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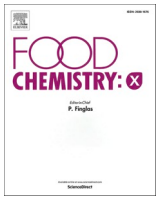

Culture condition optimization of naturally lacto-fermented cucumbers based on changes in detrimental and functional ingredients

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ABSTRACT

A two-step trial was used to optimize the culture condition of naturally lacto-fermented cucumbers. In the first trial, changes in pH values and total biogenic amines were measured to optimize the pickling juice formula. A 15% crystal sugar solution with low-salt brine at 4 °C was proved to be the best formula. In the second trial, pH values, organic acids, total phenolics, flavonoids, saponins and free amino acids, as well as biogenic amines and nitrites under the optimal pickling formula were measured. The optimal fermentation day was suggested at around 8 days. During the cucumber's fermentation process, the pH value was quickly lowered to <4.6. Meanwhile, the functional ingredients increased significantly. In contrast, total biogenic amines and nitrites did not exceed the risk limit, evidencing the safety and functional characteristics for the naturally lacto-fermented cucumbers. The two-step trial has evidenced the possibility to develop desirable lacto-fermented cucumbers.

1. Introduction

Fermented foods, that is one kind of traditional foods using fermentation to preserve foods for a longer time and enhance flavor, have become a vital part of the world diet (Sanlier, Gokcen, & Sezgin, 2019). During the aging process, particular lacto-fermented foods may produce beneficial constituents including different lactic acid bacteria (LAB) (probiotics) and chemicals (prebiotics) including lactic acid, organic acids, free amino acids, alcohols, esters, sulfides, aldehydes, antioxidants, gamma-aminobutyric acid, conjugated linoleic acids (CLA), biologically active peptides, exopolysaccharides, etc. depending on the differential fermentation types, temperature and foodstuffs (Li et al., 2017; Ragul et al., 2020; Rao et al., 2020; Sanlier, Gokcen, & Sezgin, 2019; Song & Yu, 2018; Wang et al., 2020; Zhao et al., 2016). In contrast, some non-nutrients and toxic components in different foodstuffs may decrease in the fermented foods (Sanlier, Gokcen, & Sezgin, 2019). The health benefits of fermented foods may be embodied in antimicrobial, anti-fungal, anti-oxidant, anti-diabetic, anti-inflammatory, anti-atherosclerotic, opioid antagonist, anti-allergenic, anti-carcinogenic and blood pressure lowering effects (Sanlier, Gokcen, & Sezgin, 2019; Behera et al., 2020). Fermentation and the resulting fermented foods have attracted scientific interest and shed a light on the health food industry over time (Anal, 2019; Sanlier, Gokcen, & Sezgin, 2019; Behera et al., 2020).

Till now the artisan technology lacking in any knowledge of the microorganisms' role was still the key way to prepare the fermented foods particularly in the traditionally and naturally fermented food products, therefore understanding the divergent changes during the fermentation process may optimize the culture condition and advance the change of craft style to modern technological systems (El Sheikha, 2018a). Particularly, the quality of the ingredients, sensory quality, and microbial safety of fermented foods such as instant low-salt naturally fermented Chinese paocai and Chinese sauerkraut are critical (Anal, 2019; Zhang et al., 2016). Biogenic amines, nitrite and other potentially harmful substances may be produced during the aging process (Dala-Paula, Starling, & Gloria, 2021; Skowron et al., 2022; Zhao et al., 2021a; Zhao et al., 2021b). Therefore, it is important to optimize fermentation conditions for different foodstuffs to increase beneficial constituents but decrease harmful substances in the fermented foods and brine (Zhao et al., 2020). Recently, modern technological systems, such as applications of molecular tools for detecting and identifying probiotic microbes, as well as analyzing their activity have been introduced to explore LAB species in various fermented foods (Kao et al., 2023; El Sheikha, 2018b).

Among different foodstuffs for fermentation, cucumber (*Cucumis*

Abbreviations: CLA, Conjugated linoleic acids; ELISA, Enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; GRAS, Generally recognized as safe; HPLC, High performance liquid chromatography; LAB, Lactic acid bacteria; TNBS, 2,4,6-Trinitrobenzenesulfonic acid.

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sativus L.) which is widely cultivated in the world has been recently proven to have anti-inflammatory activity with no dose-dependent adverse side effects *in vivo* (Agatemor, Nwodo, & Anosike, 2015). The relatively fast expiration of fresh cucumber fruit results from its 96.4% water content; cucumbers therefore are extensively consumed in fresh salads or vegetables and in preserved form, such as naturally fermented sour pickled cucumbers (Zielinski, Surma, & Zielinska, 2017). To enhance cucumber's shelf life and health protection, a spontaneously fermented cucumber product was recently developed and proved a lacto-fermented type (Kao et al., 2023). However, optimizing the culture conditions is still required for naturally fermented sour pickled cucumbers based on different geographical distribution to increase the beneficial constituents while decreasing the harmful substances in the fermented cultures (Zhao et al., 2020).

To develop a new naturally lacto-fermented cucumber product in Taiwan, immature cucumbers were chosen as a fermentation target using traditional fermentation pickling juice formulas and naturally occurring bacteria in the present study. A two-step trial was performed to optimize the pickling juice formula and fermentation days for developing naturally lacto-fermented cucumbers based on changes in divergent chemical parameters during the aging process. The two-step trial has finished most scientific analyses on naturally lacto-fermented cucumbers and evidenced the possibility to develop desirable lacto-fermented vegetables. Our results concerning the developed naturally lacto-fermented cucumber product will be important and useful for establishing a scientific research and the future exploitation of vegetables to develop functional foods or nutraceuticals for safeguarding health of the most people in the world.

2. Materials and methods

2.1. Materials

Immature cucumber (*Cucumis sativus* L.) fruits were purchased from a local supermarket in Taichung City, Taiwan. The cucumbers were washed with tap water and cut into 3–5 cm long quadrants for use (Kao et al., 2023).

2.2. Determination of the optimal pickling juice formula for naturally fermented cucumbers in the first trial

2.2.1. Pickling juice formulas prepared for naturally fermented cucumbers

Throughout the preliminary experiments (Kao et al., 2023), three pickling juice formulas were designed for fermenting cucumbers. These formulas included 15% crystal sugar (sucrose, >99%, Taiwan Sugar Corporation) solution with salt water (brine) dehydrated from cucumbers by 2.5% salt (NaCl, >99%, TaiYen Iodized Superior Fine Salt, Taiwan) of sample weight (w/w) (formula A), 30% crystal sugar solution with brine dehydrated from cucumbers by 2.5% NaCl of sample weight (w/w) (formula B), and 30% crystal sugar solution without brine (formula C). Briefly, aliquots of 200 g of cucumbers were added with 5 g of NaCl, thoroughly mixed, and dehydrated for 1 h at room temperature, respectively. The brine from cucumbers dehydrated by NaCl in the pickling juice formulas A and B was preserved with cucumbers. However, the brine in pickling juice formula C was discarded. The dehydrated cucumbers with/without brine were placed into a 500 ml fermented glass can. Aliquots of 150 ml of 15% (for pickling juice formula A), 30% (for pickling juice formula B), and 30 % crystal sugar solution (for pickling juice formula C) were added to the corresponding glass bottles to cover the cucumbers. The contents were mixed and the lids opened for 30 min to expose the contents to natural airborne microorganisms. To avoid the spoilage of low-salt (2.5% NaCl of sample weight, w/w) fermented cucumbers by environmental bacteria at the higher room temperature in Taiwan, the fermented glass cans were placed into a 4 °C refrigerator for fermentation after the lids were screwed tight. During the fermentation period, 12 ml aliquots of fermented cucumbers in pickling juice were sampled for determining pH values and total biogenic amines at 0, 5, 10 and 15 days, respectively. At each sampling point, an aliquot of 2 ml of the fermented pickling juice sample was used directly for measuring pH values using a pH meter (Suntex sp-701, Taiwan). An aliquot of 10 ml of the fermented pickling juice sample was lyophilized into powder using a freeze dryer (Panchum CT-5000D, Panchum Scientific Corp., Kaohsiung, Taiwan, ROC). The yield was calculated and the powder sample was subjected to detection for total biogenic amines levels. Based on changes in pH values and total biogenic amines in the fermented juice through 15 days at 4 °C refrigerator. Pickling juice formula A (15% crystal sugar solution with brine) was selected as an optimal pickling juice formula for naturally fermented cucumbers. Formula A was further subjected to the following experiments to determine the optimal number of fermentation days.

2.2.2. Changes in pH values in the first trial

The fermented cucumber pickling juices were sampled at the stated intervals to measure changes in pH values using a pH meter (Suntex sp-701, Taiwan) (Kao et al., 2023).

2.2.3. Total biogenic amines analysis in the first trial

In this study, the total levels of biogenic amines in fermented cucumber pickling juices were determined using traditionally non-aqueous acid-base titrations of weak bases with perchloric acid ($HClO_4$ at anhydrous state (Ekeblad & Erne, 1954) in place of detecting each individual scarce and diverse amine in fermented vegetables using high performance liquid chromatography (HPLC) (Dala-Paula, Starling, & Gloria, 2021; Zhao et al., 2021a; Zhao et al., 2021b). The total biogenic amine measurement may provide a rapid determination for various but scarce biogenic compounds, including amines and heterocyclic nitrogen compounds, alkali, amino acids, and organic salts of hydrogen halides and of weak acids in the fermented vegetables (Ekeblad & Erne, 1954). Briefly, total biogenic amines in the lyophilized fermented samples (anhydrous state) were titrated with perchloric acid-acetic acid standard solution. The acetic acid used in the perchloric acid-acetic acid standard solution served as a solvent which should not exceed 0.1 to 0.2 % water content. Therefore, glacial acetic acid which is water-free (anhydrous) acetic acid was used in the titration. The perchloric acid standard solution (usually 0.05–0.1 N) can be prepared from 70% (w/w) perchloric acid aqueous solution (density = 1.68 g/ml, ca. 11.706 M, Showa, SK2645T, Tokyo, Japan). In this study, 0.10 N perchloric acid in acetic acid standardized solution was prepared to measure total biogenic amines. To prepare the 0.10 N perchloric acid in acetic acid standardized solution, an aliquot of 4.3 ml of 70% perchloric acid was first diluted with 250 ml glacial acetic acid. After the dilution, an aliquot of 12 ml acetic anhydride was added, mixed and finally converted to 500 ml with glacial acetic acid. The 0.10 N perchloric acid in acetic acid solution was then standardized with potassium hydrogen phthalate (KHP, molecular weight: 204.2) (J.T. Baker, 2958–00, USA). Firstly, a quantity of KHP was heated in an oven at 110 °C for 3 h and allowed it to cool in a desiccator. About 0.3 g (m, g) of KHP was weighed to the nearest 0.1 mg and dissolved in 20 ml glacial acetic acid (Honeywell, UN2789, Germany). Three drops of 0.5% crystal violet (Acros organic, A0420270, Morris Plains, NJ, USA) was dissolved in glacial acetic acid and added as a titration indicator (violet color). The resultant mixture (violet color) was titrated with the 0.10 N perchloric acid in acetic acid solution and recorded the titration volume (V, ml) when the titration end point (blue color). A blank titration on just 20 ml glacial acetic acid with the corresponding 0.10 N perchloric acid in acetic acid solution was performed and recorded (B, ml). The actual normality (N) of perchloric acid in acetic acid solution was calculated as follows:

$$N(HClO_4) = (1,000 \times m)/[204.2 \times (V - B)].$$

Test sample (anhydrous state, > 0.3 g) was weighed (S, g), dissolved in 30 ml of glacial acetic acid–ethanol solution [glacial acetic acid and 99.5 % ethyl alcohol (Himakyu's pure chemicals, Japan) at a fixed ratio

of 2:1] for 30 min, added with three drops of 0.5% crystal violet indicator and finally subjected to titration with perchloric acid-acetic acid standard solution. When the titration end point (blue color) was reached, the used volume of perchloric acid-acetic acid standard solution was recorded (C, ml). Total biogenic amines are defined as total base numbers per g sample and generally expressed as mg equivalent of KOH per g sample. Therefore, total biogenic amines were calculated using the equation, total biogenic amines (mg KOH/g test sample) = (C × N × 56.11)/S (Swider et al., 2020). Based on the lyophilized powder yield, the total levels of biogenic amines in the fermented cucumber juice were calculated and expressed as mg KOH equivalent/ml fresh sample.

2.3. Optimal culture period determination for naturally fermented cucumbers under the optimal pickling juice formula in the second trial

Throughout the experiments to optimize pickling juice formula for naturally fermented cucumbers (Kao et al., 2023), the optimal pickling juice formula (15% crystal sugar solution with brine) was selected for further cucumber fermentation to determine the optimal culture period for naturally fermented cucumbers. The fermented glass cans were placed into a 4 °C refrigerator for fermentation for 0, 4, 8, 12, and 16 days, respectively. After fermentation, fermented cucumbers and pickling juices were separated and weighted. A part of the fresh fermented cucumbers was weighed, stirred for 1 min using a blender and immediately used for nitrite content determination. A part of the fresh fermented pickling juices was directly used for pH values, organic acids, and nitrites analyses. The remaining fermented cucumbers and the fermented pickling juices were lyophilized and weighed, respectively. The lyophilized fermented cucumbers were ground into powder for determining total biogenic amines and functional ingredients including total phenolics, flavonoids, saponins and free amino acids. The lyophilized fermented pickling juices were used for analyzing total biogenic amines.

2.4. Organic acid analyses using HPLC

The fresh cucumber fermented pickling juices were sampled with intervals to analyze changes in organic acids using HPLC (Kishore et al., 2013). Briefly, fresh cucumber fermented pickling juices were sampled and appropriately diluted with 0.005 M sulfuric acid (Sigma, 30743, St. Louis, MO, USA) to prepare stock solutions, respectively. Aliquots of 10 mg standards, including lactic acid (Sigma, 46937, analytical standard, Belgium), citric acid (Sigma, C0759, 99%, St. Louis, MO, USA), DL-malic acid (Sigma, 240176, >99%, St. Louis, MO, USA), oxalic acid (Chem service, N-12733-1G, West Chester, PA, USA), succinic acid (Chem service, N-134234-1G, West Chester, PA, USA), acetic acid (Acros organics, 220892500, for analysis, anhydrous, >99%, Morris Plains, NJ, USA), and tartaric acid (Chem service, N-12302-1G, West Chester, PA, USA), were respectively dissolved in 10 ml deionized water to prepare 1 mg/ml stock solutions. The stock solution was filtered through a 0.22 μm filter and appropriately diluted with 0.005 M sulfuric acid to prepare standards at the fixed concentrations of 31.25, 62.5, 125, 250, 500, and 1000 μg/ml for assay and calibration. Before operation, all of sample solutions, standards and HPLC reagents was degassed ultrasonically. The HPLC machine equipped with autosampler (Hitachi, L-2200, Tokyo, Japan), UV–Visible detector (Hitachi, L-2400, Tokyo, Japan) and chromatographic separation column (Mightysil RP-C18 GP250-4.6 (5 μm), Kanto Chemicals, 25415-96) were used. The mobile phase was 0.005 M H_2SO_4 and controlled under a solvent pump (Hitachi, model L-2130, Tokyo, Japan) maintained at a fixed flow rate of 0.8 ml/min. The UV detection wavelength was set at 210 nm. An aliquot of 20 μl sample solution was loaded for each individual analysis using the autosampler. Data acquisition was controlled under software (Hitachi, model D-2000 Elite Chromatography Data Station Software). Seven pure organic acid compounds were selected as standards. Individual organic acid compound quantification levels in the fresh fermented cucumber pickling juices were calibrated using the standards curve at the same analysis condition. Results were computed and expressed as the mean ± SD (3 replicates).

2.5. Total phenolic, flavonoid, and saponin contents in fermented cucumber determination

To determine total phenolic, flavonoid, and saponin contents in fermented cucumbers, the test sample ethanol extracts should be prepared for assay. Briefly, an aliquot of 50 g lyophilized fermented cucumber powder was weighed, added with 250 ml 95% ethanol, and gently stirred for 4 h at room temperature. The mixture was centrifuged at 600 × g using a centrifuge machine (automatic high speed refrigerated centrifuge, Hitachi, CR20B2, Tokyo, Japan) for 10 min. at room temperature. The extract was carefully collected and evaporated using a vacuum evaporator (EYELA rotary vacuum evaporator, Tokyo Rikakika Co., N-1200AVF, Japan) aided with an aspirator (EYELA Aspirator, Tokyo Rikakika Co., A-1000S, Japan) to remove the solvent. The residue was weighed and re-dissolved in 95% ethanol to prepare a 25 mg/ml stock solution. The stock solution was filtered using a 0.22 μm pore size filter (Acrodisc Syringe Filters, DMSO-Safe, Sterile, Pall Life Sciences) and stored at −30 °C until use.

Total phenolic and flavonoid contents were respectively analyzed using the Folin-Ciocalteu and aluminum chloride assay methods with slight modification (Chandra et al., 2014; Kupina et al., 2018; Lin & Tang, 2007; Lukiati et al., 2020). For total phenolic content assay, an aliquot of 0.1 ml of the stock solution (ethanol extracts) was added with 2 ml of 2% sodium carbonate (Na_2CO_3, Wako Pure Chemical Industries, Ltd., 199-01585, Osaka, Japan) for 2 min, and then 0.1 ml of 50% Folin-Ciocalteau reagent (Wako, 277-08891, Osaka, Japan). The resultant mixture was incubated in the dark for 30 min at room temperature to develop the color. The absorbance at 750 nm was measured using a UV/VIS spectrophotometer (Hitachi-U2900 UV–vis spectrophotometer, Tokyo, Japan) or an aliquot of 200 μl of the reaction mixture was pipetted into a 96 well enzyme-linked immunosorbent assay (ELISA) plate to measure at 750 nm using an ELISA reader (Microplate reader FLUOstar-Omega, 415–1103, Germany). Gallic acid (Sigma-Aldrich Co., G-7384, St. Louis, MO, USA) that is a universal phenolic compound in vegetables and fruits, was chosen as a phenolic compound standard. Based on a seven-points standard curve, total phenolic contents in the samples were determined in triplicate. The results were computed according to differential conversion factors and expressed as mg gallic acid equivalents/g lyophilized fermented cucumbers powder sample or mg gallic acid equivalents/g fresh fermented cucumbers (Lin & Tang, 2007; Lim, Park, & Yoon, 2020).

For total flavonoid content assay, an aliquot of 0.1 ml of the stock solution (25 mg/ml) was pipetted into a test tube and mixed with 1.5 ml of 95% ethanol. The solution was then sequentially added with 0.1 ml of 10% aluminum chloride (Wako, 013-01875, Osaka, Japan), 0.1 ml of 1 M potassium acetate (Wako, 160-03175, Osaka, Japan), and 2.8 ml deionized water. The resultant mixture was incubated in the dark for 30 min at room temperature to develop the color. Finally, the absorbance at 415 nm was measured using a UV/VIS spectrophotometer (Hitachi-U2900 UV–vis spectrophotometer, Tokyo, Japan) or an aliquot of 200 μl of the reaction mixture was pipetted into a 96 well ELISA plate to measure at 415 nm using an ELISA reader (Microplate reader FLUOstar-Omega, 415–1103, Germany). Quercetin (Sigma, Q0125, Steinheim, Switzerland) is a universal flavonoid compound in vegetables and fruits, and was chosen as a flavonoid compound standard. Based on a seven-points standard curve, total flavonoid contents in the samples were determined in triplicate. The results were computed according to differential conversion factors and expressed as mg quercetin equivalents/g lyophilized fermented cucumbers powder sample or mg quercetin equivalents/g fresh fermented cucumbers (Lim, Park, & Yoon, 2020).

For the total saponin content assay, total saponin content in the samples were estimated using the colorimetric method with slight

modification. Briefly, an aliquot of 25 μl of the stock solution (25 mg/ml) was pipetted into a microcentrifugation tube. The solution was then sequentially added with 25 μl of 8% vanillin (Acros, 140822500, Morris Plains, New Jersey, USA) in 95% ethanol, and 250 μl of 72% sulfuric acid (Sigma, 95–97%, 30743, St Louis, MO, USA). The mixture was incubated in a water bath at 60 °C for 10 min to develop the color. After cooling to room temperature, the absorbance at 544 nm was measured using a UV/VIS spectrophotometer (Hitachi-U2900 UV–vis spectrophotometer, Tokyo, Japan). Additionally, an aliquot of 200 μl of the reaction mixture was pipetted into a 96 well ELISA plate to measure at 544 nm using an ELISA reader (Microplate reader FLUOstar-Omega, 415–1103, Germany). Oleanolic acid, (Tokyo Chemical Industry, O0317, Japan) a universal saponin compound in plants, was chosen as a saponin compound standard. Based on a seven-points standard curve (ca. 0 – 1,000 μg/ml), total saponin content in the samples was determined in triplicate. The results were computed according to differential conversion factors and expressed as mg oleanolic acid equivalents/g lyophilized fermented cucumbers powder sample or mg oleanolic acid equivalents/g fresh fermented cucumbers (Lim, Park, & Yoon, 2020).

2.6. Total free amino acids content determination in fermented cucumbers

To determine total free amino acids content in fermented cucumbers, water extracts from the test lyophilized samples should be prepared. Briefly, an aliquot of 50 g lyophilized fermented cucumbers powder sample was added with 500 ml deionized water and gently stirred for 6 h at room temperature. The mixture was centrifuged at 600 × g using a centrifuge machine (automatic high speed refrigerated centrifuge, Hitachi, CR20B2, Tokyo, Japan) for 15 min at room temperature. The extract was carefully collected and evaporated using a vacuum evaporator (EYELA rotary vacuum evaporator, Tokyo Rikakika Co., N-1200AVF, Japan) aided with an aspirator (EYELA Aspirator, Tokyo Rikakika Co., A-1000S, Japan) to concentrate the water extracts. The concentrated water extracts were lyophilized to powder using a freeze dryer (Panchum CT-5000D, Panchum Scientific Corp., Kaohsiung, Taiwan, ROC) for 72 h. The lyophilized powder was weighed and re-dissolved in deionized water to prepare a 100 mg/ml stock solution. The stock solution was filtered using a 0.22 μm pore size filter (Merck Millipore Ltd., Tullagreen Carrigtwohill Co., Cork, IRL) and stored at −30 °C until use. Following the experiment, an aliquot of 40 μl of the stock solution (100 mg/ml) was pipetted into a 1.5 ml microcentrifuge tube. The solution was then sequentially added with 320 μl of 2 M pH 8.0 phosphate buffer (SHOWA, KFT-055B, Tokyo, Japan) and 320 μl of 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) in deionized water. The resultant mixture was incubated in the dark in a water bath at 50 °C for 60 min to develop the color. After cooling to room temperature, an aliquot of 640 μl of 0.1 N hydrochloric acid (HCl, 35–37%, Wako, 080–01066, Osaka, Japan) was added into the microcentrifuge tube to stop the reaction. Finally, the absorbance at 340 nm was measured within 15 min using a UV/VIS spectrophotometer (Hitachi-U2900 UV–vis spectrophotometer, Tokyo, Japan) or a 200 μl aliquot of the reaction mixture was pipetted into a 96 well ELISA plate to measure at 340 nm using an ELISA reader (Microplate reader FLUOstar-Omega, 415–1103, Germany). Leucine (Sigma, L8125, St. Louis, MO, USA) is a universal amino acid in free amino acids, and was chosen as a free amino acid standard. Based on a seven-points standard curve, the total free amino acids content in the samples were determined in triplicate. The results were computed according to differential conversion factors and expressed as mg leucine equivalents/g lyophilized fermented cucumbers powder sample or mg leucine equivalents/g fresh fermented cucumbers (Quyang et al., 2020).

2.7. Total nitrite content determination in fresh fermented cucumbers and fermented pickling juices

Total nitrite contents in fresh fermented cucumbers and fermented pickling juices were treated to measure, respectively. The fresh fermented cucumbers were stirred for 1 min using a blender and immediately weighed 80 g homogenized cucumbers sample into a 250 ml Erlenmeyer flask. An aliquot of 5 ml of 5 % sodium tetraborate decahydrate (99%, Sigma, 221732, St. Louis, MO, USA) in distilled water and 100 ml 80 °C hot distilled water were then added. The mixture solution was incubated in a 100 °C water bath for 15 min. After cooling to room temperature, an aliquot of 2 ml of Carrez reagent I solution and 2 ml of Carrez reagent II solution were added into the flask to clarify the solution by removing interfering compounds during food testing. The resultant solution was allowed to stand for 30 min and quantitatively transferred into a 250 ml volumetric flask. The solution was filtered through filter paper into a 250 ml volumetric flask with the total amount made up with distilled water. The filtrate was collected for total nitrites assay. In this experiment, Carrez reagent I solution consisted of 2.65 g potassium ferrocyanide (Hanawa, 18698, Osaka, Japan) and 25 ml distilled water. Carrez reagent II solution consisted of 5.5 g zinc acetate (anhydrous) (99.9%, Thermo, H33734, Heysham, Lancashire, UK), 0.75 ml glacial acetic acid (Honeywell, UN2789, Germany) and 25 ml distilled water. As to the fermented pickling juices sample, an aliquot of 100 ml of the liquid sample was pipetted and treated the same as the fresh fermented cucumbers. Finally, a 10 ml aliquot of the filtrate was pipetted into a 100 ml volumetric flask, then added with 40 ml distilled water, 10 ml Griess reagent I and 3 ml hydrochloric acid. The resultant solution was incubated in the dark at room temperature for 5 min. The resultant mixture was then added with 2 ml Griess reagent II and incubated in the dark at room temperature for 15 min to develop the color. The resultant solution in the 100 ml volumetric flask was made up with distilled water. The absorbance at 540 nm was measured within 15 min using a UV/VIS spectrophotometer (Hitachi-U2900 UV–vis spectrophotometer, Tokyo, Japan). In the experiment, Griess reagent I solution consisted of 0.2 g sulfanilamide (98%, Thermo, A13001, Heysham, Lancashire, UK), 80 ml of hot distilled water, 10 ml of hydrochloride acid (HCl, Wako, 080-0106, Osaka, Japan) and the total was finally made up to 100 ml with distilled water. Griess reagent II solution consisted of 0.035 g N-(1-naphthyl ethylenediamine (Sigma, N9125, St. Louis, MO, USA), and 35 ml distilled water. Griess reagent II solution should be freshly prepared and stored in dark. Sodium nitrite (Sigma, S2252, St. Louis, MO, USA) at 1,000 μg/ml was chosen as a standard stock and was appropriately diluted to 1 μg/ml when used. Total nitrite contents in the samples were determined in triplicate based on a five-points standard curve. The results were computed according to differential conversion factors and expressed as μg nitrite (NO_2^-) equivalents/g sample or μg NO_2^- equivalents/ml sample (Stachniuk, Szmagara, & Stefaniak, 2018).

2.8. Statistical analysis

Results are shown as means ± SD (3 replicates). Data were first analyzed using one-way analysis of variance (ANOVA), if justified by the computed probability ($P < 0.05$), and followed by Duncan's new multiple range test using SPSS version 20.0. $P < 0.05$ was considered as statistically significant differences.

3. Results and discussion

3.1. Optimal pickling juice formula for naturally fermented cucumbers

3.1.1. Changes in pH values using three pickling juice formulas

To optimize the pickling juice formula, changes in pH values using three pickling juice formulas were measured. The results showed that pH values in the three selected pickling juice formulas decreased as the fermentation time was extended (Fig. 1). However, pH values in the pickling juice formula of 15% crystal sugar solution with brine (formula A) quickly and significantly ($P < 0.05$) decreased compared to those of the other two formulas (Table S1) after 5-days fermentation. The pH

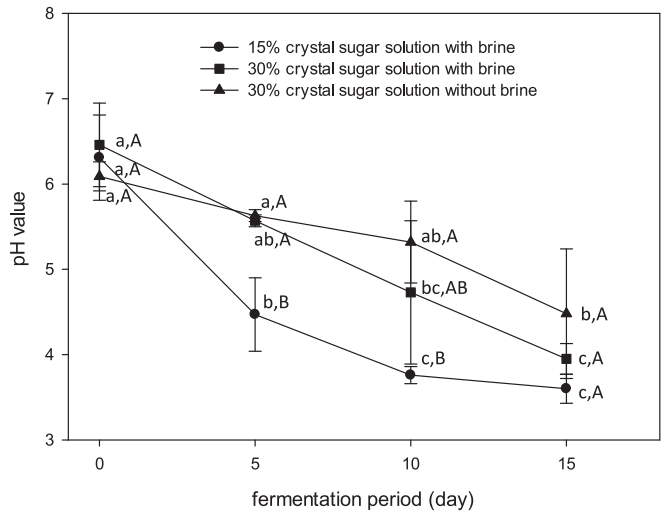

Fig. 1. Change trends of pH values in fermented cucumbers pickling juices using three different pickling juice formulas at 4 ℃ for different days. Values are means ± SD (n = 3 replicates). Values within the same plot not sharing a common lowercase letter (a, b, c), and at the same fermentation period not sharing a common uppercase letter (A, B, C) are significantly different ($P < 0.05$), respectively, from each other analyzed by one-way ANOVA, and followed by Duncan's multiple range test.

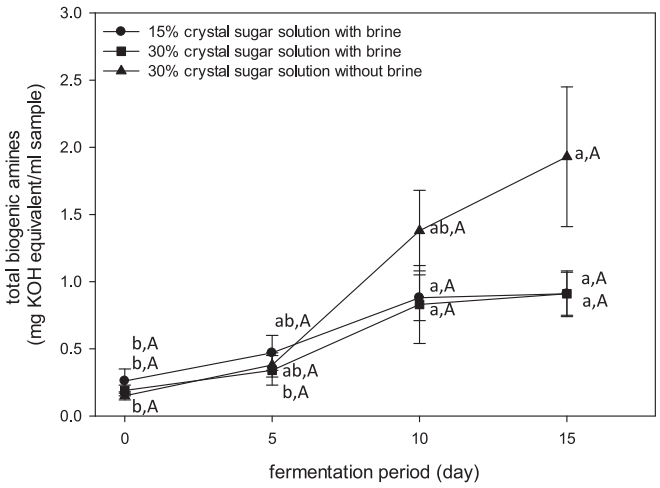

Fig. 2. Changes in total biogenic amines content in fermented cucumbers pickling juices using three different pickling juice formulas at 4 ℃ for different days. Values are means ± SD (n = 3 replicates). Values within the same plot not sharing a common lowercase letter (a, b), and at the same fermentation period not sharing a common uppercase letter (A) are significantly different ($P < 0.05$), respectively, from each other analyzed by one-way ANOVA, and followed by Duncan's multiple range test.

values in the pickling juice formula of 15% crystal sugar solution with brine (formula A) were 6.31 ± 0.50, 4.47 ± 0.43, 3.76 ± 0.10, and 3.60 ± 0.17 through 0, 5, 10 and 15 days fermentation, respectively. The lower pH values (particularly pH < 4.6, also known as high-acid foods) are very resistant to deleterious bacteria such as *Clostridium botulinum* but not LAB in fermented foods (Odlaug & Pflug, 1978). However, the acidity of the spontaneously fermented cucumbers have been suggested to be appropriately decreased to prevent a too strong taste to draw more acceptance or liking from most consumers (Kao et al., 2023). The pH value of foods is an important indicator for preservation and safety, therefore pH values ranging from 3.5 – 4.5 might be a suitable indicator for judging the fermentation maturity and quality of fermented cucumbers (Kao et al., 2023). Unfortunately, both pickling juice formulas B and C lowered the acidity to pH < 4.6 up to 10-days fermentation (Fig. 1; Table S1). This is still too long to be considered safe and economical for food manufacturing, suggesting that these two pickling juice formulas may be not suitable for naturally fermented cucumbers. Moreover, comparisons with changes in pH values using the three pickling juice formulas indicated that low-salt brine which may help to inhibit detrimental bacteria is essential for naturally fermented cucumbers (Fig. 1; Table S1). Our results suggest a low-salt and low sugar content (15% crystal sugar solution) as an optimal pickling juice formula for naturally fermented cucumbers. Based on the changes in pH values, the fermented cucumbers maturity period using the optimal pickling juice formula estimated within 5–10 days (pH 4.47 – pH 3.76).

3.1.2. Changes in total biogenic amines using three pickling juice formulas

To optimize the pickling juice formula, total levels of biogenic amines in fermented cucumbers pickling juices using the three pickling juice formulas through 0, 5, 10 and 15 days, were determined using the traditional method. The results showed that the total levels of biogenic amines in the three fermented cucumbers pickling juices were significantly ($P < 0.05$) increased time-dependently, particularly through 15-days fermentation (Fig. 2). Total levels of biogenic amines in the pickling juice formula of 30% crystal sugar solution without brine were slightly, but not significantly ($P > 0.05$), higher than those in the other two pickling juice formula. However, it was higher than the safety limit of 1000 mg/kg sample (=1.000 mg/g sample) in foods, suggesting that the pickling juice formula of 30% crystal sugar solution without brine is not suitable for naturally fermented cucumbers (Penas et al., 2010). In contrast, total levels of biogenic amines in the other two fermented pickling juices did not exceed the 1.000 mg/g sample limit, suggesting their safety for inhibiting the production of total biogenic amines in naturally fermented cucumbers. There were no significant differences in total biogenic amines between 15% crystal sugar solution with brine formula and 30% crystal sugar solution with brine formula, suggesting that low-salt brine is essential for inhibiting the production of total biogenic amines in naturally fermented cucumbers. Economically, our results suggest that a low-salt and low sugar content (15% crystal sugar solution) pickling formula is an optimal formula for naturally fermenting cucumbers. The optimal pickling juice formula may alleviate the production of total biogenic amines, possibly via inhibiting deleterious bacteria but favoring LAB to produce acidity in the naturally fermented cucumbers (Fig. 1 and Fig. 2). Biogenic amines may be produced by different deleterious bacteria possibly via decarboxylating free amino acids during natural fermentation process, resulting in the spoilage of fermented foods and eliciting adverse effects such as allergies in consumers (Penas et al., 2010). Therefore, total biogenic amines are a universal negative indicator for fermented foods, even though they are relatively scarce in fermented vegetables and fruits. According to the changes in total levels of biogenic amines, the naturally fermented period for cucumbers using the optimal pickling juice formula is suggested around 5 to 10 days.

In the first trial, we evidenced that a low-salt and low sugar content (15% crystal sugar solution) pickling juice formula is an optimal formula for naturally fermenting cucumbers through quickly decreasing the acidity to pH < 4.6 (Fig. 1), possibly via increasing the acidity but inhibiting the production of total biogenic amines (Fig. 2). In comparison with changes in pH values and total biogenic amines, the fermentation maturity period for cucumbers using the optimal pickling juice formula is estimated within 5–10 days. To be commercialized in the food industry, the optimal fermentation days for naturally fermenting cucumbers according to more divergent chemical parameters during the aging process were further confirmed in the second trial.

3.2. Optimal culture period for naturally fermented cucumbers using the optimal pickling juice formula

3.2.1. Changes in pH values in fermented cucumbers using the optimal pickling juice formula

The pH values in the fermented cucumbers pickling juices using the optimal pickling juice formula (15% crystal sugar solution with brine) were 6.06 ± 0.02, 5.54 ± 0.05, 4.27 ± 0.02, 3.82 ± 0.10, and 3.68 ± 0.09 through 0, 4, 8, 12, and 16 days, respectively (Table S2). The pH values significantly ($P < 0.05$) and time-dependently decreased throughout the fermentation period. Importantly, the pH values were lower than pH 4.6 through 8-days fermentation, suggesting that the maturity period for naturally fermented cucumbers using the optimal pickling juice formula is at around 8 days (pH 4.27) or slightly shorter.

3.2.2. Changes in total phenolic, flavonoid, and saponin contents in the fermented cucumbers using the optimal pickling juice formula

The results showed that the total number of phenolics in the fermented cucumbers (Fig. 3 (A, B)) and fermented pickling juices (Table S3 (A)) using the optimal pickling juice formula of 15% crystal sugar solution with brine significantly ($P < 0.05$) increased compared to those of fresh cucumbers, particularly through 8-days fermentation (Fig. 3 (A, B), Table S3 (A)). The results indeed evidenced that 8-days natural fermentation process increased functional phenolic ingredients in the fermented cucumbers. The increased phenolic contents might be produced by bacteria or released from bound phenolics in the naturally fermented cucumbers during the aging process. Dietary fermented cucumbers abundant in phenolics may contribute to health for their potent antioxidant functions (Wijewardhana, Gunathilaka, & Navaratne, 2019).

Total flavonoid in the fermented cucumbers and fermented pickling

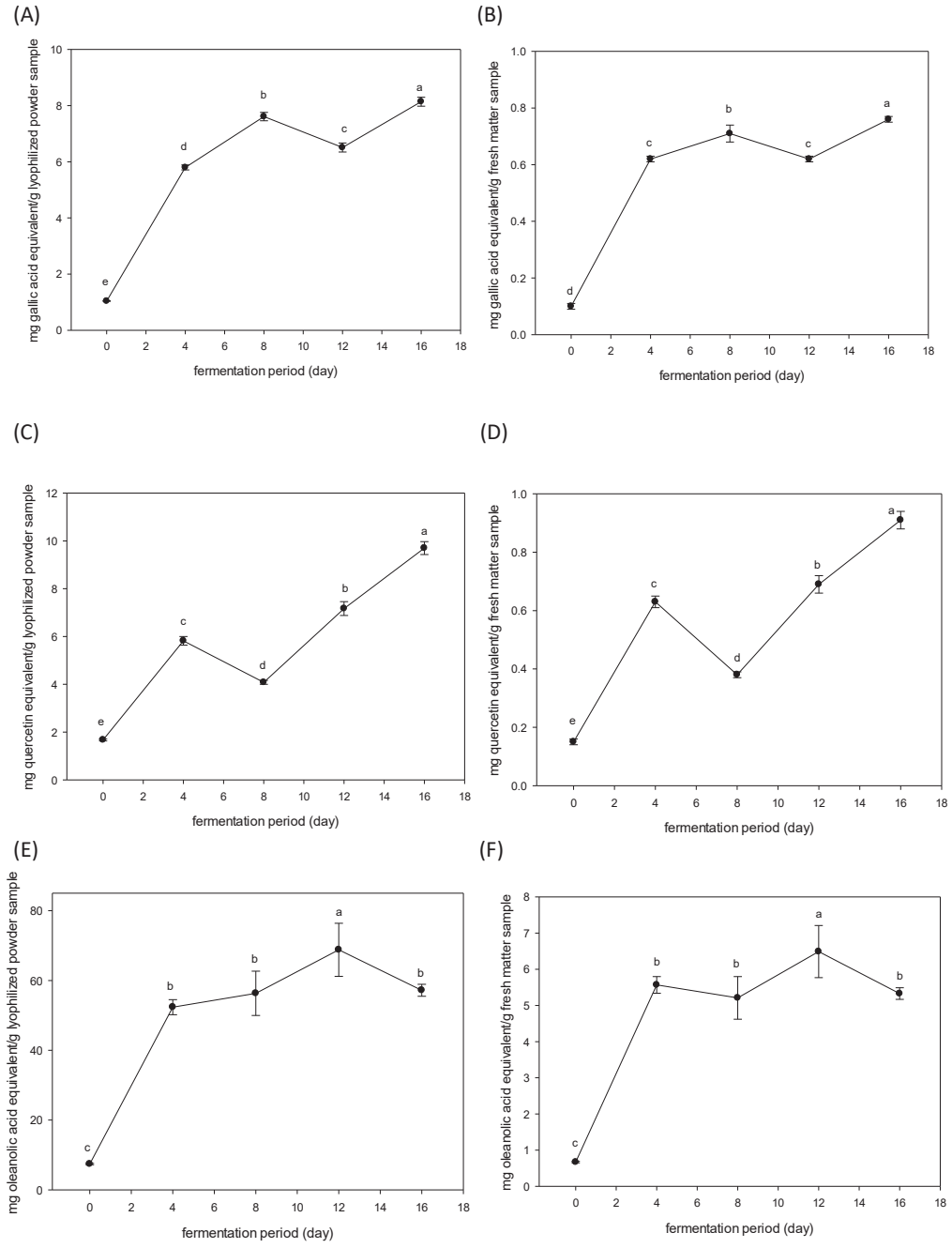

Fig. 3. Changes in total phenolic (A, B), flavonoid (C, D) and saponin (E, F) contents in fermented cucumbers using a 15% crystal sugar solution with low-salt brine pickling juice formula at 4 ℃ for different days. Values are means ± SD (n = 3 replicates). Values within the same plot not sharing a common letter are significantly different ($P < 0.05$) from each other analyzed by one-way ANOVA, followed by Duncan's multiple range test. Lyophilized powder moisture content: 0%; all of fresh fermented cucumber moisture content: 91%.

juices using the optimal pickling juice formula of 15% crystal sugar solution with brine significantly ($P < 0.05$) increased as compared to those in fresh cucumbers as the fermentation days extended (Fig. 3 (C, D), Table S3 (B)). Our results evidenced that the natural fermentation process increased functional flavonoids ingredients in the fermented cucumbers that may enhance health benefits due to their potent antioxidant potential (Chandra et al., 2014). The increased flavonoid contents in the fermented foods might be attributed by bacteria in the naturally fermented cucumbers or bound flavonoids in the cucumbers hydrolyzed during the aging process. The maturity period of naturally fermented cucumbers for enhancing their flavonoid contents using the optimal pickling juice formula is suggested around 8—16 days. Attractively, a significant decrease of total flavonoids was observed in 8-days lacto-fermented cucumbers compared to those in the 4-days product. Meanwhile, we found that there was a significant increase in total bacteria in 8-days naturally fermented cucumber pickling juices compared to those in the 4-days sample (Kao et al., 2023). We hypothesized that increases in large numbers of total bacteria including LAB may further metabolize flavonoids, transiently surpassing the amounts they produced (Kao et al., 2023). However, the complicated mechanisms of functional ingredients produced in fermented foods by diverse microorganisms should be investigated in the future.

Interestingly, changing trends in total saponin contents in the fermented cucumbers were quite different to those in the fermented juices (Fig. 3 (E, F); Table S3 (C)). We supposed that naturally fermented bacteria might further make use of saponins for biosynthesis, resulting in a slight decrease of total saponins in the fermented pickling juices. Importantly, our results evidenced that the natural fermentation process significantly ($P < 0.05$) increased functional saponin ingredients in the fermented cucumbers that may enhance health benefits due to their antioxidant activity and potent anticancer potential (Lim, Park, & Yoon, 2019). The increased saponin contents in the fermented cucumbers might be attributed by bacteria in the naturally fermented cucumbers to digest bound saponins to be the form of unbound saponins during the aging process. However, the saponin content conversion mechanism in the fermented foods should be further investigated. The maturity period of naturally fermented cucumbers for enhancing total saponin contents using the optimal pickling juice formula is suggested around 8–12 days.

3.2.3. *Changes in total free amino acids in the fermented cucumbers using the optimal pickling juice formula*

Our results showed that total free amino acids either in the fermented cucumbers or pickling juices using the optimal pickling juice formula of 15% crystal sugar solution with brine significantly ($P < 0.05$) increased compared to those in 0-day fermented cucumbers, particularly through 4-days fermentation (Fig. 4). The results indicate that the

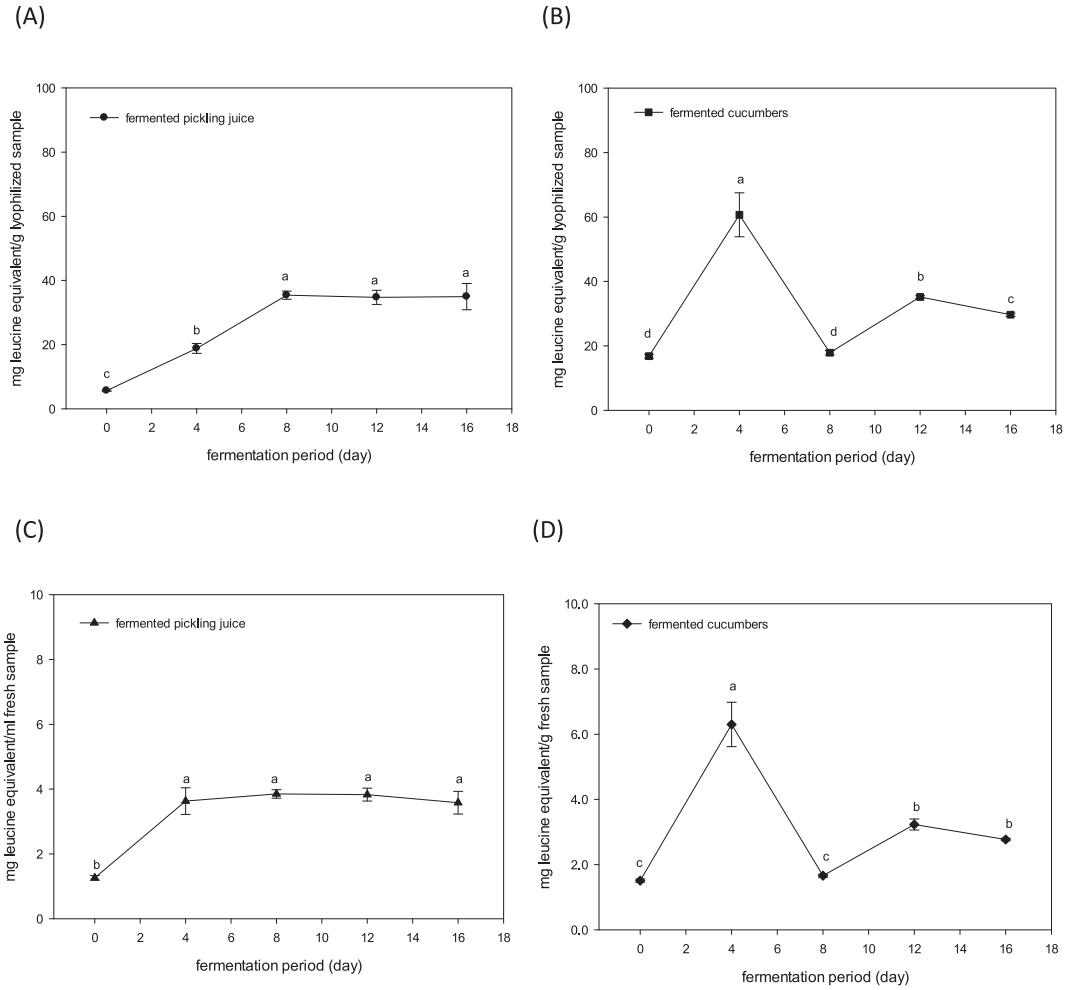

Fig. 4. Changes in total free amino acids content in lyophilized fermented pickling juices (A), lyophilized fermented cucumbers (B), fresh fermented pickling juices (C), and fresh fermented cucumbers (D) using a 15% crystal sugar solution with low-salt brine pickling juice formula at 4 °C for different days. Values are means ± SD (n = 3 replicates). Values within the same plot not sharing a common letter are significantly different ($P < 0.05$) from each other analyzed by one-way ANOVA, followed by Duncan's multiple range test. Lyophilized powder moisture content: 0%; all fresh fermented cucumber moisture content: 91%. 0 day: 22% total solids in the fermented pickling juice; 4 days: 18% total solids in the fermented pickling juice; 8 days: 11% total solids in the fermented pickling juice; 12 days: 11% total solids in the fermented pickling juice; 16 days: 10% total solids in the fermented pickling juice.

natural fermentation process for 4 days increased total free amino acids content in the fermented cucumbers (Fig. 4 (B, D)). The increased free amino acids might be produced by bacteria via digesting proteins in the naturally fermented cucumbers during the aging process. Fermented cucumbers abundant in total free amino acids may provide biologically active peptides and enhance the flavor for consumers (Sanlier, Gokcen, & Sezgin, 2019). The maturity period of naturally fermented cucumbers using the optimal pickling juice formula was around > 4 days.

3.2.4. Changes in different organic acids in the fermented cucumbers using the optimal pickling juice formula

Levels and diversities in organic acids in fermented foods may contribute to the acidity (pH values), flavor, and taste in matured fermented foods (Kranenburg et al., 2002). Particularly, lactic acids are pleasant organic acids produced by lactic acid bacteria during aging process (Kranenburg et al., 2002). To optimize fermentation days, changes in different organic acids, including lactic acid, acetic acid, tartaric acid, citric acid, succinic acid, oxalic acid, and malic acid, in the fermented cucumbers using the optimal pickling juice formula of 15% crystal sugar solution with brine were measured using HPLC based on the standards and standard curves (Figs. S1–S3). The results showed that tartaric acid, citric acid, succinic acid, oxalic acid and malic acid were very scarce relative to the contents of lactic acid and acetic acid in the fermented cucumbers pickling juices using the optimal pickling juice formula of 15% crystal sugar solution with brine (Table 1). Both lactic acids and acetic acids presented the most amounts among seven determined organic acids and significantly ($P < 0.05$) increased during the aging process. Importantly, the highest lactic acid and acetic acid levels using the optimal pickling juice formula of 15% crystal sugar solution with brine were found at 12 fermentation day. Most importantly, our results suggest that a low-salt and 15% crystal sugar solution (low sugar content) optimal formula may favor the growth of lactic acid and acetic acid bacteria to produce lactic acids and acetic acid for lowering pH values to inhibit other deleterious bacteria in naturally fermented cucumbers (Table S2). Based on the changes in lactic acid and acetic acid levels, the fermented cucumber maturity period using the optimal pickling juice formula was suggested to be within 12 days.

The malic acid standard used in this study is DL-malic acid. There is a mixture of D-malic acid and L-malic acid in the DL-malic acid standard (Fig. S1 (G)). L-malic acid that is unstable and may form dimer is generally present in natural plants, however both D-malic acid and L-malic acid were recently found in fermented foods such as wines (Onozato et al., 2022). According to computer-indicated retention time (RT) and corresponding peak area on each individual peak in HPLC chromatograms in the present study, the content of each individual organic acid can be easily calculated (Table 1). Interestingly, trace L-malic acid (RT = 5.4 min) and D-malic acid (RT = 12.3 min) might exist in fresh cucumbers (Fig. S1 (G) and Fig. S2 (A)). However, the results may be further confirmed by HPLC-MS/MS in the future study.

3.2.5. Changes in total biogenic amines and nitrites in the fermented cucumbers using the optimal pickling juice formula

Total biogenic amines and nitrites are generally selected as negative indicators for fermented foods, even though they are relatively scarce in fermented vegetables and fruits (Yu et al., 2021). Levels of total biogenic amines in the fermented cucumbers pickling juices were significantly ($P < 0.05$) increased through the 16-days fermentation (Table 2). However, it was still<1000 mg/kg sample (=1.000 mg/g sample, the safety limit) within 8-days fermentation, suggesting that 8-days fermentation is suitable for naturally fermenting cucumbers using the optimal pickling juice formula of 15% crystal sugar solution with brine (Penas et al., 2010). Importantly, total biogenic amines in the fermented cucumbers were too scarce to be detectable, suggesting that the safety of naturally fermented cucumbers. Economically, our results suggested a low-salt and low sugar content optimal pickling juice formula for 8-days to naturally ferment cucumbers.

Our results showed that nitrites in the fermented cucumbers and pickling juices using the pickling juice formula of 15% crystal sugar solution with brine markedly increased, particularly in the fermented cucumbers pickling juices, compared to those in the fresh sample (day 0) (Table 2). The natural fermentation process might increase hazardous nitrites in the fermented cucumbers pickling juices. However, the total nitrite contents in the fermented cucumbers were still scarce and did not exceed the risk limit, suggesting the safety of naturally fermented cucumbers. The acceptable daily intake (ADI) of nitrite ion is 0.07 mg/kg body weight for consumers (Elias et al., 2020). Particularly, total nitrites

Table 2
Changes in total biogenic amines and nitrites contents in fermented cucumbers and fermented pickling juices using a 15% crystal sugar solution with low-salt brine pickling juice formula at 4 ℃ for different days.

Fermentation days	Total biogenic amines	
	Fermented pickling juices (mg KOH equivalent/ml sample, fresh matter)	Fermented cucumbers (mg KOH equivalent/g sample, lyophilized powder)
0 day	0.37 ± 0.04^{c}	ND
4 days	0.69 ± 0.19^{b}	ND
8 days	0.95 ± 0.26^{a}	ND
12 days	1.37 ± 0.16^{a}	ND
16 days	1.27 ± 0.24^{a}	ND
fermentation days	nitrites	
	fermented pickling juices (μg NO_2^- equivalent /ml sample)	fermented cucumbers (μg NO_2^- equivalent /g sample)
0 day	1.72 ± 0.04^{c}	0.51 ± 0.03^{b}
4 days	1.73 ± 0.07^{b}	ND
8 days	2.33 ± 0.35^{a}	1.37 ± 0.06^{a}
12 days	1.77 ± 0.21^{b}	0.26 ± 0.09^{c}
16 days	2.45 ± 0.11^{a}	0.55 ± 0.11^{b}

Values are means ± SD (n = 3 replicates). Values within the same column in the same item not sharing a common letter are significantly different ($P < 0.05$) from each other analyzed by one-way ANOVA, and followed by Duncan's multiple range test. ND, not detectable.

Table 1
Changes in organic acids contents in fermented cucumbers pickling juices using a 15% crystal sugar solution with low-salt brine pickling juice formula at 4 ℃ for different days.

Fermentation days	Organic acids in fermented pickling juices (μg/ml)						
	Lactic acid	Acetic acid	Tartaric acid	Citric acid	Succinic acid	Oxalic acid	Malic Acid
0 day	ND	ND	ND	ND	ND	ND	5.29 ± 0.58^{a}
4 days	10.40 ± 1.40^{b}	8.36 ± 0.25^{b}	ND	ND	ND	ND	3.80 ± 0.64^{b}
8 days	18.67 ± 5.50^{ab}	52.15 ± 12.19^{a}	ND	ND	ND	ND	2.84 ± 0.03^{b}
12 days	39.15 ± 8.72^{a}	64.64 ± 9.81^{a}	ND	ND	4.21 ± 1.38^{a}	0.63 ± 0.12^{a}	ND
16 days	36.85 ± 8.67^{a}	58.67 ± 5.21^{a}	ND	ND	ND	0.62 ± 0.17^{a}	ND

Values are means ± SD (n = 3 replicates). Values within the same column not sharing a common letter are significantly different ($P < 0.05$) from each other analyzed by one-way ANOVA, followed by Duncan's multiple range test. ND, not detectable.

content in the fermented cucumbers through 12-days fermentation significantly ($P < 0.05$) decreased compared to that at day 0. Based on the changes in total levels of nitrites, the naturally fermented period for cucumbers using the optimal pickling juice formula is suggested within 12 days.

In the second trial for developing naturally fermented cucumbers, we found that the optimal number of fermentation days is about 8 days using the low-salt and low sugar content optimal pickling juice formula. Using this optimal culture condition for naturally fermented cucumbers, pH values quickly decreased to<4.6 (aka high-acid foods) in the fermented cucumber pickling juices, but the functional ingredients contents of total phenolics, flavonoids, saponins, free amino acids, and individual organic acid particularly lactic acid and acetic acid in the fermented cucumbers significantly increased. Importantly, total biogenic amines and nitrites contents in the fermented cucumbers or pickling juices did not exceed the risk limit, indicating the safety and functional characteristics of the naturally lacto-fermented cucumbers. In addition, sensory and microbial analyses using molecular techniques have further revealed more characteristics of the naturally lacto-fermented cucumbers using the established fermentation condition (El Sheikha & Hu, 2020; Kao et al., 2023). The overall sensory property of the fermented cucumbers product markedly improved during the optimal fermentation process, while *Leuconostoc mensenterosides* which is a lactic acid bacterium that is generally recognized as safe (GRAS) approved by US Food and Drug Administration (FDA) was found to significantly elevate from 33.64% to 80.72% after 8-days optimal aging process (Kao et al., 2023; Ray, El Sheikha, & Kumar, 2014). Meanwhile, detrimental *Enterobacter* unclassified bacteria decreased significantly from 35.45% to 3.34% (Kao et al., 2023). The present study has unraveled some important evidences for naturally lacto-fermented cucumbers to increase shelf life and enhance human health benefits, however developing fermented foods still face serious challenges in the world because of the lack of standardized international legislation (El Sheikha, 2022). Till now, it is still difficult to be standardized to harvest the aged naturally fermented product with the same and stable quality in some other environment (El Sheikha, 2022; Kao et al., 2023). Single or combined species of probiotic lactic acid bacteria particularly isolated from the same naturally fermented foods used for co-fermentation may greatly improve the difficulty of fermentation consistency. Therefore, *Leuconostoc mesenteroides* and *Lactiplantibacillus plantarum* co-fermented cucumbers are under being evolving with the established optimization fermentation condition. Mixed-culture fermentations are quite common in the preparation of fermented foods in the world, particularly in countries in the Far East including China, India, Indonesia, Japan, Korea, Pakistan, Philippines, Taiwan, Thailand and the encompassing areas, arising in probiotics and other diverse functional ingredients such as antioxidants, while lacking the dairy allergens in the oriental fermented foods (Ray, El Sheikha, & Kumar, 2014). Even though, the revolution history of fermented foods still indicates that some common factors may hinder the development and uptake of traditional fermented foods, including inadequate raw materials, rough handling and processing techniques, the shorter preservation time (shelf life), the lack of homogeneity, and an unattractive package of the final product (El Sheikha, 2018a, 2018b). The two-step trial experimental design in the present study has provided most scientific analyses on naturally lacto-fermented cucumbers based on the changes of divergent chemical parameters during the aging process, suggesting the possibility to develop desirable lacto-fermented vegetables and industrialization of traditional fermented foods.

In the present study we tried to identify and minimize detrimental ingredients during cucumber fermentation that is crucial for food safety and quality, however these fermentation changes may also include the production of undesirable compounds or the degradation of beneficial components. Lacto-fermentation involves complex microbial and biochemical interactions, thus optimizing culture conditions solely based on changes in detrimental and functional ingredients might oversimplify the process. Therefore, we have assessed microbial diversity, texture, color, and sensory attributes that are important for consumer acceptance to optimize cucumber's culture condition and obtained the similar culture condition optimization based on chemical parameter changes such as detrimental and functional ingredients (Kao et al., 2023). Most importantly, the naturally lacto-fermented cucumber product got superior scores than those of a commercial pickling cucumber product in the sensory evaluation of consumer acceptance test that would provide valuable insights into the real-world implications of the optimization (Kao et al., 2023). The 8-days fermentation process using the established 15% crystal sugar solution with low-salt brine has been evidenced the optimal choice (Kao et al., 2023). The result concluded in the present study is identical to the published literature (Kao et al., 2023).

In the beginning of this lacto-fermented experiment, the variability in cucumber sources and the impact of different processing techniques should be considered for fermentation. Even though the same cucumber source was purchased for this fermented experiments to lessen confounding factors, cucumbers from different regions or varieties may have inherent differences in their composition and microbial profile, which can influence the fermentation process and the effectiveness of culture condition optimization. Considering this variability would provide a more comprehensive understanding of the optimization process.

Although we have obtained some achievements, there are limitations in the present work and they should be investigated in details in the future. While the optimization of culture conditions for lacto-fermented cucumbers based on changes in detrimental and functional ingredients is an important area of research, the critical side lies in the potential oversimplification of the fermentation process, limited analysis, narrow focus on detrimental changes, and variability in cucumber sources and processing. In addition, the components in the fermented cucumber juices are so complicated that may interfere with organic acids determination using HPLC. The resolution of chromatograms can be improved and the results may be further confirmed by HPLC-MS/MS in the future. Exploring these limitations and accumulating more research data will contribute to a more robust and comprehensive understanding of culture condition optimization for lacto-fermented cucumbers.

4. Conclusion

A two-step trial for naturally fermented cucumbers was performed to optimize culture conditions in the present study. In the first trial, simple chemical targets including changes in pH values and total biogenic amines were measured to optimize the pickling juice formula. The 15% crystal sugar solution with brine was evidenced as the best pickling juice formula. In the second trial, more divergent chemical parameters were evaluated to optimize the fermentation days. The optimal number of fermentation days was suggested at around 8 days. During the fermentation process, the pH value quickly decreased to<4.6, possibly via producing large amounts of lactic and acetic acids in the fermented cucumber pickling juice. Meanwhile, total functional ingredients, such as total phenolics, flavonoids, saponins and free amino acids, markedly increased in the fermented cucumbers. The detrimental ingredients, including total biogenic amines and nitrites in the fermented cucumbers did not exceed the risk limit, indicating the safety of naturally fermented cucumbers. The present study concluded that 8-days naturally fermented cucumbers using 15% crystal sugar solution with low-salt brine at 4 °C may be developed as a new functional fermented product. The experimental design using a two-step trial in this study has finished most scientific analyses on naturally lacto-fermented cucumbers based on the changes of divergent chemical parameters during the aging process, suggesting the possibility to develop desirable lacto-fermented vegetables and industrialization of traditional fermented foods.

CRediT authorship contribution statement

Chien-Chia Kao: Data curation, Formal analysis, Investigation, Writing – original draft. **Jin-Yuarn Lin:** Conceptualization, Investigation, Methodology, Validation, Writing – review & editing, Funding acquisition.

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.fochx.2023.100839.

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