Prev. Nutr. Food Sci. 2023;28(4):502-513
https://doi.org/10.3746/pnf.2023.28.4.502
ISSN 2287-8602

Multi-Strain Probiotics Enhance the Bioactivity of Cascara Kombucha during Microbial Composition-Controlled Fermentation

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ABSTRACT: Kombucha is a widely consumed fermented tea beverage with diverse health benefits. In a previous study, we demonstrated that the use of cascara as a substrate results in a special kombucha beverage with high bioactivity. Traditional kombucha fermentation using a symbiotic culture of bacteria and yeast (SCOBY) can lead to inconsistent product quality because of the lack of control over microbial composition. We successfully isolated and identified yeast and bacteria, including *Saccharomyces cerevisiae*, *Komagataeibacter rhaeticus*, and *Lactobacillus brevis* that are appropriate starter cultures for cascara kombucha fermentation. We also demonstrated that a supplementation with lactic acid bacteria (LAB) and a mixture of *S. cerevisiae* and *K. rhaeticus* resulted in higher total polyphenol and flavonoid content of cascara kombucha compared with the traditionally fermented product using SCOBY as the inoculum. The free radical scavenging activity, inhibitory effects on α-amylase, tyrosinase activity, and antibacterial properties of cascara kombucha were also enhanced as a result of LAB supplement. These findings provide valuable insights into the controlled microbiological composition required for the fermentation of cascara kombucha, thereby ensuring consistent quality and enhanced bioactivity of the product. Further, the use of cascara as a substrate for kombucha production not only offers various health benefits and biological effects, but also repurposes by-products from the coffee industry, which contributes to sustainable development and is eco-friendly.

Keywords: antibacterial, antioxidant, fermentation, probiotics, sustainable development

INTRODUCTION

Kombucha is a beverage produced by fermenting sweetened black or green tea using a symbiotic culture of bacteria and yeast (SCOBY) (Chakravorty et al., 2019). Kombucha is widely regarded for its health benefits, which include improved digestion, immune function, and antioxidant activity (Dutta and Paul, 2019). Recently, there has been increased interest in the identification of alternative substrates for kombucha fermentation, such as apple, grape, and pomegranate, as well as herbal infusions, to create diverse flavor profiles; thus, expanding the choices available to consumers (Ayed et al., 2017; Emiljanowicz and Malinowska-Pańczyk, 2020). Cascara, which is the dried husk of coffee cherries, is of interest because it contains an abundance of phenolic compounds and bioactive components, making it a promising substrate for kombucha production (Heeger et al., 2017). In a previous study, we found that cascara kombucha exhibits high levels of antioxidant and antimicrobial activities, which is comparable to that of black tea-based kombucha (Van et al., 2023). In addition, the use of cascara offers numerous benefits, including the development of novel fermented beverages with a distinctive flavor as well as the potential to reduce waste within the coffee industry, thereby contributing to sustainable agriculture (Van et al., 2023).

SCOBY is a biofilm that develops on the surface of the liquid during kombucha fermentation. It consists of a diverse microbial community, including acetic acid bacteria (AAB), lactic acid bacteria (LAB), and various yeast species (Antolak et al., 2021). During kombucha fermentation, yeast species including *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* play an important role in converting sugar to ethanol and carbon dioxide (CO_2) via the glycolysis pathway (Jayabalan et al., 2017). AAB species, including *Acetobacter* spp., *Gluconacetobacter* spp., and *Ko-*

Received 8 August 2023; Revised 18 September 2023; Accepted 25 September 2023; Published online 31 December 2023
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magataeibacter spp., metabolize ethanol into acetic acid, which is responsible for the distinct flavor of kombucha (Kumar and Joshi, 2016). AAB also participates in the synthesis of gluconic acid and glucuronic acid through fermentation (Jayabalan et al., 2014). LAB species, particularly *Lactobacillus* spp. and *Bifidobacterium* spp., contribute to fermentation by producing lactic acid and bioactive compounds, such as vitamins B, polyphenols, and antioxidant substances that are associated with the health benefits of kombucha (Chong et al., 2023). Furthermore, LAB in kombucha fermentation generates other bioactive compounds, including organic acids, antimicrobial agents, and beneficial metabolites, which play an important role in the sensory characteristics, preservation, and health benefits of kombucha (Ibrahim et al., 2021; Silva et al., 2021). A previous study demonstrated that incorporating *Lacticaseibacillus casei* and *Lactobacillus plantarum*, derived from kefir, into the kombucha fermentation process increased glucuronic acid levels, enhanced antioxidant activity, and improved antimicrobial effectiveness against pathogenic bacteria (Nguyen et al., 2015).

SOCBY represents a complex microbial community consisting of numerous bacterial and yeast species (Antolak et al., 2021). The interactions and synergistic effects of these microorganisms within the SCOBY contribute to the fermentation process as a whole and the distinctive characteristics of kombucha (Villarreal-Soto et al., 2018). However, the microbial composition of SCOBY varies considerably based on many factors, such as batch variation, geographic location, and tea substrates, making it challenging to establish standardized microbial profiles for kombucha (Mayser et al., 1995). The variability in the microbial community, which is influenced by environmental conditions, fermentation techniques, and SCOBY origin, can result in significant variations in the flavor, aroma, and quality of the final product (Suffys et al., 2023). Moreover, the presence of contaminants or undesirable microorganisms may threaten the quality and safety of kombucha (Villarreal-Soto et al., 2018; de Simone, 2019). Therefore, isolating starter bacteria for fermentation is essential to ensure consistent fermentation, improve efficiency, develop the desired flavors and fragrances, control spoilage microorganisms, optimize nutrient utilization, and facilitate product differentiation and innovation (Sharma et al., 2020).

Despite the importance of starter bacteria for kombucha fermentation, there have been few studies on the isolation of specific microbial strains for use as starter cultures. Therefore, we isolated and identified the yeast and bacterial strains responsible for cascara kombucha fermentation. Furthermore, LAB have been recognized for their beneficial probiotic effects on the host and their ability to enhance the beneficial properties of fermented foods (Ayivi et al., 2020). Therefore, we determined the effect of LAB supplementation of cascara kombucha fermentation under controlled microbial composition, with the goal of improving the biological activity, quality, and health-promoting activities of these products.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains, *Lactobacillus rhamnosus* ATCC 9595, *Staphylococcus aureus* MRSA ATCC 43300, *Salmonella enterica* ATCC 14028, and *Escherichia coli* ATCC 25922, were purchased from the American Type Culture Collection (ATCC). *Weizmannia coagulans* (isolated from fermented milk) was obtained from the Microbiology Laboratory, Department of Biotechnology, NTT Hi-Tech Institute, Nguyen Tat Thanh University.

Isolation of bacteria and yeast from SCOBY

SCOBY was purchased from Foodplus Ltd., Hanoi, Vietnam. To isolate bacteria and yeast, 1 g of SCOBY was homogenized in 10 mL of phosphate-buffered saline (PBS), serial diluted, and plating on appropriate selection media. AAB were isolated on YPGD agar containing 5 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 5 g/L glycerol, 4% ethanol, and 2% agar (Wu et al., 2017). Yeast isolation was done using a yeast extract-peptone-dextrose (YPD) medium (Sigma-Aldrich) supplemented with 1% chloramphenicol to suppress bacterial growth (Qvirist et al., 2016). LAB were isolated on DeMan, Rogosa, Sharpe (MRS) agar (HiMedia Laboratories) (Reuben et al., 2019). Subsequently, single colonies were selected and subcultured to obtain pure cultures.

Morphological and biochemical characterization of bacteria and yeast isolates

The primary identification of isolates was based on culture characteristics, Gram staining, and microscopic observation. Yeast strains were characterized by spherical or ovoid morphology (Hossain et al., 2020). AAB strains were characterized by Gram-negative staining and the ability to convert ethanol to acetic acid (indicated by a color change from green to yellow in Carr medium) and produce bacterial cellulose on Hestrin-Schramm (HS) medium (Semjonovs et al., 2017). LAB strains were characterized by Gram-positive characteristics and negative for catalase and coagulase activity (Reuben et al., 2019).

Molecular identification of the selected isolates

The molecular identification of yeast isolates was done by amplification of the internal transcribed spacer (ITS) region using universal primers ITS1 (5'-GTTTCCGTAGGT GAACTTGC-3') and ITS4 (5'-TCCTCCGCTTATTGATA TGC-3') (Sebastiani et al., 2002). For bacterial identifica-

tion, the 16S ribosomal DNA was amplified using primers E517F (5'-GCCAGCAGCCGCGGTAA-3') and E106R (5'-CTCACGRCACGAGCTGACG-3') (Wang et al., 2022). Polymerase chain reaction (PCR) products were conducted on a T100 Thermal Cycler (Bio-Rad) and the resulting PCR products were visualized using 0.8% agarose gel electrophoresis with GelRed Loading Buffer (TBR). Subsequently, the PCR products were purified and sequenced by DNA SEQUENCING Ltd. The resulting Sanger sequencing data were edited using BioEdit software and aligned with full-length sequences in the NCBI Basic Local Alignment Search Tool (BLAST) databases to retrieve references. Phylogenetic analysis based on the ITS rDNA or 16S rDNA sequence was performed using the ClustalW function and the neighbor-joining method implemented in MEGA 6.0.

Kombucha fermentation

Cascara was extracted in hot water for 20 min at a concentration of 10 g/L (1%). Then, 100 g/L sucrose was added, transferred to a 100 mL bottle, and autoclaved for sterilization. The cascara solution was cooled to room temperature and inoculated with either SCOBY or the bacterial mixture, as shown in Table 1 at 30°C for 12 days.

Determination of pH and total acid in cascara kombucha

The pH of cascara kombucha was measured using a Mettler Toledo J12683 pH meter. Total acidity was measured by acid-base titration using a 0.1 N sodium hydroxide (NaOH) solution as the titrant and phenolphthalein as the color indicator.

Quantification of total polyphenol content (TPC) in cascara kombucha

The determination of polyphenols was done using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). The reaction mixture consisted of 500 μL of cascara kombucha, 500 μL distilled water, and 500 μL of 10% Folin-Ciocalteu reagent, which was thoroughly mixed. Next, 500 μL of a 10% Na_2CO_3 solution was added and the mixture was incubated at 40°C for 30 min. The absorbance of the reaction mixture was determined at a wavelength of 765 nm. The TPC content was quantified using a gallic acid standard curve with a concentration range of 0～100 μg/mL.

Quantitation of total flavonoid content (TFC) in cascara kombucha

The TFC content was quantified using the method described by (Pękal and Pyrzynska, 2014). The reaction mixture consisted of 0.5 mL of cascara kombucha or quercetin standard (QE) solution in 1.5 mL 99% ethanol and was incubated for 5 min. Next, 0.1 mL of 10% aluminum chloride ($AlCl_3$) was introduced and maintained at room temperature for 5 min. Then, 0.1 mL of 1 M potassium acetate (CH_3COOK) was added to the mixture and incubated for 45 min at room temperature. The absorbance was measured at a wavelength of 415 nm. The TFC content was determined based on a QE standard curve with a concentration range of 0～200 μg/mL.

Pretreatment of cell-free supernatants (CFS) derived from cascara kombucha

CFS was obtained by centrifugation of the fermented cascara at 10,000 *g* and subsequently filtered through a 0.22 μm filter. The CFS were adjusted to a pH of 7 using a 1 N NaOH solution. The CFS were incubated at 95°C for 15 min and subsequently used for further experiments.

Evaluation of antioxidant activity of cascara kombucha

The antioxidant activity of CFS derived from cascara kombucha was evaluated by spectrophotometric methods using the synthetic free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Pekkarinen et al., 1999) or 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Re et al., 1999). The reaction mixture consisted of 50 μL of pH-neutralized CFS or heat-treated CFS derived from cascara kombucha in 1 mL of DPPH (Cas 1898-66-4; TCI Chemicals) or ABTS (Cool Chemical Science and Technology) in the dark at room temperature for 30 min. The group re-

Table 1. The inoculum received in each experimental group

Group	Inoculum
Control	None
SCOBY	3g/L SCOBY
SK	1×10^6 CFU/mL of *S. cerevisiae* and 1×10^6 CFU/mL of *K. rhaeticus*
SKLR	1×10^6 CFU/mL of *S. cerevisiae*, 1×10^6 CFU/mL of *K. rhaeticus*, and 1×10^6 CFU/mL of *L. rhamnosus*
SKW	1×10^6 CFU/mL of *S. cerevisiae*, 1×10^6 CFU/mL of *K. rhaeticus*, and 1×10^6 CFU/mL of *W. coagulans*
SKLB	1×10^6 CFU/mL of *S. cerevisiae*, 1×10^6 CFU/mL of *K. rhaeticus*, and 1×10^6 CFU/mL of *L. brevis*
SK Multi-Lab	1×10^6 CFU/mL of *S. cerevisiae* and 1×10^6, CFU/mL of *K. rhaeticus*, 1×10^6 CFU/mL of *L. rhamnosus*, 1×10^6 CFU/mL of *W. coagulans*, and 1×10^6 CFU/mL of *L. brevis*

SCOBY, symbiotic culture of bacteria and yeast; SK, *Saccharomyces cerevisiae* and *Komagataeibacter rhaeticus*; SKLR, *S. cerevisiae*, *K. rhaeticus*, and *Lactobacillus rhamnosus*; SKW, *S. cerevisiae*, *K. rhaeticus*, and *Weizmannia coagulans*; SKLB, *S. cerevisiae*, *K. rhaeticus*, and *Lactobacillus brevis*; SK Multi-Lab, *S. cerevisiae*, *K. rhaeticus* and mixture of *L. rhamnosus*, *W. coagulans*, and *L. brevis*.

ceiving only distilled water was considered the control group. The absorbance was measured at a wavelength of 517 nm for the DPPH assay and 734 nm for the ABTS assay. The experiment was conducted in triplicate with three independent replicates and changes in the reaction were calculated by comparison with the control groups.

Evaluation of the α-amylase inhibitory activity of cascara kombucha

The α-amylase inhibitory effect of the CFS derived from cascara kombucha was determined by adding 10 μL of pH-neutralized CFS or heat-treated CFS derived from cascara kombucha to a mixture of 90 μL of PBS and 50 μL of 4 μM α-amylase (Sigma-Aldrich) solution in a 96-well microplate (Gu et al., 2021). The control group received distilled water, whereas the group receiving acarbose was considered a positive control. The plate was then incubated at 37°C for 10 min. Then, 50 μL of a 0.5 mg/mL starch solution was added, followed by incubation at 37°C for 10 min. Subsequently, 10 μL of 1 M HCl was added and the color reaction was initiated by adding 100 μL of 3,5-dinitrosalicylic acid (DNS) (Sigma-Aldrich) color reagent and boiled for 20 min. The absorbance of each reaction was measured spectrophotometrically at a wavelength of 590 nm. The experiment was conducted in triplicate with three independent replications and changes in the reaction were calculated by comparison with the control groups.

Evaluation of the tyrosinase inhibitory activity of cascara kombucha

The tyrosinase inhibitory effect of CFS derived from cascara kombucha was measured as described previously (Batubara et al., 2015) using L-DOPA as the substrate, with kojic acid serving as the positive control. Briefly, 70 μL of pH-neutralized or heat-treated CFS derived from cascara kombucha was aliquoted into 96-well plates. Then, 30 μL of tyrosinase (Sigma-Aldrich) at a concentration of 300 U/mL in PBS was added and the mixture was incubated for 5 min. Following the incubation, 100 μL of 2 mM L-DOPA was added and the solution was incubated at 37°C for 30 min. The absorbance of each reaction was measured spectrophotometrically at a wavelength of 492 nm. The experiment was performed in triplicate with three independent replicates and changes in the reaction were calculated by comparison with the control groups.

Evaluation of antibacterial activity of cascara kombucha

The antibacterial ability of CFS derived from cascara kombucha was determined using an agar diffusion assay (Balouiri et al., 2016). The pathogenic bacterial strains were cultured overnight in Mueller Hinton Broth (HiMedia Laboratories), suspended in PBS, and diluted to a final concentration of 1×10^7 CFU/mL). A total of 100 μL of each bacterial suspension was spread onto Mueller Hinton agar (MHA) culture medium. Three wells with a diameter of 5 mm were created on each MHA plate, equidistant from one another. Subsequently, 100 μL of the CFS, pH-neutralized CFS, or heat-treated CFS derived from cascara kombucha were added to each well, whereas the control group received only distilled water. The MHA plates were incubated at 37°C and the inhibition zones were measured after a 24-h incubation.

Statistical analysis

A completely randomized design with three replicates was used for each treatment. Data were analyzed using SAS 9.4 software (SAS, Inc.) and presented as the mean± standard error of the mean of triplicate readings. Statistical significance between groups was determined using Duncan's test with $P<0.05$ indicating significance.

RESULTS

Isolation of bacteria and yeast involved in SCOBY formation

Yeast isolates were cultured on YPD medium containing chloramphenicol, whereas AAB and LAB were isolated on YPGD and MRS agar, respectively. The yeast colonies obtained from the YPD medium exhibited a circular shape and a creamy white color. Microscopic analysis revealed that these colonies had a spherical or ovoid morphology (Fig. 1A). A biochemical evaluation confirmed negative urease activity and positive CO_2 production. AAB colonies grown on YPGD medium exhibited a round shape with slightly wrinkled and rough surfaces. Rod-shaped Gram-negative bacteria were observed by microscopic examination (Fig. 1B). The production of acetic acid was assessed by inoculating the isolate onto Carr agar supplemented with ethanol and bromocresol green, which resulted in a color transition from green to yellow (Fig. 2A). Bacterial cellulose production was evaluated using HS medium, which resulted in the development of a thick cellulose layer (Fig. 2B). The LAB strains isolated on MRS medium exhibited a Gram-positive rod shape (Fig. 1C) with negative catalase activity.

Molecular identification of bacteria and yeast responsible for kombucha fermentation

The PCR product for the rDNA region derived from each isolate was amplified and subsequently purified to obtain the nucleotide sequence. Phylogenetic analysis was conducted using reference species from Gene Bank to determine the taxonomic classification of the isolates. A BLAST analysis revealed that the yeast strain shared 99.81% similarity with *S. cerevisiae*, the AAB strain exhibited 99.85% similarity with *Komagataeibacter rhaeticus*, and the LAB

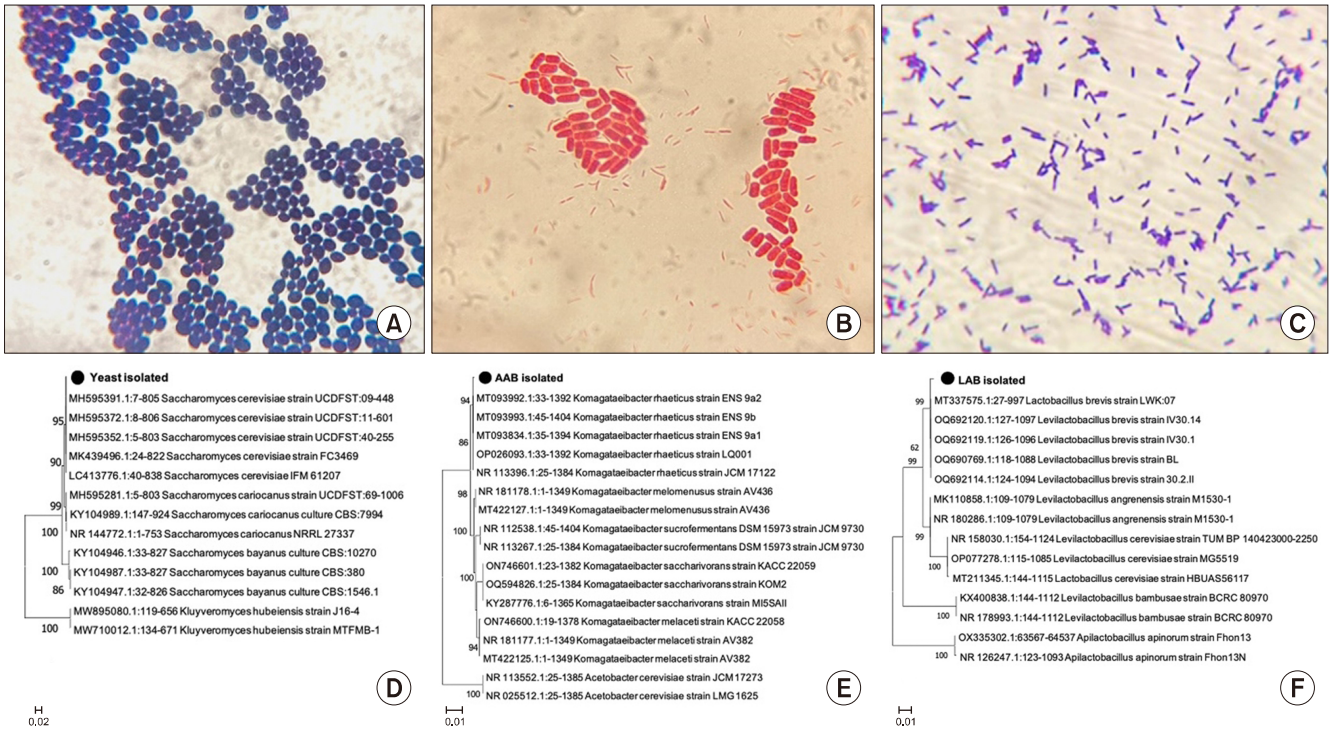

Fig. 1. Characterization of the microbial strain responsible for kombucha fermentation. Gram stain microscopic morphology of yeast (A), acetic acid bacteria (AAB) (B), and lactic acid bacteria (LAB) (C) isolated from symbiotic culture of bacteria and yeast under a microscope at 1,000× magnification. A phylogenetic tree based on internal transcribed spacer gene sequences of yeast (D), AAB (E), and LAB (F) isolates and reference strains in the Gene Bank database.

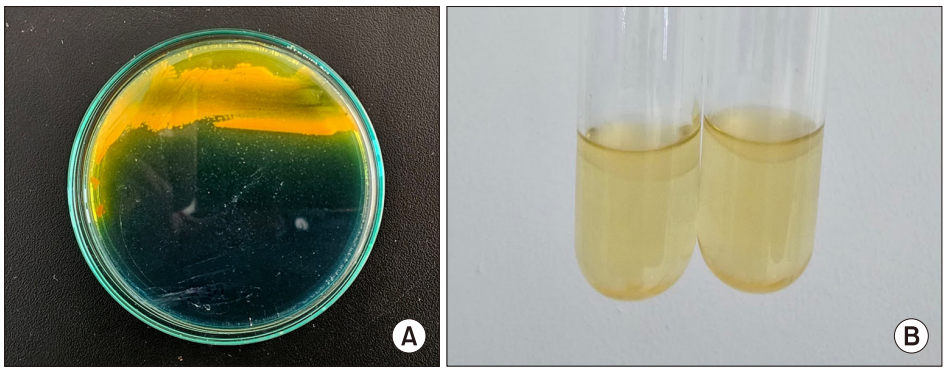

Fig. 2. (A) The growth of acetic acid bacteria (AAB) on Carr agar supplemented with ethanol and bromocresol green resulted in a color change from green to yellow. (B) Bacterial cellulose production of AAB in Hestrin-Schramm medium.

strain showed 99.99% similarity with *Lactobacillus brevis*. Internal phylogenetic trees were constructed using MEGA 4.1, based on ITS rDNA for yeast strains and 16S rDNA for bacterial strains. The phylogenetic analysis revealed that the yeast isolates and *S. cerevisiae* belonged to the same clade, with other *Saccharomyces* species showing a greater divergence from *S. cerevisiae* (Fig. 1D). The AAB strain clustered together with *K. rhaeticus* (Fig. 1E), whereas the LAB strain was grouped with *L. brevis* (Fig. 1F). Therefore, the yeast, AAB, and LAB isolates were identified as *S. cerevisiae*, *K. rhaeticus*, and *L. brevis*, respectively.

Effect of inoculation on nascent pellicle SCOBY formation on cascara substrates

Kombucha fermentation was carried out by inoculating the cascara substrate with either SCOBY alone or a mixture of bacteria and yeast, followed by incubation at 30°C. All groups exhibited nascent pellicle formation after 10 days, which indicated SCOBY development. The group that only received *S. cerevisiae* and *K. rhaeticus* as starter cultures had considerably lower nascent pellicle mass compared with that of the control group that received the "mother" SCOBY (Fig. 3A). In contrast, LAB supplementation to the *S. cerevisiae* and *K. rhaeticus* mixture restored the nascent pellicle mass, indicating a positive interaction between LAB, yeast, and AAB (Fig. 3A).

Determination of pH and total acid accumulating in the cascara kombucha

The pH of non-fermented cascara was initially measured at 4.78; however, after 12 days of fermentation, significant differences in pH were observed among the different

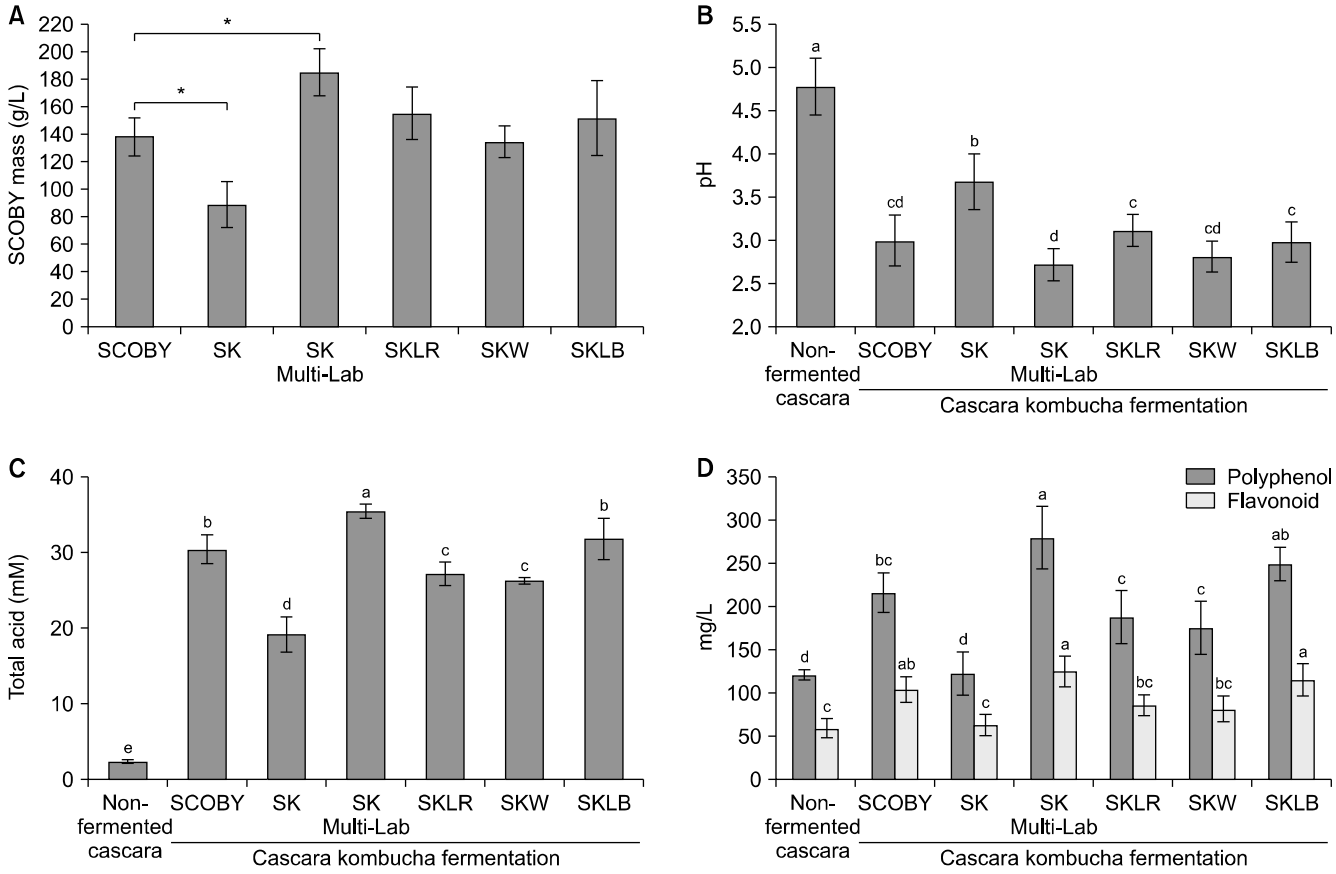

Fig. 3. Effects of the inoculum on the growth of nascent pellicles symbiotic culture of bacteria and yeasts (SCOBYs) using a cascara substrate (A), pH values (B), total acid accumulation (C), and total polyphenol content (TPC)/total flavonoid content (TFC) (D) in cascara kombucha. Data are presented as the means of triplicate analysis±SD. *Indicate the significant difference from the SCOBY group (P<0.05). Lowercase letters (a-e) indicate significant differences between groups according to Duncan's test (P<0.05). SK, *Saccharomyces cerevisiae* and *Komagataeibacter rhaeticus*; SK Multi-Lab, *S. cerevisiae*, *K. rhaeticus* and mixture of *Lactobacillus rhamnosus*, *Weizmannia coagulans*, and *Lactobacillus brevis*; SKLR, *S. cerevisiae*, *K. rhaeticus*, and *L. rhamnosus*; SKW, *S. cerevisiae*, *K. rhaeticus*, and *W. coagulans*; SKLB, *S. cerevisiae*, *K. rhaeticus*, and *L. brevis*.

groups depending on the inoculation. The pH of the SK group was 3.68, which was significantly higher compared with the pH values of the cascara kombucha groups with SCOBY or SK supplemented with LAB as inoculum (Fig. 3B). The presence of LAB, whether naturally occurring in the original SCOBY or as a supplement, enhanced acidification, as evidenced by total acid quantitation and resulting in a reduction in pH levels between 2.7~3.1 (Fig. 3B and 3C).

Effect of inoculation on total polyphenol and flavonoid content of the cascara kombucha beverage

The levels of TPC and TFC in cascara kombucha were measured to determine the effect of different inoculums. Using only *S. cerevisiae* and *K. rhaeticus* as starter cultures, no significant effect on TPC and TFC levels was observed compared with the non-fermented group (Fig. 3D). However, the use of SCOBY as the starter culture resulted in a significant increase in TPC and TFC in cascara kombucha after 12 days of fermentation (Fig. 3D). Furthermore, inclusion of *L. brevis* in the mixture of *S. cerevisiae* and *K. rhaeticus* resulted in a significant increase in TFC and TPC levels, which could overcome those achieved with the SCOBY inoculum (Fig. 3D) and comparable to that of the SK Multi-Lab group. These results indicate a positive effect of LAB, specifically *L. brevis*, which enhances the levels of phenolic and flavonoid compounds in cascara kombucha.

Effect of inoculation on the antioxidant activities of cascara kombucha beverage

The antioxidant activity of cascara kombucha was examined (Fig. 4). Our data indicate that, with the exception of the KS group, the fermentation of cascara kombucha resulted in significant DPPH and ABTS free radical scavenging activities compared with the non-fermented group (Fig. 4). In addition, *L. brevis* or SK Multi-Lab administration resulted in greater free radical scavenging abilities compared with the group that received SCOBY as the inoculum (Fig. 4). In addition, heat treatment did not affect the antioxidant activity of cascara kombucha (Fig. 4). These results indicate that LAB, particularly *L. brevis*, is involved in the fermentation cascara kombucha, which produces thermostable bioactive compounds with antioxidant activity.

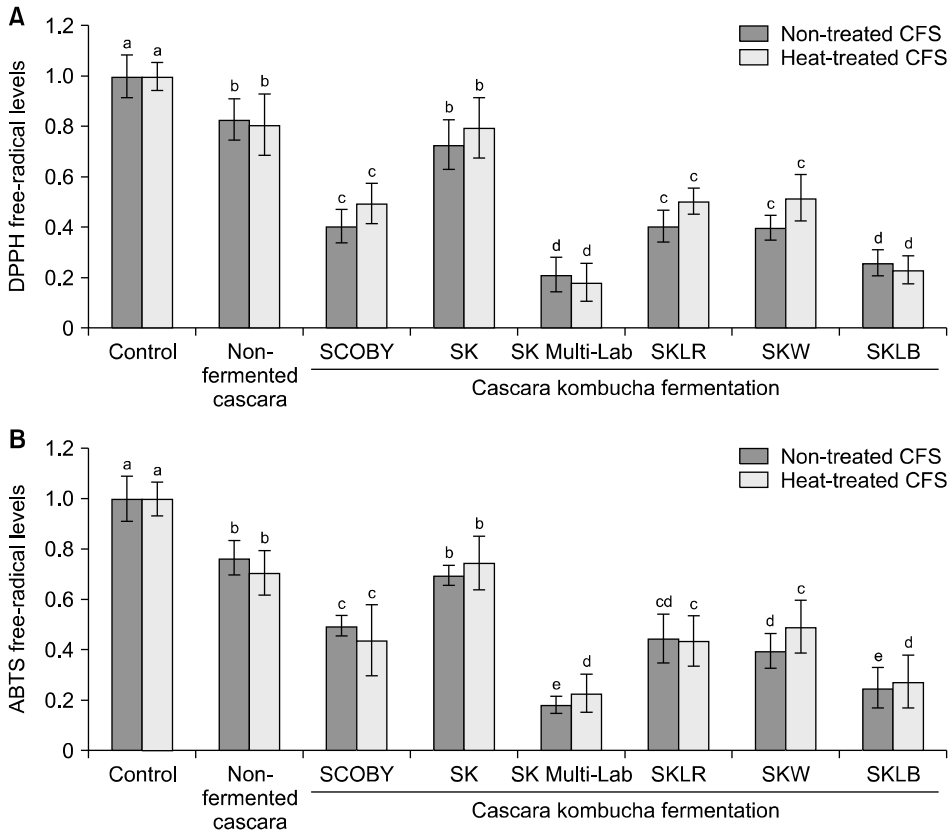

Fig. 4. Effects of the inoculum on the free radical scavenging activities of cascara kombucha expressed by DPPH (A), and ABTS (B). Data are presented as the means of triplicate analysis±SD. Lowercase letters (a–e) indicate significant differences between groups according to Duncan's test ($P<0.05$). CFS, cell-free supernatants; SCOBY, symbiotic culture of bacteria and yeast; SK, *Saccharomyces cerevisiae* and *Komagataeibacter rhaeticus*; SK Multi-Lab, *S. cerevisiae*, *K. rhaeticus* and mixture of *Lactobacillus rhamnosus*, *Weizmannia coagulans*, and *Lactobacillus brevis*; SKLR, *S. cerevisiae*, *K. rhaeticus*, and *L. rhamnosus*; SKW, *S. cerevisiae*, *K. rhaeticus*, and *W. coagulans*; SKLB, *S. cerevisiae*, *K. rhaeticus*, and *L. brevis*.

Effect of inoculation on α-amylase inhibition ability of the cascara kombucha beverage

Alpha-amylase and α-glucosidase are essential enzymes involved in the hydrolysis of starch into disaccharides and their subsequent conversion into glucose (Masasa et al., 2022). The inhibition of α-amylase is an important strategy in the management of diabetes (Koh et al., 2010). Thus, we determined whether cascara kombucha could inhibit α-amylase activity. The results (Fig. 5A) indicated that the α-amylase activity was not affected by non-fermented cascara. However, CFSs derived from cascara kombucha fermented by SCOBY or SK with LAB supplementation inhibited α-amylase activity, even those that were heat-treated. These results indicate an important role for LAB supplement of cascara kombucha, which enhances alpha-amylase inhibition; therefore, providing novel strategies for blood sugar regulation and contributing to the management of type 2 diabetes.

Effect of inoculation on tyrosinase inhibition of the cascara kombucha beverage

Tyrosinase, an indispensable enzyme in melanin biosynthesis, converts tyrosine into dihydroxyphenylalanine (DOPA). DOPA then undergoes subsequent enzymatic reactions resulting in melanin formation (Chen et al., 2017). Overexpression of tyrosinase activity causes hyperpigmentation disorders associated with skin aging, including loss of elasticity and wrinkles (Pintus et al., 2022). In addition, tyrosinase activity may be associated with neuromelanin synthesis in the brain and neurodegenerative disorders, such as Parkinson's disease (Carballo-Carbajal et al., 2019). The results indicated that cascara kombucha derived from SCOBY or SK supplemented with LAB fermentation reduced tyrosinase activity (Fig. 5B). Moreover, *L. rhamnosus* or SK Multi-Lab supplement exhibited a marked inhibitory effect compared with the group receiving SCOBY as an inoculum (Fig. 5B). These findings indicate that LAB releases unknown compounds which are potent tyrosinase inhibitors during cascara kombucha fermentation. Thus, cascara kombucha with LAB supplement, particularly *L. rhamnosus*, are valuable for ameliorating aging-associated and neurodegenerative processes mediated by tyrosinase activity.

Effect of inoculation on antibacterial activity of the cascara kombucha beverage

The antibacterial activities of cascara kombucha were evaluated to determine its effect against pathogenic bacteria. Non-fermented cascara showed no antibacterial activity; however, cascara kombucha demonstrated inhibitory effects against *S. aureus*, *E. coli*, and *S. enterica*, which were dependent on the initial inoculum (Table 2). The antibacterial activities of the SK group were weak and undetectable after pH neutralization and heat treatment (Table 2). These results indicate that the antibacterial activity of the SK group is primarily associated with acetic acid (Kundukad et al., 2020). In contrast, SK Multi-Lab or *L. brevis* supplementation into the SK group resulted in

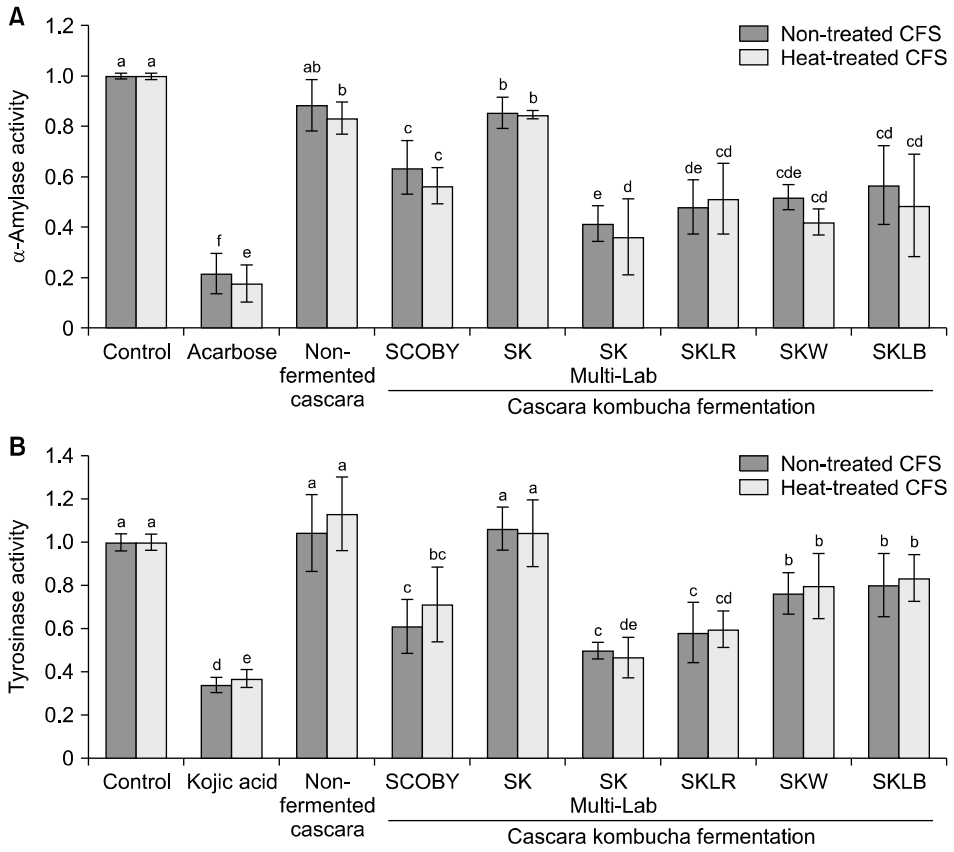

Fig. 5. Effects of the inoculum on α-amylase inhibition activity (A) and tyrosinase inhibition activity (B) of cascara kombucha. Data are presented as the means of triplicate analysis±SD. Lowercase letters (a-f) indicate significant differences between groups according to Duncan's test (P<0.05). CFS, cell-free supernatants; SCOBY, symbiotic culture of bacteria and yeast; SK, *Saccharomyces cerevisiae* and *Komagataeibacter rhaeticus*; SK Multi-Lab, *S. cerevisiae*, *K. rhaeticus* and mixture of *Lactobacillus rhamnosus*, *Weizmannia coagulans*, and *Lactobacillus brevis*; SKLR, *S. cerevisiae*, *K. rhaeticus*, and *L. rhamnosus*; SKW, *S. cerevisiae*, *K. rhaeticus*, and *W. coagulans*; SKLB, *S. cerevisiae*, *K. rhaeticus*, and *L. brevis*.

Table 2. Effect of inoculation on the antibacterial activity of cascara kombucha using an agar diffusion assay

	Inhibition zone diameter (mm)						
	Control	SCOBY	SK	SK Multi-Lab	SKLR	SKW	SKLB
Staphylococcus aureus							
CFS	0.00±0.00^{e}	15.67±2.25^{c}	10.25±1.47^{d}	22.00±3.03^{a}	16.00±1.41^{c}	17.17±1.47^{bc}	19.50±2.74^{ab}
pH-neutralized CFS	0.00±0.00^{c}	14.17±1.17^{b}	0.00±0.00^{c}	20.17±3.66^{a}	14.00±1.26^{b}	15.50±3.08^{b}	19.67±3.01^{a}
Heat-treated CFS	0.00±0.00^{c}	15.50±3.27^{b}	0.00±0.00^{c}	21.00±2.82^{a}	14.17±1.60^{b}	14.27±1.83^{b}	18.67±3.67^{a}
Escherichia coli							
CFS	0.00±0.00^{e}	13.33±1.03^{c}	11.33±2.34^{d}	22.17±2.64^{a}	14.50±1.22^{bc}	14.17±0.75^{c}	16.33±1.03^{b}
pH-neutralized CFS	0.00±0.00^{e}	14.07±0.77^{d}	0.00±0.00^{e}	21.17±2.56^{b}	16.17±0.75^{c}	13.33±1.03^{d}	23.17±2.32^{a}
Heat-treated CFS	0.00±0.00^{d}	14.50±1.05^{c}	0.00±0.00^{d}	19.67±1.97^{a}	13.83±2.14^{c}	13.67±1.75^{c}	17.00±2.53^{b}
Salmonella enterica							
CFS	0.00±0.00^{c}	17.33±2.16^{a}	11.17±1.17^{b}	19.67±1.63^{a}	13.50±0.83^{b}	13.67±1.75^{b}	19.83±3.54^{a}
pH-neutralized CFS	0.00±0.00^{c}	12.00±1.41^{b}	0.00±0.00^{c}	18.67±1.75^{a}	11.33±1.51^{b}	13.17±2.13^{b}	16.83±2.48^{a}
Heat-treated CFS	0.00±0.00^{d}	13.17±1.47^{c}	0.00±0.00^{d}	19.67±1.97^{a}	14.17±1.17^{c}	14.33±1.63^{bc}	18.17±1.60^{ab}

The inhibition zone calculated in the diameter around the well.
Values are presented as the mean of triplicate analysis±SD.
Lowercase letters (a-e) within the line indicate significant differences between groups according to Duncan's test (P<0.05).
CFS, cell-free supernatants; SCOBY, symbiotic culture of bacteria and yeast; SK, *Saccharomyces cerevisiae* and *Komagataeibacter rhaeticus*; SK Multi-Lab, *S. cerevisiae*, *K. rhaeticus* and mixture of *Lactobacillus rhamnosus*, *Weizmannia coagulans*, and *Lactobacillus brevis*; SKLR, *S. cerevisiae*, *K. rhaeticus*, and *L. rhamnosus*; SKW, *S. cerevisiae*, *K. rhaeticus*, and *W. coagulans*; SKLB, *S. cerevisiae*, *K. rhaeticus*, and *L. brevis*.

higher antibacterial activity compared with the group that received SCOBY as an inoculum (Table 2). Moreover, the antibacterial activity remained even after pH neutralization or heat treatment, indicating the presence of heat-resistant antibacterial compounds originating from LAB (Table 2).

DISCUSSION

Traditional kombucha production using the entire "mother SCOBY" as the initial inoculum has limitations because of its inability to control various microorganisms, which results in inconsistent product quality, a negative

effect on fermentation efficiency, and potential biological safety concerns (Freer et al., 2003). In the present study, a microbial symbiosis model consisting of isolated and selected yeast, AAB, and LAB strains was developed to ensure safe production. Furthermore, our previous study indicated the potential of cascara to serve as a suitable substrate for the production of kombucha beverage containing various health-promoting compounds. The product exhibited sensory properties comparable to that of traditional black tea-based kombucha (Van et al., 2023). Therefore, in the present study, we used cascara as a substrate for kombucha fermentation with controlled microbial composition to produce a consistently high-quality fermented beverage with enhanced nutritional benefits for consumers.

Bacteria and yeast strains responsible for kombucha fermentation that were isolated and identified included *S. cerevisiae*, *K. rhaeticus*, and *L. brevis*. Kombucha fermentation was carried out by inoculating a mixture of bacteria and yeast on a cascara substrate, which resulted in the formation of a nascent pellicle SCOBY mass that was equivalent to using "mother SCOBY." In addition, including LAB along with kombucha fermentation yielded a higher nascent pellicle SCOBY mass compared with yeast and AAB alone. This suggests that LAB also has a role in promoting SCOBY formation. This finding is consistent with that of a previous study indicating that co-cultivation of AAB and LAB results in the increased formation of bacterial cellulose compared with AAB monoculture (Brugnoli et al., 2023). Quorum sensing (QS), a density-dependent signaling mechanism, facilitates the interaction between LAB and AAB, leading to bacterial cellulose formation (Zhang et al., 2021). Moreover, kombucha fermentation caused a significant change in pH because of the accumulation of organic acids resulting from microbial activity (Chakravorty et al., 2019). The controlled fermentation of cascara kombucha with specific microorganisms yielded a pH range of 2.7∼3.1, resembling the pH levels observed in the group that used SCOBY as an inoculum. The recommended pH value for kombucha products is 2.5 to 4.2, which is considered microbiologically safe (Nummer, 2013). Consequently, the use of AAB, yeast, and LAB in cascara kombucha fermentation is appropriate with the recommended safety standard for kombucha.

Our study emphasized the significant contribution of LAB for enhancing the bioactivity of cascara kombucha. The inclusion of *L. brevis* or SK Multi-Lab during cascara kombucha fermentation resulted in higher concentrations of total polyphenols and flavonoids compared with the group that used the conventional "mother SCOBY" as an inoculum. Furthermore, LAB was shown to express β-glucosidase that cleaves glycosidic bonds in polyphenol compounds, resulting in the liberation of aglycones (De Montijo-Prieto et al., 2023). Moreover, LAB may secrete esterase that breaks down larger polyphenol molecules into smaller fragments and converts them into a bioactive form (Esteban-Torres et al., 2015). The enhancement of total polyphenol and flavonoid content during fermentation may result in increased free radical scavenging capacity of cascara kombucha. In addition, LAB may synthesize peptides, which are responsible for antioxidant *in vitro* (Taha et al., 2017). Moreover, exopolysaccharides derived from *Lactobacillus* spp. modulate antioxidants in mice cause by 1,2-dimethyl hydrazine (Deepak et al., 2021). Thus, the inclusion of *L. brevis* in cascara kombucha during fermentation may represent a promising strategy to enrich polyphenols and flavonoids, thereby enhancing the antioxidant capability of the final product.

Inhibition of α-amylase activity shows the potential for managing type 2 diabetes *in vitro* and *in vivo* (Kumari et al., 2022; Liu et al., 2022). Furthermore, lowering tyrosinase activity contributes to the neuroprotective potential in mice (Lee et al., 2023). Moreover, dietary supplementation of probiotics reduces locomotor impairment in mice with Parkinson's disease (Sun et al., 2022a). Our results suggest that LAB supplementation elevates the bioactivity of cascara kombucha and inhibits α-amylase activity. Interestingly, *L. rhamnosus* or SK Multi-Lab supplementation has a profound effect on reducing the tyrosinase activity of the product; however, the specific compounds responsible for the observed bioactivities in cascara kombucha have not been identified. These bioactive properties remained present even after undergoing heat treatment, which suggests that LAB releases various heat-stable bioactive compounds during fermentation, which may contribute to the overall health benefits of the final product.

The antibacterial activity of kombucha is attributed to the accumulation of acetic acid during fermentation (de Miranda et al., 2022). The inhibitory effect was lost when the pH was neutralized in the group that only received yeast and AAB as the inoculum. LAB supplementation during fermentation improved the antibacterial effectiveness. In addition, the antibacterial activity was unaffected by pH neutralization or heat treatment. In addition to organic acids, LAB releases heat-resistant molecules with antibacterial activity during fermentation. LAB inhibits the growth of pathogenic bacteria by generating antimicrobial exopolysaccharides, peptides, small molecules, and bacteriocins (Perez et al., 2014; Sun et al., 2022b; Yang et al., 2023). Furthermore, acetic acid modulates QS in *Lactobacillus* spp., which results in the increased production of bacteriocins and enhanced antibacterial effects (Meng et al., 2021). Thus, the co-cultivation of AAB and LAB may increase antibacterial activity by regulating bacteriocin production through an acid acetic-mediated pathway (Meng et al., 2021). Further studies are needed to better

understand the mechanisms involved in this interaction, which will facilitate the enhanced control over the fermentation process and improve the bioactivity quality of cascara kombucha.

In summary, the present study highlights the significance of effectively managing the microbial composition for ingredient production to ensure consistent and high-quality kombucha production, while minimizing the risk of contamination. We successfully isolated and identified *S. cerevisiae*, *K. rhaeticus*, and *L. brevis* as suitable starter strains for kombucha fermentation. The inclusion of LAB during cascara kombucha fermentation, specifically *L. brevis*, resulted in a marked increase of polyphenol and flavonoid levels, thereby enhancing the antioxidant capacity of the product. In contrast, *L. rhamnosus* produces unknown bioactive compounds that significantly lower tyrosinase activity *in vitro*. These effects remain in the SK Multi-Lab group, suggesting the absence of inhibitory interactions among the LAB strains. Although *W. coagulans* exhibits lower biological activity compared with *Lactobacillus* spp., it still contributes to α-amylase inhibition and the antibacterial activity of the product *in vitro*. Therefore, the improving *W. coagulans* is necessary for its application to kombucha production. The bioactive compounds produced by LAB during cascara kombucha fermentation remain unknown and require further analysis. Although these findings suggest potential health benefits *in vitro*, additional studies using animal models are necessary to better understand the physiological and biochemical mechanisms underlying the potential health benefits of cascara kombucha. Utilizing cascara, a coffee industry by-product, as a substrate for kombucha production with a high bioactive content, also contributes to reduced waste and encourages environmentally friendly practices, thereby contributing to sustainable agriculture.

ACKNOWLEDGEMENTS

The authors are especially grateful to Nguyen Tat Thanh University for providing all the resources needed for this study.

FUNDING

None.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: ADD, TPV. Analysis and interpretation: ADD, TPV, HPQ, QKP, GBP, NHNT, HTTT. Data collection: HPQ, QKP, GBP, NHNT, HTTT. Writing the article: ADD, TPV. Critical revision of the article: ADD. Final approval of the article: all authors. Statistical analysis: ADD. Overall responsibility: ADD, TPV.

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