}	-2.65 ± 0.10^{ABc}	-2.04 ± 0.19^{Ab}	-1.80 ± 0.15^{Aa}	-1.85 ± 0.13^{Ba}
	ABY	-3.11 ± 0.19^{Ce}	-2.79 ± 0.015^{Bd}	-2.61 ± 0.09^{Bc}	-2.00 ± 0.17^{Ab}	-1.29 ± 0.04^{Aa}
	YXC	-2.66 ± 0.38^{Bb}	-2.59 ± 0.59^{ABb}	-2.06 ± 0.44^{Aa}	-1.98 ± 0.09^{Aa}	-1.95 ± 0.14^{Ba}
	YC	-2.40 ± 0.03^{Ab}	-2.09 ± 0.80^{Aab}	-2.13 ± 0.55^{Aab}	–	–
b	YFH	22.77 ± 2.18^{Ad}	23.38 ± 0.90^{Acd}	24.52 ± 2.86^{Abc}	24.97 ± 0.46^{Aab}	25.87 ± 0.48^{Aa}
	ABY	21.01 ± 1.75^{Bc}	22.97 ± 0.98^{Ab}	22.99 ± 1.80^{Ab}	23.31 ± 0.08^{Cbc}	24.75 ± 0.14^{Ba}
	YXC	20.30 ± 0.98^{Bc}	22.00 ± 2.26^{ABb}	22.93 ± 0.99^{Aab}	22.83 ± 0.30^{Cab}	28.55 ± 0.57^{Ca}
	YC	18.24 ± 0.74^{Cd}	21.51 ± 0.42^{Bc}	22.90 ± 2.27^{Ab}	23.75 ± 0.82^{Bab}	24.58 ± 0.69^{Ba}

[a–d] Means with different letters in the same row differ significantly at $p < 0.05$

[A–C] Means with different letters in the same column differ significantly at $p < 0.05$

CHK: *Citrus hybrid Kanpei,* YFH: YoFlex Harmony 1.0, ABY: ABY-3; YXC: YC-X11; YC: YC-180

19–22%. As the fermentation process progressed, the brix values of all samples decreased, suggesting that LAB consumed sugars (represented by brix) to produce lactic acid and other components [5]. Hunter's color values (*L*, *a*, and *b*) of the CHK extracts during fermentation are shown in Table 2 and showed little overall change. The *L* values decreased with increasing fermentation time, whereas the *a* and *b* values increased in all samples during fermentation. The *a* value was highest after 36 h of fermentation, whereas the highest *b* value was observed after 48 h.

Determination of organic acid content

Organic acids are naturally occurring compounds in fruits and their presence and composition play crucial roles in determining the organoleptic properties and stability of fruit juices [10]. The organic acid content of the LAB-fermented CHK extracts was compared between before and after 24 h of fermentation, and the results are shown in Table 3. Before fermentation, oxalic, tartaric, malic, lactic, acetic, citric, succinic, and ascorbic acid were detected in CHK (control). Citric acid was identified as the major organic acid in CHK (2.18 mg/mL), which was consistent with the results for organic acids in *Citrus sinensis L* previously studied [11]. After 24 h of fermentation, the total organic acid content increased in all the four sample groups, with the highest content observed in YFH (15.52 mg/mL). Furthermore, acetic acid, which was not detected in CHK, was present in high amounts

Table 3 Organic acid contents of fermented *Citrus hybrid kanpei* (CHK) extracts

mg/mL	Oxalic acid	Tartaric acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid	Ascorbic acid	Total organic acid content
CHK	0.13 ± 0.02^{Df}	1.28 ± 0.11^{Cb}	0.27 ± 0.02^{Ce}	0.73 ± 0.09^{Dc}	N.D[1)]	2.18 ± 0.04^{Aa}	0.44 ± 0.03^{Ad}	0.11 ± 0.09^{Bf}	5.12 ± 0.13^{D}
YFH	1.31 ± 0.02^{Ad}	2.90 ± 0.05^{Bc}	3.54 ± 0.19^{Ab}	5.29 ± 0.31^{Ba}	3.07 ± 0.25^{Bc}	0.22 ± 0.05^{Ce}	0.39 ± 0.00^{ABe}	0.24 ± 0.03^{Ae}	15.52 ± 1.59^{A}
ABY	1.04 ± 0.06^{Bd}	3.32 ± 0.15^{Bb}	2.84 ± 0.21^{Bb}	5.83 ± 0.01^{Aa}	1.77 ± 0.42^{Cc}	1.26 ± 0.35^{Bd}	0.51 ± 0.16^{Ae}	0.15 ± 0.00^{Be}	14.92 ± 0.31^{B}
YXC	0.65 ± 0.01^{Ce}	5.31 ± 0.09^{Aa}	3.45 ± 0.40^{Ab}	2.71 ± 0.21^{Cc}	1.76 ± 0.01^{Cd}	0.30 ± 0.02^{Cf}	0.52 ± 0.01^{Aef}	0.26 ± 0.02^{Af}	14.14 ± 1.61^{B}
YC	0.62 ± 0.05^{Cd}	5.00 ± 0.38^{Aa}	2.82 ± 0.16^{Bc}	2.67 ± 0.18^{Cc}	3.92 ± 0.33^{Ab}	0.27 ± 0.02^{Cd}	0.28 ± 0.01^{Bd}	0.25 ± 0.06^{Ad}	12.87 ± 2.85^{C}

YFH • ABY • YXC • YC = 24 h fermented CHK extracts

[a–f] Means with different letters in the same row differ significantly at $p < 0.05$

[A–E] Means with different letters in the same column differ significantly at $p < 0.05$

[1)] ND, not detected

CHK: *Citrus hybrid Kanpei,* YFH: YoFlex Harmony 1.0, ABY: ABY-3; YXC: YC-X11; YC: YC-180

after fermentation (1.77–3.92 mg/mL). The lactic acid content also increased significantly, with the highest levels observed in the YFH and ABY samples (at 5.29 mg/mL and 5.86 mg/mL, respectively). The increase in lactic acid levels during fermentation may be attributed to the metabolic activity of the probiotics, which can inhibit the activity of spoilage microorganisms [12]. Lactic acid is a natural preservative that can enhance the shelf life of foods and serve as a flavoring agent [13].

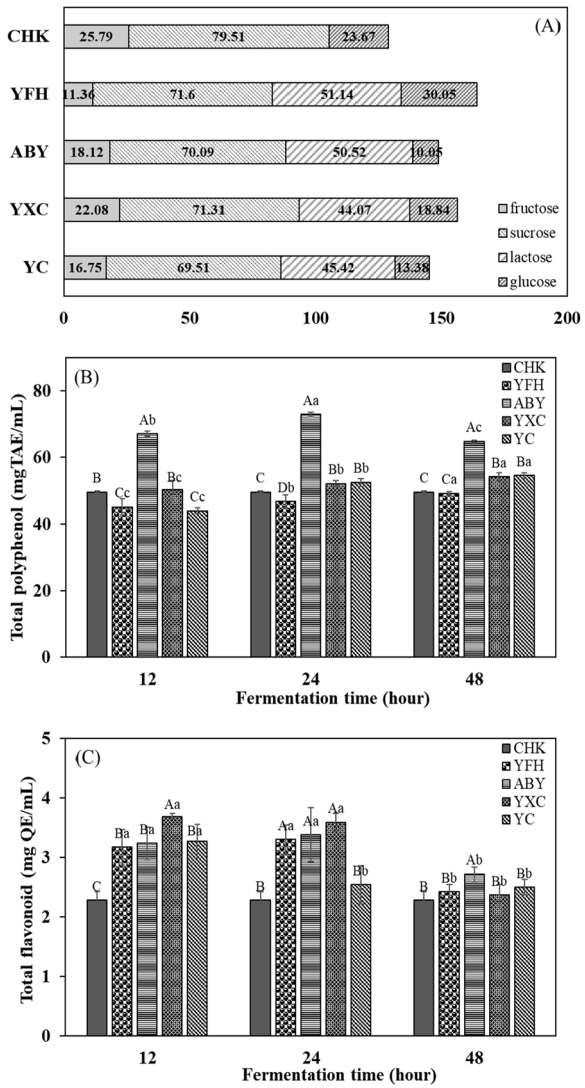

Fig. 3 Free sugar contents of 24 h fermented CHK extracts (**A**) and total polyphenol (**B**) and total flavonoid content (**C**) of CHK extracts during fermentation. Values followed by different small letters (a–c) and by different capital letters (A–D) are significantly different at $p < 0.05$ according to the fermentation time and the group, respectively

Determination of free sugar content

Free sugars have a significant impact on sweetness, which is a crucial aspect of the sensory quality of fruits. Fructose, glucose, and sucrose are the primary sugars in citrus fruits [14]. The free sugar content of the LAB-fermented CHK extracts is shown in Fig. 3A. The free sugar content of CHK before fermentation was 128.97 mg/g, and fructose, sucrose, and glucose were detected. After 24 h of fermentation, the free sugar content of the LAB-fermented CHK extract increased to 145.06–164.15 mg/g. With the addition of skimmed milk powder for LAB fermentation, lactose was detected after fermentation, and YFH showed the highest lactose content (51.14 mg/g). In contrast, the contents of glucose, fructose, and sucrose decreased, which was consistent with previous studies showing that glucose and fructose were consumed during fermentation [15].

Total polyphenol and flavonoid content

Polyphenols are considered promising natural antioxidants that can be used to develop functional foods [16]. The TPC in natural products can be measured using the Folin–Ciocalteu reagent, which is involved in the basic mechanism of oxidation/reduction reactions and can indicate the presence of any substance with antioxidant properties [17]. As shown in Fig. 3B, the polyphenol content of the CHK before fermentation was 49.62 mg/mL, which increased to 52.14–72.93 mg/mL after 24 h fermentation (up to 46% increase). The TPC of the ABY sample was significantly higher than other samples, with a highest content of 72.93 mg/mL at 24 h.

Citrus fruits are one of the most abundant dietary sources of flavonoids, which have various therapeutic effects, such as radical scavenging, antioxidant, and anti-inflammatory activities [18]. All four samples exhibited a higher total flavonoid content than the unfermented CHK extract (2.28 mg/mL) at all fermentation times (Fig. 3C). The highest value (3.17–3.69 mg/mL) was observed in all four samples at 12 h of fermentation, and the total flavonoid content decreased after 24 h. Ricci, Cirlini [19] reported that hydrolytic enzymes produced during LAB fermentation, such as glucosidase, amylase, and cellulase produced during LAB fermentation promote the production of phenolic compounds by decomposing plant cell walls. The increase in the total polyphenol and flavonoid contents of the CHK extract through LAB fermentation

has excellent nutritional properties and is expected to have beneficial effects on human health.

Antioxidant activity

Antioxidant activity is an important mechanism to promote human health and aid in the prevention and treatment of diseases [20]. In this study, the antioxidant activity of CHK extracts during LAB-based fermentation was evaluated using DPPH and ABTS radical scavenging assays, as well as the SOD-like activity method. As shown in Fig. 4A, YFH and ABY demonstrated significantly higher DPPH radical-scavenging activities than the pre-fermentation extract (60.69%). The ABY (77.31%) and YFH (74.00%) samples showed the highest DPPH radical scavenging activity after 24 h of fermentation, whereas YXC (76.84%) showed the maximum DPPH radical scavenging activity at 12 h of fermentation. Only YC showed a lower DPPH radical-scavenging activity than the unfermented CHK extract (not fermented). The different DPPH antioxidant activities and optimum fermentation times may be attributed to variations in the fermentation products generated by different types of LAB.

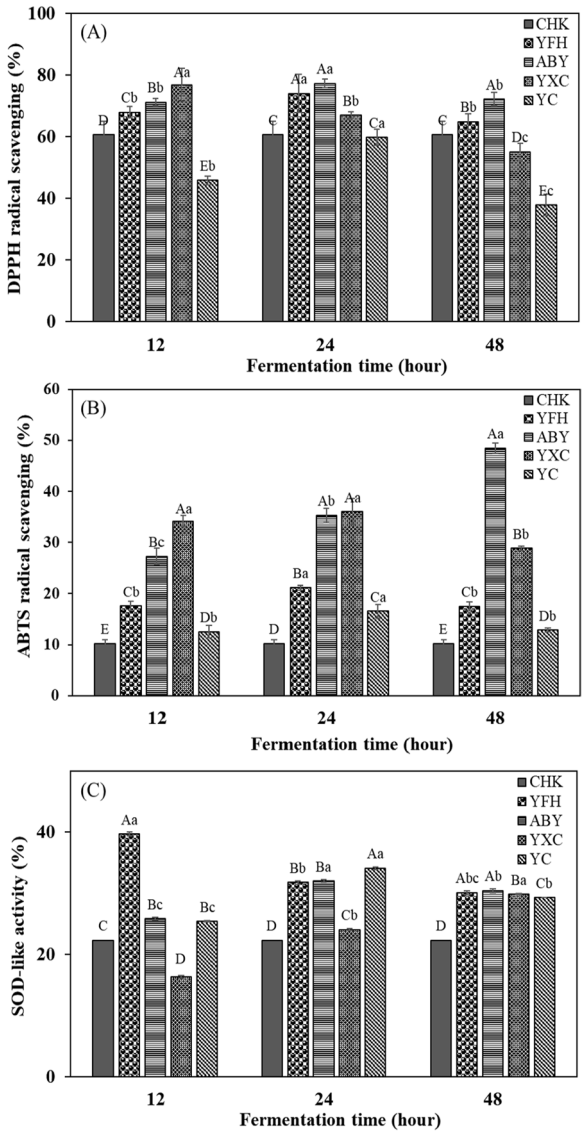

Fig. 4 Antioxidant activities of *Citrus hybrid kanpei* (CHK) extracts during fermentation by DPPH (**A**), ABTS (**B**), and SOD-like activity (**C**). Values followed by different small letters (a–c) and by different capital letters (A–E) are significantly different at $p < 0.05$ according to the incubation time and the group, respectively

ABTS radical scavenging ability increased significantly after lactic acid fermentation in all samples (Fig. 4B). The ABTS radical scavenging ability of the CHK stock solution before fermentation was 10.20%; ABY showed the highest activity of 48.36% after 48 h of fermentation, and YC showed the lowest activity at all fermentation times. YFH, YXC, and YC showed maximum values of 21.16%, 36.02%, and 16.59%, respectively, at 24 h of fermentation, and then decreased. DPPH and ABTS radical scavenging activities were the highest at 24 h and then decreased, which corresponded to the trend in the number of LAB.

All living organisms have an antioxidant system that includes various antioxidant enzymes such as SOD and catalase. An assay of SOD-like activity, based on its ability to inhibit the auto-oxidation of pyrogallol, was used to predict antioxidant capability [21]. The SOD-like activity of LAB-fermented CHK extracts is shown in Fig. 4C. The SOD-like activity of CHK before fermentation was 23.30%, which increased to 24.02–34.11% after 24 h of fermentation. These results indicate that the CHK extract obtained through lactic acid fermentation has excellent antioxidant activity and a high commercial value. In the fermentation process, microorganisms alter the molecular structure of raw materials through their metabolic capabilities, generating complex intermediate and cross-metabolites. This leads to an increase in antioxidant activity. At the 24-h fermentation time, the ABY sample exhibited the highest antioxidant activity among the four samples, consistent with the its highest total polyphenol (flavonoids) content. This can be attributed to the interactions among the diverse strains present in ABY (*Staphylococcus thermophilus, Lactobacillus delbrueckii. Bulgaricus, Bifidobacterium animalis spp., and Lactobacillus acidophilus)*, and further research on their interactions is deemed necessary for future investigations.

Consumer acceptance

Table 4 presents the results of the sensory evaluation of probiotic CHK juice prepared with the four different types of LAB-fermented CHK extracts. Significant differences were observed in the scores for color, flavor,

Table 4 Sensory evaluation of *Citrus hybrid kanpei* (CHK) fermented beverages

	Visual appearance	Color	Flavor	Sweetness	Sourness	Swallowability	Overall acceptability
YFH	6.52 ± 0.67^{a}	6.56 ± 0.69^{b}	4.68 ± 0.63^{b}	5.97 ± 0.82^{c}	6.57 ± 0.68^{a}	6.70 ± 0.54^{a}	5.58 ± 0.58^{b}
ABY	6.59 ± 0.66^{a}	6.59 ± 0.63^{b}	4.16 ± 0.69^{c}	6.40 ± 0.63^{b}	5.93 ± 0.66^{b}	6.52 ± 0.59^{a}	5.38 ± 0.49^{c}
YXC	6.46 ± 0.69^{a}	6.87 ± 0.62^{ab}	5.26 ± 0.53^{a}	6.87 ± 0.69^{a}	6.63 ± 0.50^{a}	6.57 ± 0.50^{a}	6.60 ± 0.65^{a}
YC	6.67 ± 0.65^{a}	7.00 ± 0.73^{a}	5.52 ± 0.57^{a}	6.94 ± 0.69^{a}	6.56 ± 0.64^{a}	6.80 ± 0.41^{a}	6.52 ± 0.63^{a}

$^{a–c}$ Means with different letters in the same row differ significantly at $p < 0.05$

CHK: *Citrus hybrid Kanpei,* YFH: YoFlex Harmony 1.0, ABY: ABY-3; YXC: YC-X11; YC: YC-180

sweetness, and sourness among the four probiotic juices. YXC and YC had the highest scores for color, flavor, and sweetness, whereas ABY had the lowest score for sourness. Overall acceptance was the highest for YXC (6.60) and YC (6.52), and the lowest for ABY (5.38). These differences can be attributed to the characteristics of LAB present in each sample [22]. Based on the antioxidant activity and number of viable LAB, YXC was identified as the most suitable for producing probiotic CHK juice.

DPPH radical scavenging activity and probiotic viability during in vitro digestion

The results of the in vitro gastrointestinal transit phase of the probiotic CHK juice showed that all sample groups showed a significant reduction in probiotic viability (Table 5), which is necessary for them to provide health benefits to consumers. These results can be ascribed to the activation of enzymes such as pepsin in the acidic environment of the simulated gastric juice, which results in the degradation of the cell membranes of the probiotics, leading to reduced viability [23]. Among the four samples, YC showed the highest survival rate during gastric digestion with a count of 5.88 log CFU/mL at the end of 60 min. However, the viability of LAB decreased significantly during intestinal digestion. Probiotics produced by LAB fermentation can provide health benefits through their bioaccessibility and bioactivity, even if they are not absorbed by the intestine [24]. Generally, the growth and viability of probiotics in fruit or vegetable beverages are related to the type of strain used, the pH of the product, and the concentrations of lactic and acetic acids [25]. Recent studies have implemented encapsulation to increase cell viability and prevent the release of probiotics

Table 5 Lactic acid bacteria viability of *Citrus hybrid kanpei* (CHK) fermented beverages in in vitro digestion

Gastric digestion	0 min	1 min	60 min
YFH	6.78 ± 0.06^{Ba}	5.44 ± 0.04^{Bb}	4.36 ± 0.07^{Cc}
ABY	6.04 ± 0.04^{Da}	4.71 ± 0.08^{Db}	4.61 ± 0.09^{Bb}
YXC	6.26 ± 0.05^{Ca}	4.84 ± 0.06^{Cb}	4.00 ± 0.02^{Dc}
YC	6.99 ± 0.13^{Aa}	5.88 ± 0.03^{Ab}	5.80 ± 0.03^{Ab}
Intestinal digestion	0 min	1 min	120 min
YFH	6.78 ± 0.06^{Ba}	4.13 ± 0.05^{Bb}	1.46 ± 0.05^{Cc}
ABY	6.04 ± 0.04^{Da}	4.62 ± 0.15^{Ab}	2.36 ± 0.32^{Bc}
YXC	6.26 ± 0.05^{Ca}	4.00 ± 0.00^{Bb}	2.49 ± 0.20^{Ac}
YC	6.99 ± 0.13^{Aa}	3.75 ± 0.05^{Cb}	1.84 ± 0.20^{Cc}

$^{a–c}$ Means with different letters in the same row differ significantly at $p < 0.05$

$^{A–D}$ Means with different letters in the same column differ significantly at $p < 0.05$

CHK: *Citrus hybrid Kanpei,* YFH: YoFlex Harmony 1.0, ABY: ABY-3; YXC: YC-X11; YC: YC-180

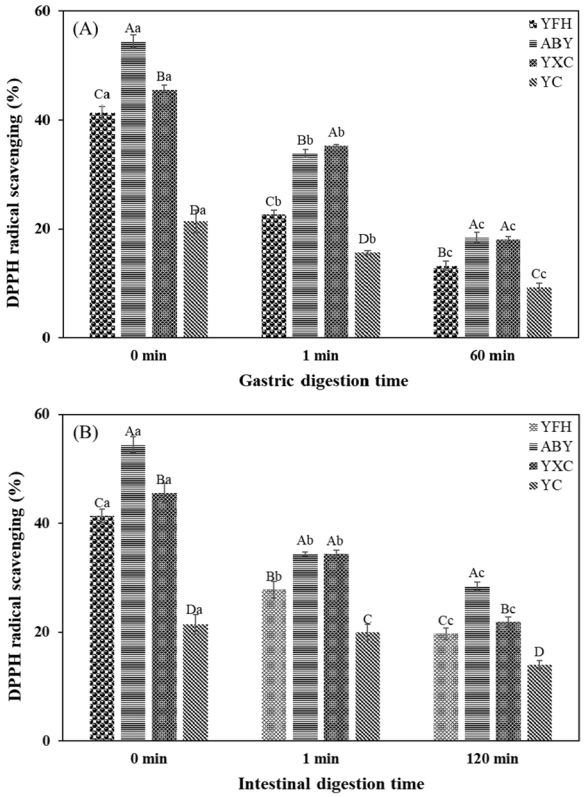

Fig. 5 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of probiotic *Citrus hybrid kanpei* (CHK) juice after in vitro digestion. Values followed by different lowercase letters (a–c) and different uppercase letters (A–D) are significantly different at $p < 0.05$, according to digestion time and group, respectively

into the body [26]. Therefore, to obtain beneficial effects from probiotics after gastrointestinal digestion, further research using various encapsulation techniques, such as spray drying, freeze drying, ion gelation, and in vivo studies, is necessary to fully elucidate the survival of probiotics in the product.

After in vitro digestion, the antioxidant capacity of the probiotic CHK juice is important as it represents the available antioxidant activity in the body. Fig. 5 reports the DPPH radical scavenging activity of probiotic CHK juice in a simulated gastrointestinal phase. Before digestion, the DPPH radical scavenging activity of the probiotic CHK juice was 21.40%–54.46%, and the highest activity was observed in ABY (54.46%). The antioxidant capacity after gastrointestinal digestion was the highest in ABY and YXC, followed by YFH. YC showed the lowest antioxidant capacity at all time points, which was consistent with the DPPH radical scavenging results of the LAB-fermented CHK extract. The overall trend in the antioty of the probiotic CHK juices after gastrointestinal digestion was as follows: ABY > YXC > YFH > YC. These results suggest that the antioxidants in these probiotic CHK juices are stable against pH changes and enzyme disruption, according to Ryan and Prescott [27]. It is important to understand the measurement principles and limitations of various antioxidant activities and to choose an appropriate method to evaluate antioxidant capacity.

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Author contributions
Conceptualization and Methodology: DK and IO, Formal analysis, Writing–original draft: DK, Validation, Investigation, Resources, Writing–review & editing, Supervision, Project administration, and Funding acquisition: IO. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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