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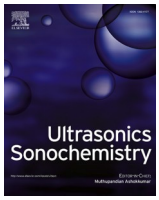

Combining thermosonication microstress and pineapple peel extract addition to achieve quality and post-acidification control in yogurt fermentation

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ABSTRACT

This work investigated the effects of the combined use of thermosonication-preconditioned lactic acid bacteria (LAB) with the addition of ultrasound-assisted pineapple peel extracts (UU group) on the post-acidification potential, physicochemical and functional qualities of yogurt products, aimed at achieving prolonged preservation and enhancing functional attributes. Accordingly, the physical–chemical features, adhesion properties, and sensory profiles, acidification kinetics, the contents of major organic acids, and antioxidant activities of the differentially processed yogurts during refrigeration were characterized. Following a 14-day chilled storage process, UU group exhibited acidity levels of 0.5–2 °T lower than the control group and a higher lactose content of 0.07 mg/ml as well as unmodified adhesion potential, indicating that the proposed combination method efficiently inhibited post-acidification and delayed lactose metabolism without leading to significant impairment of the probiotic properties. The results of physicochemical analysis showed no significant changes in viscosity, hardness, and color of yogurt. Furthermore, the total phenolic content of UU-treated samples was 98 μg/mL, 1.78 times higher than that of the control, corresponding with the significantly lower IC50 values of DPPH and ABTS radical scavenging activities of the UU group than those of the control group. Observations by fluorescence inverted microscopy demonstrated the obvious adhesion phenomenon with no significant difference found among differentially prepared yogurts. The results of targeted metabolomics indicated the proposed combination strategy significantly modified the microbial metabolism, leading to the delayed utilization of lactose and the inhibited conversion into glucose during post-fermentation, as well as the decreased lactic acid production and a notable shift towards the formation of relatively weak acids such as succinic acid and citric acid. This study confirmed the feasibility of thermosonication-preconditioned LAB inocula, in combination with the use of natural active components from fruit processing byproducts, to alleviate post-acidification in yogurt and to enhance its antioxidant activities as well as simultaneously maintaining sensory features.

1. Introduction

Fermented milk is an essential carrier for viable probiotics to consumers [1]. As a popular fermented dairy product, yogurt has a unique flavor and is rich in nutrients. It has various health benefits, such as improving immune function, alleviating lactose intolerance, relieving constipation, reducing cholesterol, and demonstrating anti-cancer properties [2]. Therefore, it has become a globally important dairy product in the entire dairy market. As probiotic carrier food, the viability of probiotics is a critical factor in determining whether fermented milk has health benefits, and a minimum viable count of at least 10^6 CFU per milliliter is required to produce beneficial effects [3].

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However, the fermentative activities of substantial lactic acid bacteria (LAB) during chilled storage lead to a gradual decrease in the pH of yogurts and induce post-acidification problems (also called post-fermentation acidification), which cause multiple negative effects including significantly shortening the shelf life of yogurt products, inducing whey precipitation, as well as the modified viability and prebiotic characteristics of LAB cells [4].

To minimize the negative effects derived from post-acidification problems on yogurts, there are various methodologies which have been developed in recent years for controlling the degree of yogurt post-acidification, including the intervention of physical processing (e.g., high pressure processing, laser pasteurization, ultraviolet-C, and thermal treatments), chemical preservatives [5], and the selection/intervention of starter culture with low potential of post-acidification. Although some mild treatments such as thermization and mechanical stress could achieve efficient post-fermentation acidification, the major challenge to develop a suitable post-fermentation strategy is related to maintain the balance between post-acidification control efficiencies and the maintenance of the viability of probiotics, physicochemical attributes and sensory scores. Particularly, the addition of chemical preservatives inhibiting the activity of lactic acid bacteria can significantly alleviate post-acidification potential in yogurt, such as the commonly used potassium sorbate, niacin, essential oil and ε-polylysine [3,4]. In spite of significant post-acidification control efficiency, these exogenous components mainly rely on the antifungal/antibacterial properties to achieve the post-acidity regulation, thus producing significant limitations including the cause of compromised culture viability and raising safety concerns for consumers due to the natural unavailability. Correspondingly, screening natural active ingredients with the potential of the targeted regulation of organic acid metabolism of microbes is a conservative and safe control strategy for delaying post-acidification. Pineapple peel is a byproduct of pineapple skin after crushing, which contains many active substances such as sugars, cellulose, lignin, phenolic acids, flavonoids and multiple enzymes. It is worth mentioning that the plant polyphenols and bromelain in pineapple peel exhibited antibacterial effects, such as disrupting cell morphology and affecting energy metabolism [6]. Hundreds of tons of pineapple peel are discarded yearly in the world, with only a small amount of peel residue being used as animal feed, natural adsorbent, and a source of functional nutrition food, causing severe economic losses and environmental pollution [7]. Reasonable utilization of pineapple peel can achieve resource recycling. Incorporating the rich bioactive components from pineapple peel into yogurt not only conforms to the environmentally conscious concept of green food processing but also contributes to environmental protection, while yielding substantial economic benefits.

Ultrasound could be used as a novel non-thermal stress adaptation technique to regulate the microbial metabolisms, thus providing the potential chance to modify the kinetics of yogurt (post)-fermentation process [8]. Simultaneously, relying on the optimization of treatment parameters and process design of a sonoreactor, high-intensity ultrasound stimuli are capable to produce multiple effects including accelerated or decelerated cell growth, the modified dynamic process of metabolites, and reshaping metabolic flux distribution [9,10] as typically exemplified by accelerated lactose hydrolysis and *trans*-galactosylation reactions in lactic acid fermentation assisted by ultrasound [11]. After treating *Lactobacillus paracasei* and skim milk medium separately, the peptide content was increased by 64.23 % in ultrasound-treated fermentation, according to Huang et al [12]. Furthermore, heating treatment has been observed to possess the potential to control the post-acidification by inducing sublethal states of microbes, while for sonication, local high temperature referred as thermosonication effects could maintain the metabolic activity of microorganisms by rebalancing metabolic flux [13,14]. In this sense, it was hypothesized that adjusting thermosonication stress-related parameters was capable to delay acid production metabolism via influencing metabolic pathways in relation to organic acid distribution, thereby alleviating post-acidification in yogurt. On the other hand, the behavior of void cavitation caused by the propagation of sound waves leads to chemical, physical, and mechanical effects, resulting in the degradation of the material matrix, promotion of the release of extractable compounds, and enhancement of mass transfer from the solvent to the sample [15]. So far, there have been no reports concerning the use of pineapple peel extract coupled with low-frequency ultrasound stress for post-acidification control of lactic acid bacteria, particularly related to the general influence on acidification curves, probiotic features and antioxidant activities.

Accordingly, to explore the feasibility of combining thermosonication stress with the use of natural components from pineapple peel extract in post-acidification control, the acidification curves, probiotic features and antioxidant activities of differentially prepared yogurt samples were characterized. Specifically, the alterations in the pH values, titratable acidity, starter culture viability, physicochemical and sensory attributes, antioxidant activities, total phenolics, adhesion properties, as well as the contents of major organic acids and carbohydrates in yogurts were determined. The results obtained from the current study are expected to alleviate the post-acidification process of yogurt in a tailored way, without compromising the probiotic properties and sensory features while simultaneously enhancing its antioxidant capacity.

2. Materials and methods

2.1. Materials, sample preparation and inoculation

Fresh powdered milk, water, and sugar were mixed and stirred until completely dissolution, followed by a water bath at 80 °C and heating for 15 min. After the mixture was cooling down to 42–45 °C, subsequently used for inoculation. The activated *Lactobacillus delbrueckii* subsp. *bulgaricus BNCC 336436* (*L. delbrueckii*) and *S. thermophilus* ABT-T were washed twice with sterile distilled water, of which the values of optical density (OD) were adjusted to about 1. The *L. delbrueckii* suspension, as the major microbe responsible for inducing post-acidification of yoghurt, was placed in a conical flask, and the ultrasonic probe was inserted into the bacterial suspension for low-frequency and low-intensity ultrasound pre-treatment (600 W, 10 min, 25 kHz) using a SIENTZ-IID device [16]. During ultrasonic stress treatment processes of the inoculated strains, the violent collapse of microbubbles and hydro-mechanical shear forces produced during the ultrasonic process induced the increase in the temperature of bacterial suspension, thus improving the overall temperature of the solutions from 20.05 to 22.95 °C (Fig. S1).

2.2. Preparation of pineapple peel extract

An appropriate amount of fresh pineapple peel was weighed, washed, and crushed. Subsequently, it was combined with 70 % volume ethanol and placed in an ultrasonic device SB-400DTY under the conditions of 33 kHz, 400 W, and 25 °C for extraction for 60 min, followed by oscillation filtration to obtain filtrate and precipitate [17]. The extraction process was repeated three times to get the precipitate. Next, the filtrates obtained from the three extractions were combined and concentrated under reduced pressure at 35 °C. The concentrate was then removed and diluted to a fixed volume with distilled water, resulting in the pineapple extract.

2.3. Preparation and refrigeration of fermented milk

The inocula, including *S. thermophilus* and *L. delbrueckii* with and without ultrasound stress, were added into reconstituted milk and fermented at 42 °C for 6 h in a constant temperature incubator. After fermentation, the concentrated pineapple peel water extract obtained from sonication/non-sonication assisted extraction was added to the fermented yogurt, and the mixture was refrigerated at 4 °C. The process

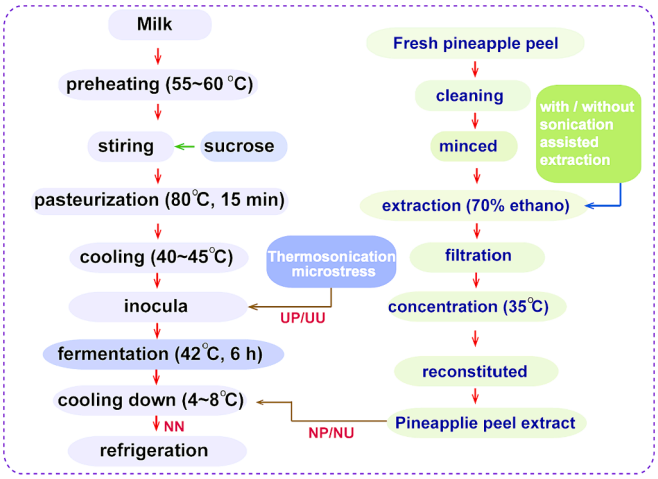

Fig. 1. A general manufacturing process of yogurts by combining ultrasonic stress and the addition of pineapple peel extract obtained from different extraction procedures. NN: the control group with unstressed inocula and without adding pineapple peel extracts; NP: unstressed inocula combined with the addition of conventional pineapple peel extract; NU: unstressed inocula with ultrasonication-assisted peel extract; UP: ultrasonically stressed inocula combined with the addition of conventional extraction solution; UU: ultrasonically stressed inocula combined with the addition of ultrasonication-assisted peel extract.

flow of fermented milk preparation is summarized in Fig. 1.

According to the different treatments of the strains and extraction methods of pineapple peel extract, they were divided into five groups. The NN group represented normal fermentation without any treatment; the NP group represented fermentation with untreated strains and normal extraction solution; the NU group represented fermentation with untreated strains and ultrasound-assisted extraction solution; the UP group represented fermentation with ultrasound-treated strains and normal extraction solution; and the UU group represented fermentation with ultrasound-treated strains and ultrasound-assisted extraction solution.

2.4. Measurement of pH and acidity in fermented milk

A calibrated desktop pH meter was used to measure the pH value and acidity changes of fermented yogurt during refrigeration at 4 °C. The acidity was determined by mixing a 10 mL yogurt sample with 20 mL distilled water, followed by titration with 0.1 M standard sodium hydroxide (NaOH) solution while stirring until the pH of the solution reached 8.2 [18]. The total volume of standard NaOH solution consumed during titration was recorded, and the titratable acidity was calculated [19].

2.5. Determination of counting in fermented milk

During refrigeration, the viable count of lactic acid bacteria in fermented milk was measured using an automatic colony counter [20]. The samples were diluted with 0.9 % sterile physiological saline to an appropriate multiple, and then 100 μL of each sample was spread onto MRS agar plates. The plates were then incubated in a 37 °C culture chamber for at least 48 h.

2.6. Physicochemical properties and sensory evaluation of fermented milk

The gel hardness and viscosity of yogurt were tested using a rheometer [21]. The whey separation was determined by centrifuging equal amounts of 5 g yogurt sample at 4000 g for 10 min at 4 °C and measuring the weight of the separated whey [22]. The color of the yogurt was observed using an SWG-2300 spectrophotometer. A sensory panel of laboratory members evaluated yogurt's aroma, color, texture, viscosity, and taste using a 5-point scoring system.

2.7. Determination of the antioxidant capacity of fermented milk

Measurement of antioxidant capacity included determination of total phenols, DPPH, ABTS+, and FRAP, with slight modifications based on the method described by literature [23–26]. 10 mL of well-mixed yogurt sample was taken, centrifuged at 7000 g, 4 °C for 10 min, and the supernatant was collected. Then, 15 mL of acidified methanol containing 0.5 % concentrated hydrochloric acid was added, vortexed for 1 min, let stand at −20 °C for 4 h, and then centrifuged (5000 g, 10 min, 4 °C). The supernatant was collected for further analysis.

2.8. Measurement of total phenolic content

1 mL of the supernatant extract was taken, and 5 mL of 10 % Folin-phenol reagent was added. The mixture was thoroughly mixed and allowed to stand for 5 min. Then, 4 mL of 7.5 % sodium carbonate solution was added, and the mixture was mixed well. The reaction proceeded at room temperature in the dark for 60 min. The absorbance at 765 nm was measured, and the total phenolic content in the yogurt was calculated for each storage day. A standard curve was established using gallic acid as a control (10–50 μg/mL), with the equation y = 0.0048x + 0.0602, R2 = 0.9989.

2.9. Measurement of DPPH radical scavenging activity

The extract was diluted with methanol to 200, 100, 50, 25, and 12.5 μg/mL concentrations. Then, 0.5 mL of the supernatant extract was mixed with 2 mL of DPPH working solution (0.0079 g of DPPH dissolved in anhydrous ethanol and made up to 200 mL). The mixture was left to stand in the dark for 30 min. Methanol was used as a blank control and recorded as A_0, while that of samples was labeled as A_1. The absorbance was measured at 517 nm, and the scavenging rate was calculated. IC50 values were calculated using SPSS. The DPPH radical scavenging rate (%) was calculated as the formula: DPPH radical scavenging rate (%) = (A_0 - A1) / A0 * 100 %.

2.10. Measurement of ABTS + radical scavenging activity

The extract was diluted with methanol to 200, 100, 50, 25, and 12.5 μg/mL concentrations. Then, 100 μL of the supernatant extract was taken and mixed with 3 mL of ABTS + working solution. The ABTS + working solution was a mixture of 7 mmol/L ABTS + solution and 2.45 mmol/L potassium persulfate solution in equal volumes, incubated at 4 °C for 14 h, and diluted with PBS phosphate buffer solution at pH 7.4 to achieve an absorbance range of 0.7 ± 0.02 at 730 nm). The mixture was left to stand in the dark for 10 min. Methanol was used as a blank control and recorded as A0. The absorbance was measured at 730 nm, and the scavenging rate was calculated. IC50 values were calculated using SPSS.

2.11. Measurement of FRAP antioxidant capacity

FRAP total antioxidant capacity was measured with slight modifications based on the method described in the reference [27]. A volume of 0.1 mL of the supernatant extract was taken and mixed with 2.9 mL of TPTZ working solution (composed of 0.3 M acetate buffer, 20 mM ferric chloride solution, and 10 mM TPTZ solution in a volumetric ratio of 10:1:1). The reaction was carried out at 37 °C for 10 min, followed by measuring the absorbance at 593 nm. The standard curve, using ferrous sulfate equivalents as the control, was obtained as y = 0.0038x + 0.0096, with a correlation coefficient (R2) of 0.9991.

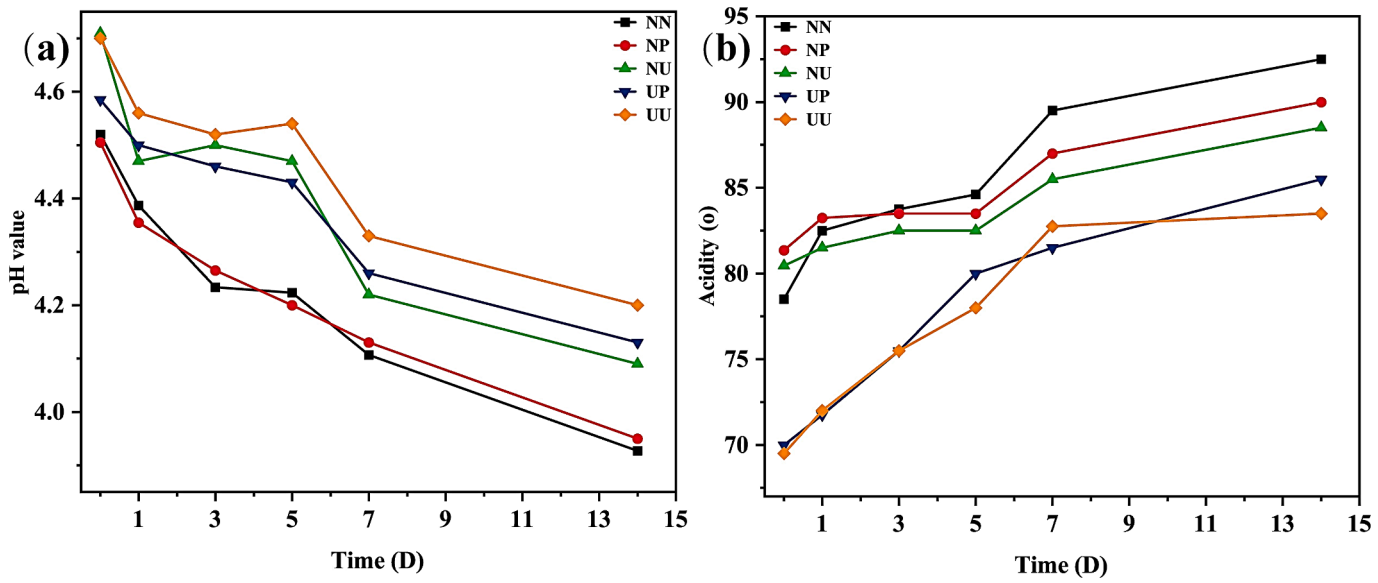

Fig. 2. Changes in pH (a) and titratable acidity (b) of fermented milk during storage; NN: the control group with unstressed inocula and without adding pineapple peel extracts; NP: unstressed inocula combined with the addition of conventional pineapple peel extract; NU: unstressed inocula with ultrasonication-assisted peel extract; UP: ultrasonically stressed inocula combined with the addition of conventional extraction solution; UU: ultrasonically stressed inocula combined with the addition of ultrasonication-assisted peel extract.

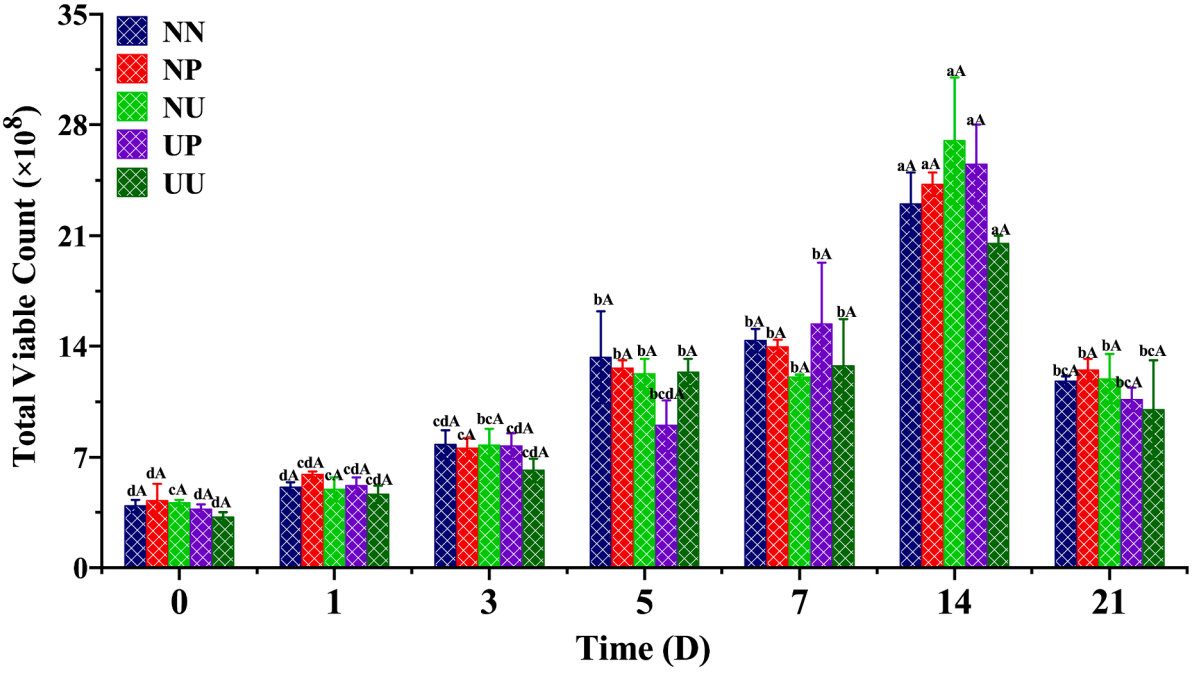

Fig. 3. Changes in the total cell numbers of viable bacteria of yogurt samples with different treatments during storage. The lower-case letters indicate that the same group has significant differences under different storage periods ($P < 0.05$); the indicates that different groups have significant differences at the same storage time ($P < 0.05$). NN: the control group with unstressed inocula and without adding pineapple peel extracts; NP: unstressed inocula combined with the addition of conventional pineapple peel extract; NU: unstressed inocula with ultrasonication-assisted peel extract; UP: ultrasonically stressed inocula combined with the addition of conventional extraction solution; UU: ultrasonically stressed inocula combined with the addition of ultrasonication-assisted peel extract.

2.12. Adhesion and fluorescence imaging

The adhesion experiment was conducted using the method described in the method of Rieu [28]. After incubating Caco-2 cells until they reached stable adhesion, the bacterial strains were adapted to pineapple peel extract at 37 °C for 30 min and then centrifuged at 5000 g for 10 min. The pellets were resuspended in sterile PBS buffer and washed three times before adjusting the OD to 1.0. FITC dye was added at a final concentration of 100 μM, and the mixture was incubated in the dark at 37 °C for 30 min. After washing three times with PBS buffer, the fluorescence intensity of the labeled bacteria was measured using a microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 538 nm [29], with the initial fluorescence value recorded as A_0. The labeled bacterial suspension was transferred into a six-well culture plate and incubated for 2 h. The culture supernatant was carefully aspirated, and the bacterial cells were washed twice with PBS

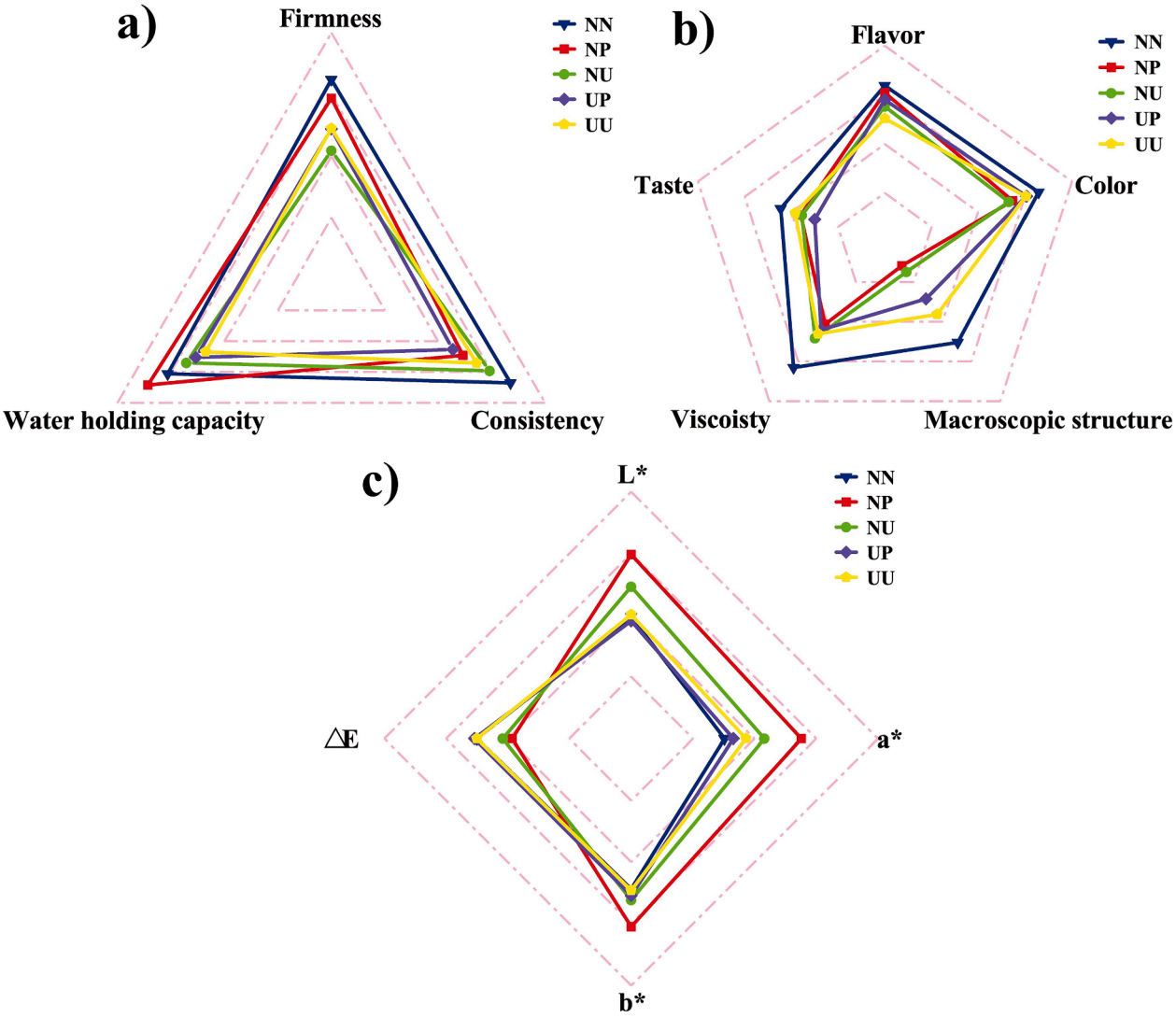

Fig. 4. Physicochemical properties and sensory evaluation of fermented milk with varied manufacturing processes: (a) physical property analysis; (b) sensory evaluation; (c) color. NN: the control group with unstressed inocula and without adding pineapple peel extracts; NP: unstressed inocula combined with the addition of conventional pineapple peel extract; NU: unstressed inocula with ultrasonication-assisted peel extract; UP: ultrasonically stressed inocula combined with the addition of conventional extraction solution; UU: ultrasonically stressed inocula combined with the addition of ultrasonication-assisted peel extract.

buffer before being detached with trypsin and measured for their fluorescence value (A_1). The bacterial adhesion rate (%) = $A_1/A_0 \times 100$ %. Fluorescence imaging was observed under a fluorescence-inverted microscope.

2.13. Targeted metabolomics for detecting carbohydrates and organic acids

1 g of collected fermented milk was weighed, and 1 mL of potassium ferrocyanide solution (106 g/L) and 1 mL of zinc acetate solution (220 g/L) were added. Water was added to make a total volume of 10 mL. The mixture was thoroughly mixed, allowed to stand for 30 min, and then centrifuged at 4000 g and 4 °C for 5 min. The supernatant was collected and filtered using a 0.45 μm membrane filter and then stored at 4 °C. Prior to measurement, the derivatization method for sugar analysis [30] was employed. 1 mL of the single sugar standard solution and the sample solution were taken. 200 μL of 0.3 mol/L sodium hydroxide solution and 150 μL of 0.5 mol/L PMP methanol solution were added to both solutions. The mixtures were well mixed and incubated in a water bath at 75 °C for 30 min. Then, they were removed and allowed to cool to room temperature. Sequentially, 200 μL of 0.3 mol/L HCl and 3 mL of chloroform were added, vortexed, and allowed to stand for 2 min. The upper aqueous phase was collected after passing through a 0.45 μm membrane filter for analysis.

The chromatographic conditions were as follows: injection volume: 20 μL, column: ZORBAX SB 5 μm C18 (4.6 x 150 mm), mobile phase: acetonitrile/0.1 mol/L acetic acid ammonium buffer (22/78 v/v) with isocratic elution, flow rate: 1 mL/min, column temperature: 25 °C, detection wavelength: 244 nm.

An appropriate amount of standard organic acid samples was dissolved in ultrapure water and made up to 100 mL to obtain standard solutions of lactic acid, citric acid, ascorbic acid, tartaric acid, propionic acid, and succinic acid at a concentration of 1 mg/mL, as well as a mixed acid solution. They were stored in a freezer at −20 °C for use. The samples were sequentially diluted with 0.1 % phosphoric acid buffer, and a mixed acid standard curve was prepared, centrifuged at 3000 g for 10 min, and the supernatant was filtered through a 0.22 μm membrane filter before chromatographic analysis. The chromatographic conditions were as follows: injection volume: 20 μL, column: ZORBAX SB 5 μm C18 column (4.6 × 150 mm), mobile phase: 0.1 % phosphoric acid solution/methanol (97.5/2.5; v/v) with isocratic elution, flow rate: 0.4 mL/min, column temperature: 40 °C, detection wavelength: 210 nm.

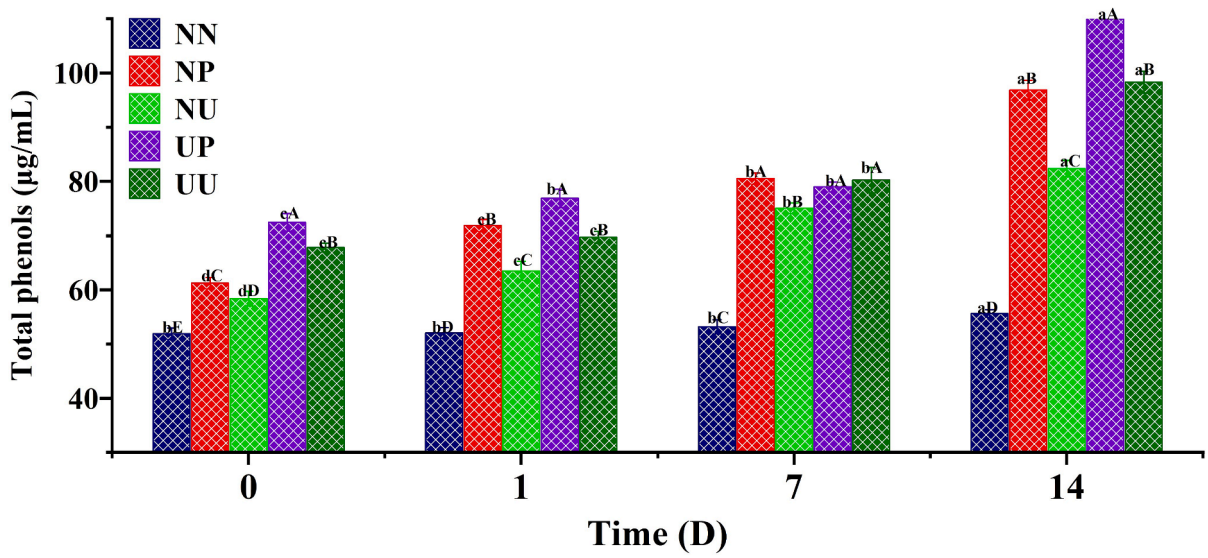

Fig. 5. Total phenolic contents of fermented milk obtained from varied manufacturing processes under different storage time; a-d indicate that the same group has significant differences at different storage times ($P < 0.05$); A-E indicate that different groups have significant differences at the same storage time ($P < 0.05$).

2.14. Statistical analysis

The results presented here are reported as the mean ± standard deviation of the average values. One-way analysis of variance and IC50 values was performed using IBM SPSS Statistics 26 software (version 26.0, SPSS Inc., Chicago, IL, USA) with a selected threshold p-value of < 0.05 for determining statistical significance.

3. Result and discussion

3.1. The pH value and acidity changes of fermented milk

According to Fig. 2, fermented milk retained metabolic activity when stored at 4 °C. The pH decreased with increasing storage time, while the acidity exhibited the opposite trend. When comparing the NN group with the NP and NU groups, it was evident that adding ultrasound-treated/untreated pineapple peel extract after fermentation resulted in a higher pH than the NN group, with an increase ranging from 0.02 to 0.25. In contrast, the acidity decreased by 1–4 °T, indicating that the addition of pineapple peel extract partially alleviated acidification. Pineapple waste could serve as a fermentable substrate, a carbon source for acid fermentation, and a favorable nutrient for bacterial growth [31]. However, the addition of pineapple peel extract during post-fermentation/storage processes exhibited a mitigating effect, possibly due to its rich content of antioxidant components such as myricetin, salicylic acid, tannic acid, vanillic acid, ferulic acid, and syringic acid, which possessed antibacterial activity [32]. Comparing the data of pH and acidity between the NU and NP groups, as well as the UU and UP groups, it was observed that the fermented milk supplemented with ultrasound-treated pineapple peel extract exhibited lower acidity (reduced by 0.5–2 °T) and a pH increase of 0.06–0.27 compared to the fermented milk supplemented with untreated pineapple peel extract. It suggested that ultrasound-treated pineapple peel extract had a more pronounced effect in inhibiting post-acidification in acid milk. The combined effect of an ultrasound-treated fermentation starter and ultrasound-assisted plant extract held great potential for addressing the issue of post-acidification in lactic acid fermentation.

3.2. The variation in microbial count of fermented milk

The variation in microbial count could visually reflect the growth of probiotic bacteria in fermented milk during refrigeration. Fig. 3 shows that the total colony count initially increased and then decreased during the storage process, indicating that the lactic acid bacteria in the fermented milk continued to grow and metabolize residual nutrients from the acid milk during storage. However, after 14 days, the growth of bacteria may have been hindered due to the decrease in pH and a significant rise in acidity, which created acidic stress. Through comparisons among different groups, no significant differences in the microbial count were observed, whether ultrasound-treated/untreated pineapple peel extract was added or ultrasound fermentation starter was used. The overall number of lactic acid bacteria was not visibly influenced. The total colony count exceeded 10^6 CFU/mL, complying with the requirements for dairy products stated in international standards [33].

3.3. Physical analysis of fermented milk

As shown in Fig. 4, compared with the NN group, the viscosity, hardness, and color of the NP, NU, UP, and UU groups were slightly reduced, but no significant differences were observed. However, water-holding capacity decreased significantly. Water-holding capacity was related to the ability of the gel structure to bind water, and the addition of pineapple peel extract after fermentation may have disrupted the gel structure of the yogurt, resulting in an uneven texture and weakened water-binding capacity [34]. The sensory evaluation results in Fig. 4c were consistent with this observation. However, adding pineapple peel extract did not weaken the flavor or reduce the palatability of fermented yogurt, maintaining its suitability for consumption.

3.4. Analysis of antioxidant capacity in fermented milk

Phenolic compounds serve as antioxidants by providing the ability to donate hydrogen or electrons, thereby terminating chain reactions or by chelating transition metal ions to terminate the Fenton reaction [35]. As shown in Fig. 5, it was evident that the total phenolic content increased continuously throughout the storage period. The NN group had an insignificant increase from 51.9 to 55.6 μg/mL, while the remaining groups with ultrasound-preconditioned or non-ultrasound-preconditioned pineapple peel extracts exhibited significantly higher total phenolic content. During refrigeration, the phenolic compounds in yogurt from pineapple peel extract addition were affected by enzymes or other biochemical substances generated by bacterial metabolism, leading to their release or transformation [36], thereby gradually increasing the total phenolic content. The difference in total phenolic content

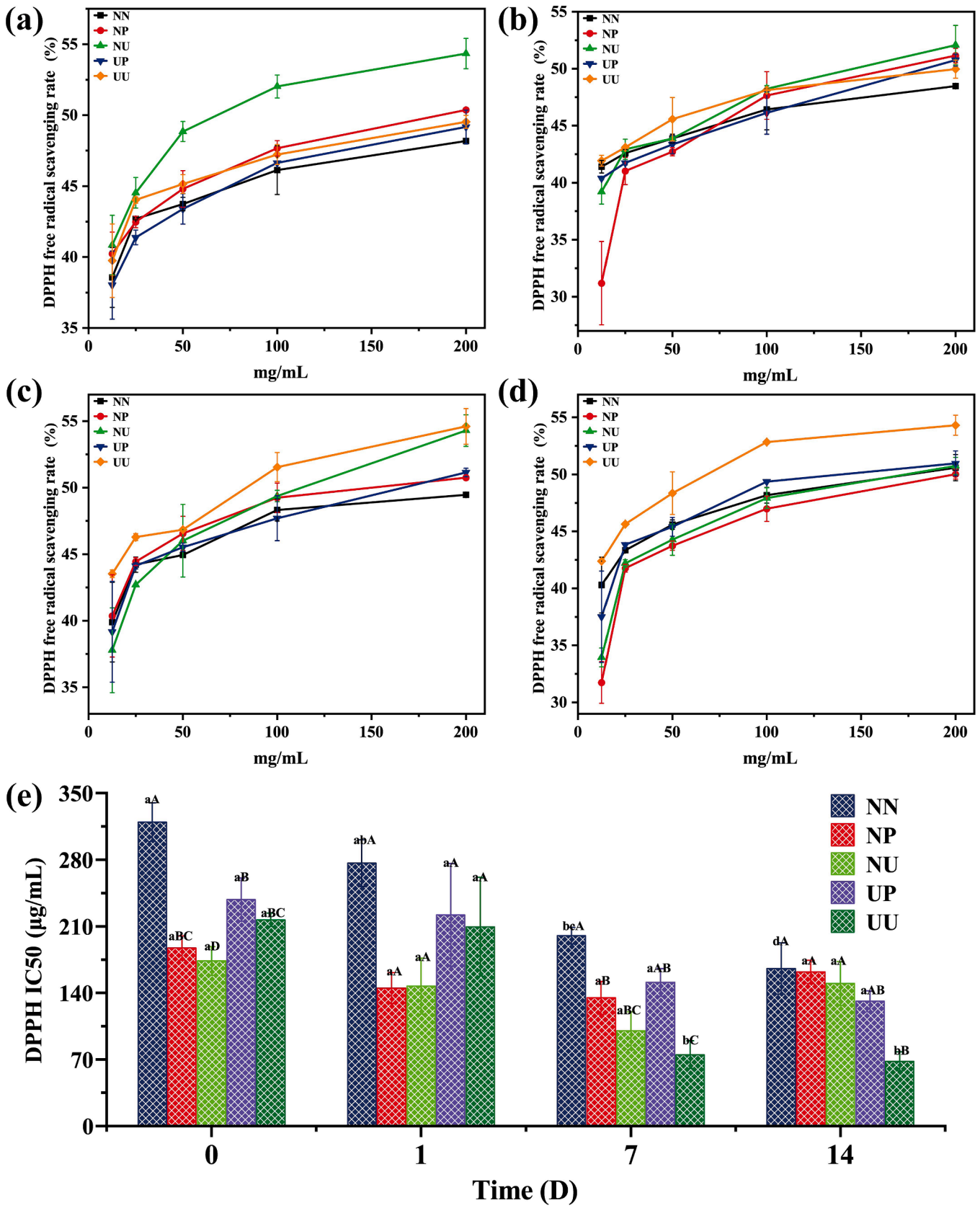

Fig. 6. DPPH free radical scavenging rate of fermented milk obtained from varied manufacturing processes under different storage time: (a) 0 days; (b) 1 day; (c) 7 days; (d) 14 days; (e) IC50 value. a-d indicates that the same group has significant differences at different storage times ($P < 0.05$); A-E indicates that different groups have significant differences at the same storage time ($P < 0.05$);

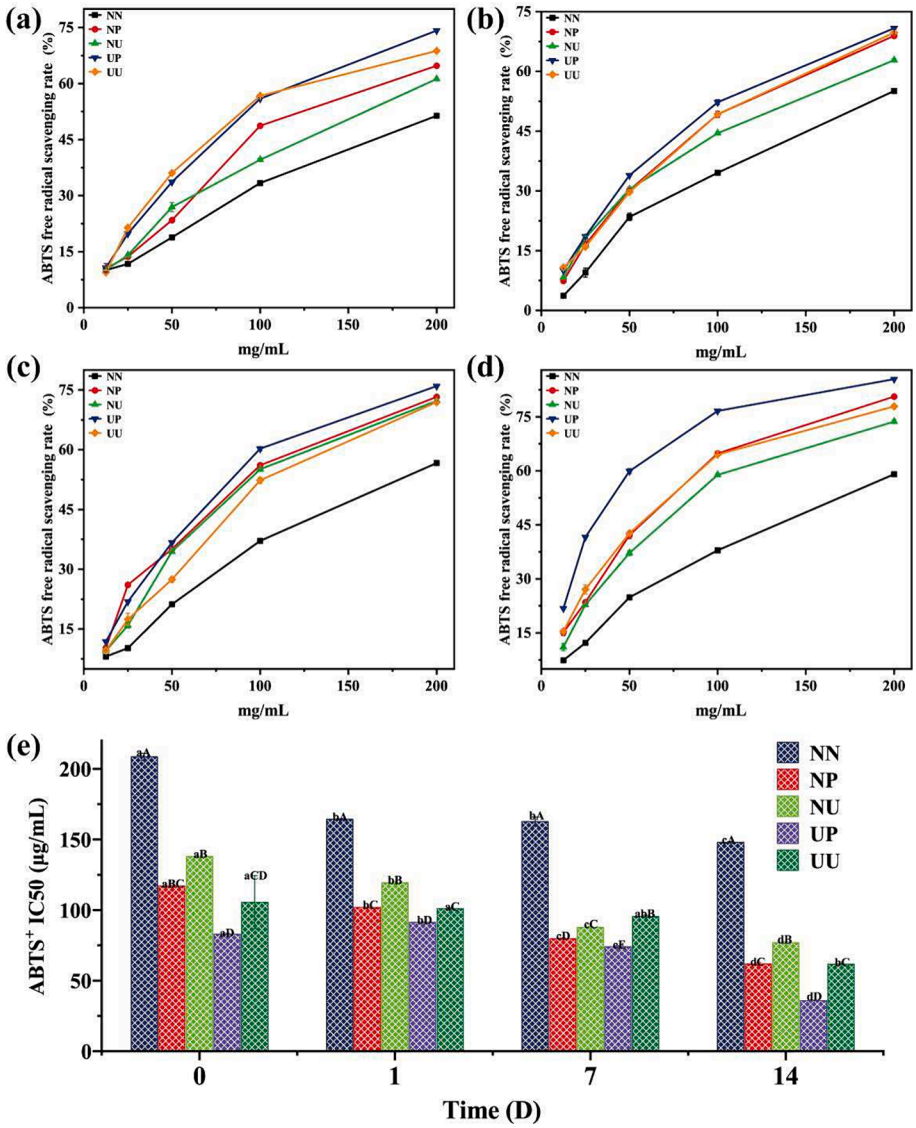

Fig. 7. ABTS radical-scavenging rates of fermented milk obtained from varied manufacturing processes under different storage points: (a) 0 day; (b) 1 day; (c) 7 days; (d) 14 days; (e) IC50 value. a-d indicate that the same group has significant differences at different storage times ($P < 0.05$); A-E indicate that different groups have significant differences at the same storage time ($P < 0.05$);

between the UP and UU groups and the NP and NU groups was 24.27 μg/mL and 15.98 μg/mL, respectively, indicating that the fermented milk with ultrasound-conditioned strains contained more phenolics. It may have been due to the ultrasound activating genes responsible for phenolic compound production within the bacteria. The addition of ultrasound and non-ultrasound pineapple peel extracts also influenced the total phenolic content. During the storage process, the NP group exhibited higher phenolic content than the NU group, with a difference of approximately 2.84–14.5 μg/mL.

Similarly, the UU group showed lower phenolic content than the UP group, with a difference of approximately 4.65–22.79 μg/mL, indicating that the phenolic content in the ultrasound-treated pineapple peel extract was lower than that in the non-ultrasound-treated extract. Ultrasound treatment has been utilized for extracting phenolic compounds from various food matrices, including citrus fruits and peels, to improve recovery rates and reduce extraction time [37]. High-intensity ultrasound treatment could effectively enhance the stability and increase the content of phenolic substances [38]. Some studies have reported that the phenolic content of yogurt containing plant materials such as garlic, goji berries, soy, and tea may be associated with high antioxidant activity [39]. The main polyphenolic compounds in pineapple peel include ferulic acid, gallic acid, catechin, and dry extracts of catechins [40].

The antioxidant activity of the fermented milk was evaluated by measuring its ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. As shown in Fig. 6, the IC50 values of DPPH radical scavenging activity decreased with increasing storage time, indicating a more vital ability to scavenge free radicals with lower IC50 values. Within two weeks, the IC50 values of the groups with ultrasound-preconditioned or non-ultrasound-preconditioned pineapple peel extracts were lower than that of the NN group (165.59 μg/mL), indicating a more robust DPPH radical scavenging capacity when ultrasound or non-ultrasound-treated pineapple peel extracts were added. On the fourteenth day, comparing the IC50 values of the NP and NU groups and the UP and UU groups, the NP group showed approximately 12.19 μg/mL higher IC50 value than the NU group. The UU group exhibited approximately 63.15 μg/mL lower IC50 value than the UP group. It indicates that the yogurt with ultrasound-extracted pineapple peel infusion has a more vital DPPH radical scavenging ability. Pineapple peel's free radical-scavenging activity was attributed to total phenolics, flavonoids, and anthocyanins [41].

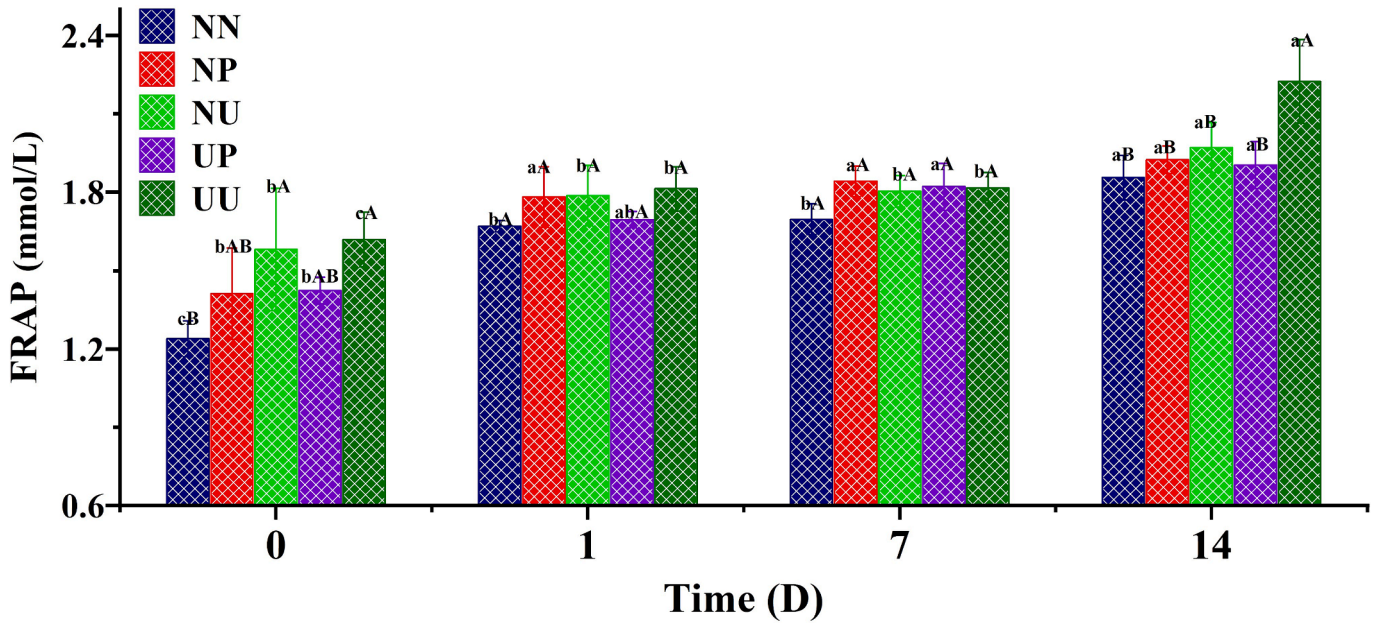

Fig. 8. FRAP values of fermented milk obtained from varied manufacturing processes in different storage time; a-c indicates that there are significant differences in the same group at different storage times ($P < 0.05$); A-B indicates that there are significant differences in different groups at the same storage time ($P < 0.05$).

The results of $ABTS^+$ radical scavenging activity are shown in Fig. 7, with a similar trend to the IC50 value of DPPH radical scavenging activity, decreasing with increasing storage time. The NN (blank) group showed the highest IC50 value of 147.98 μg/mL, indicating the weakest ability to scavenge $ABTS^+$ radicals. In contrast, the other groups with added pineapple peel extracts exhibited more potent free radical scavenging abilities. The IC50 value difference between the NP and NU groups was 15.06 μg/mL, and between the UP and UU groups was 25.7 μg/mL. The IC50 value differences suggested that non-ultrasound-assisted pineapple peel extracts had better $ABTS^+$ radical scavenging ability than ultrasound-assisted ones. It may have been due to the change in the gel network structure of whey protein caused by the addition of ultrasound-assisted pineapple peel extracts [42], which affected the difference in $ABTS^+$ radical scavenging activity due to changes in hydrophobicity.

The FRAP assay characterized the antioxidant activity by measuring the reducing power, with higher FRAP values indicating more significant antioxidant activity. Fig. 8 shows the results of FRAP antioxidant capacity measurements of yogurt at various storage times, with a similar trend to total phenols. Prolonged storage time increased the ability to scavenge DPPH radicals and FRAP, with an increase observed within 14 days of storage. The NN group showed the lowest FRAP value of 1.85 mmol/L, indicating the weakest antioxidant activity. In contrast, the UU group exhibited the most potent antioxidant activity with a value of 2.22 μg/mL, possibly due to phytochemicals and microbial metabolic activities in the pineapple peel extract [43]. Additionally, the study showed that the antioxidant properties of yogurt were attributed to the involvement of many different amino acids, small molecule peptides, and biologically active peptides produced during fermentation [44]. Moreover, polyphenols from plant sources greatly enhanced the antioxidant properties of yogurt.

3.5. Changes in adhesion and imaging of fermented milk

To investigate the impact of adding pineapple peel extract on the probiotic properties of bacterial cells, in vitro adhesion characterization was performed on five groups of bacterial suspensions, and overall adhesion was observed using fluorescence inverted microscopy. The results of the adhesion experiment are shown in Fig. 9a. Compared to the NN group, adding extract-grown strains (NP/NU) did not exhibit a significant change in adhesion, and the ultrasound-treated strains (UP/UU) showed a slight increase in adhesion but not significantly. Observations using fluorescence inverted microscopy revealed the obvious adhesion phenomenon in different groups (Fig. 9), with no distinct differences among them, consistent with the adhesion testing results, indicating that the addition of pineapple peel extract during cultivation did not affect adhesion, and the ultrasound-treated strains demonstrated good adhesion. Similar results were previously observed when using ultrasound to treat the strains of bifidobacteria and lactic acid bacteria [45].

3.6. Changes in carbohydrate content in fermented milk

The consumption of fermentation substrates was the basis for microbial metabolic activities. As shown in Fig. 10, the contents of lactose decreased with storage time while glucose increased. It was because lactic acid bacteria continued to undergo normal fermentation under low-temperature conditions during storage. Lactic acid bacteria, through enzymatic action, broke down lactose into galactose and glucose, resulting in a decrease in lactose and an increase in glucose. Adding pineapple peel extract led to a higher lactose content than the NN group. The rate at which lactic acid bacteria utilized lactose slowed while the glucose content increased. Pineapple peel extract may contain abundant glucose, fructose, and sucrose [7]. Compared to the NP and NU groups, the UP and UU groups had higher lactose content, indicating that the lactose consumption rate in the fermentation by ultrasound-treated strains was slower than that of regular strains. The metabolic activity of fermented strains was reduced after ultrasound treatment, exhibiting a decelerated effect. Compared to the NP group, the lactose content was 0.05 mg/mL higher, and the glucose content was 0.02 mg/mL lower. Between the UU and UP groups, the lactose content was 0.07 mg/mL higher, and the glucose content was 0.01 mg/mL lower. It suggested that adding ultrasound extract had an inhibitory effect on strain metabolism. Coupling ultrasound-assisted extract addition and ultrasound-pretreated strains for fermentation could effectively delay lactose metabolism.

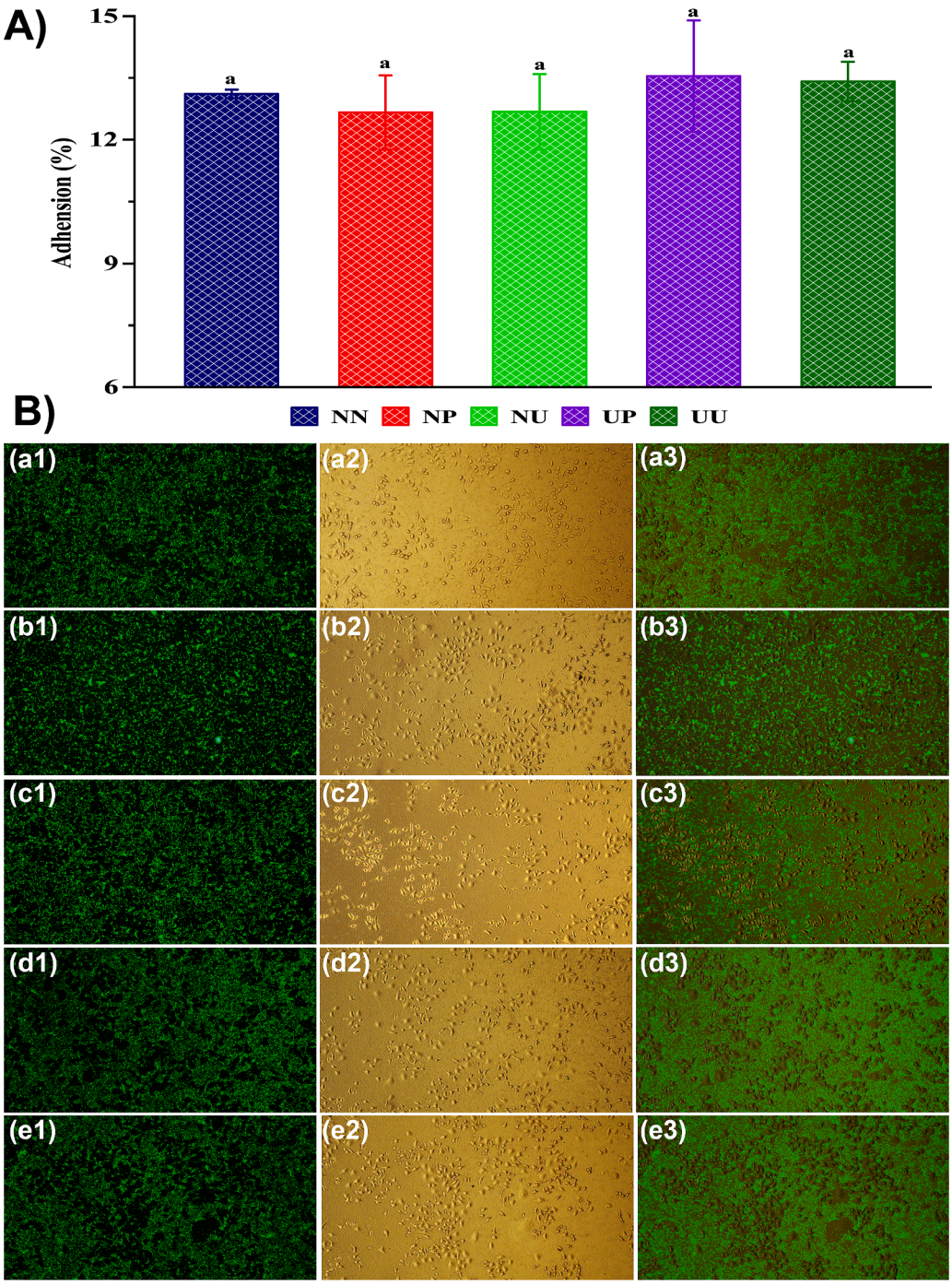

Fig. 9. Adhesion properties (A) of bacteria in fermented milk obtained from varied manufacturing processes under different fermentation conditions; superimposition and bright field images of differentially treated bacteria observed under a fluorescence-inverted microscope (B): a1-a3, NN group; b1-b3, NP group; c1-c3, NU group; d1-d3, UP group; e1-e3, UU group. a1, b1 and c1 represent fluorescence images; a2, b2, c2 mean bright-field images; a3, b3, and c3 indicate superimposed images of the fluorescence and bright field. NN: the control group with unstressed inocula and without adding pineapple peel extracts; NP: unstressed inocula combined with the addition of conventional pineapple peel extract; NU: unstressed inocula with ultrasonication-assisted peel extract; UP: ultrasonically stressed inocula combined with the addition of conventional extraction solution; UU: ultrasonically stressed inocula combined with the addition of ultrasonication-assisted peel extract.

3.7. Changes in organic acid content in fermented milk

The continuous accumulation of organic acids is the key factor leading to post-acidification [46]. During storage, the changes in organic acids, including lactic acid, citric acid, ascorbic acid, tartaric acid, propionic acid, succinic acid, and malic acid, were monitored in fermented milk, as shown in Fig. 11. The lactate content continuously increased trend during storage, as lactate was the main product of lactose metabolism. The lactate content in the NN group reached 0.49 mg/mL after 14 days of storage, while the contents in the NP, NU, UP, and UU groups were significantly lower than the control group, ranging from 0.04 to 0.19 mg/mL.

Citric acid, an essential organic acid in organisms, was one of the significant contributors to acidity. It also showed an increasing trend during storage but with differences among groups. The NN group had the lowest citric acid content, only 0.37 mg/mL. The UU group had

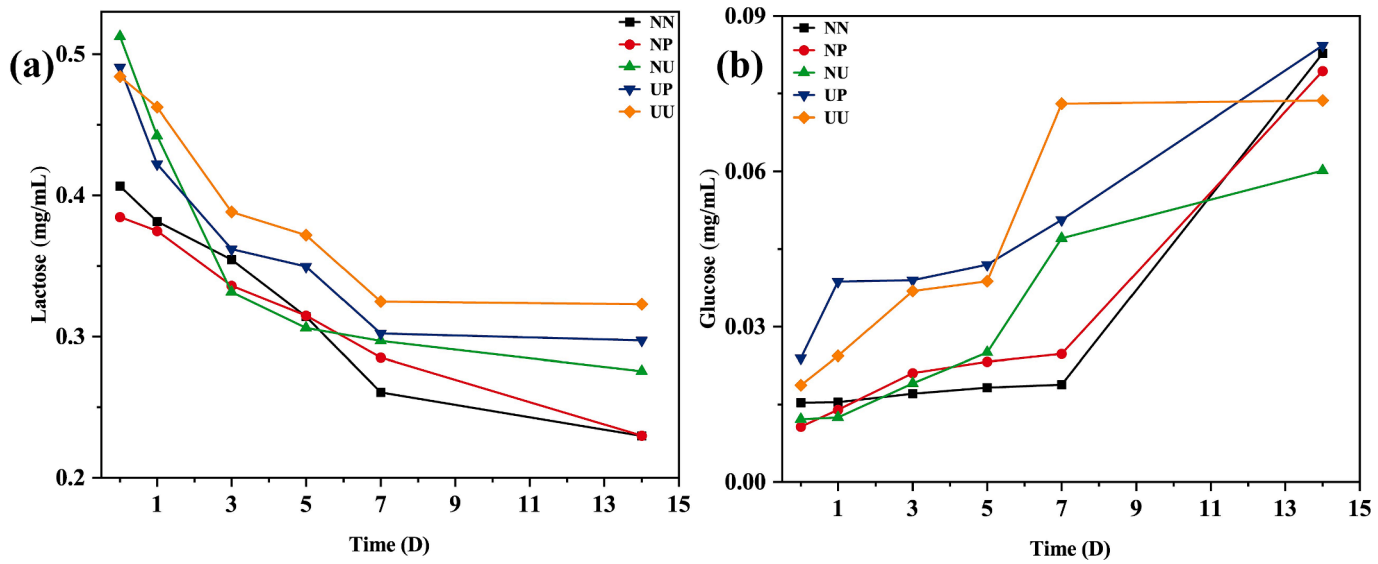

Fig. 10. Changes of carbohydrate contents in fermented milk obtained from varied manufacturing processes during the chilled storage: (a) lactose; (b) glucose.

lower citric acid content than the UP group, and the NU group had lower content than the NP group. It may have been due to citric acid in the extraction solution or a higher utilization rate during refrigeration.

Ascorbic and tartaric acid are organic acids with specific antioxidant properties [47]. However, their trends during storage were the opposite. The ascorbic acid content increased while the tartaric acid content decreased. The increase in ascorbic acid may be attributed to the synthesis of vitamin C by the *L. delbrueckii* in yogurt and the high antioxidant capacity of ascorbic acid in pineapple peel extract [48]. There was no significant effect on propionic and succinic acids after 14 days of storage. Malic acid was not detected during storage.

Fig. 12 demonstrates that LAB strains fermented lactose and glucose, producing pyruvic acid and lactic acid through a series of enzymatic reactions [49]. The distribution of various acids in fermented milk could be observed by detecting the organic acids. Particularly, it could be concluded that the combination strategy significantly delayed the consumption of lactose and its decreased hydrolysis into glucose, as well as the low contents of lactic acid. The decrease in the concentrations of lactic acid in the samples was attributed to the inhibited lactose metabolism into glucose, as well as the accelerated transformation and incorporation of lactic acid into tricarboxylic acid cycle, which produced relative weak acids to lactic acid (e.g., succinic acid, citric acid), thus obviously attenuating the post-acidification potential [50]. The storage of fermented milk with an ultrasound fermentation agent and pineapple peel extract could regulate acidification mainly by changing the concentration of lactic acid and citric acid. It alleviated post-acidification issues and enhanced fermented milk's antioxidant capacity and storage stability.

Ultrasound could be utilized to redesign the metabolic behavior of biological systems by altering various process parameters, thereby regulating the mechanism of post-acidification of strains [51,52]. Under thermosonication microstress, specific enzyme activity and metabolic pathways of strains were affected, leading to alterations in the distribution of organic acids [53]. Moreover, the cell walls of pineapple peel could be disrupted by ultrasonic-assisted extraction, promoting the release of bioactive compounds and enhancing extraction efficiency, resulting in the generation of higher concentrations of bioactive components such as polyphenols and bromelain. These compounds could improve the overall antioxidant activity of products by scavenging free radicals, reducing oxidative stress, and inhibiting lipid peroxidation [2]. By subjecting strains to ultrasound stress for fermentation and incorporating pineapple peel extract, yogurt products reduced the rate of acidification and exhibited improved antioxidant properties due to the combined effects of ultrasound attenuation and the bioactive compounds present in pineapple peel extract. However, further investigation was needed to understand the impact of ultrasound stress on strain fermentation and acid metabolism pathways when coupled with pineapple peel extract.

4. Conclusion

The pH and acidity results indicated a significant decrease in the acidification ability of fermented milk during refrigeration, due to the treatments of thermosonication microstress and the addition of pineapple peel extracts. The measured levels of lactose, glucose, and organic acids confirmed a slower rate of sugar fermentation and lactic acid production process. The texture and sensory evaluation results demonstrated that fermented milk's structure and sensory experience remained intact. The investigation of probiotic properties through adhesion studies also revealed enhanced effects. After adding pineapple peel extract, fermented milk's total phenolic content increased during storage. The DPPH and $ABTS^{+}$ radical scavenging capacities were enhanced, and the FRAP value increased, indicating a substantial improvement in antioxidant capacity. Coupling pineapple peel extract with low-frequency ultrasound stress on lactic acid bacteria fermentation enhanced the antioxidant activity of fermented milk products and alleviated the post-acidification problems. The synergistic effects of ultrasound and strain stress combined with pineapple peel extract had the potential to simultaneously improve the functional properties of yogurt and alleviate post-acidification issues. Although the combined approach of fermentation using thermosonication microstress strains with the addition of pineapple peel extract showed reliability in controlling post-acidification issues, there was a slight decrease in the viscosity, texture, and flavor of the fermented product, thus demonstrating that it is required to optimize the operational details. The potential impact of this combined strategy on other related metabolic pathways of the strains, as well as its relationship with final product quality, remained unknown. Further research was under preparation to clarify the influence of thermosonication microstresses on the strain fermentation performance and acid metabolism pathways when coupled with pineapple peel extract.

CRediT authorship contribution statement

Xiaohui Zhang: Methodology, Investigation, Formal analysis, Writing – original draft. **Yuanrong Zheng:** Funding acquisition,

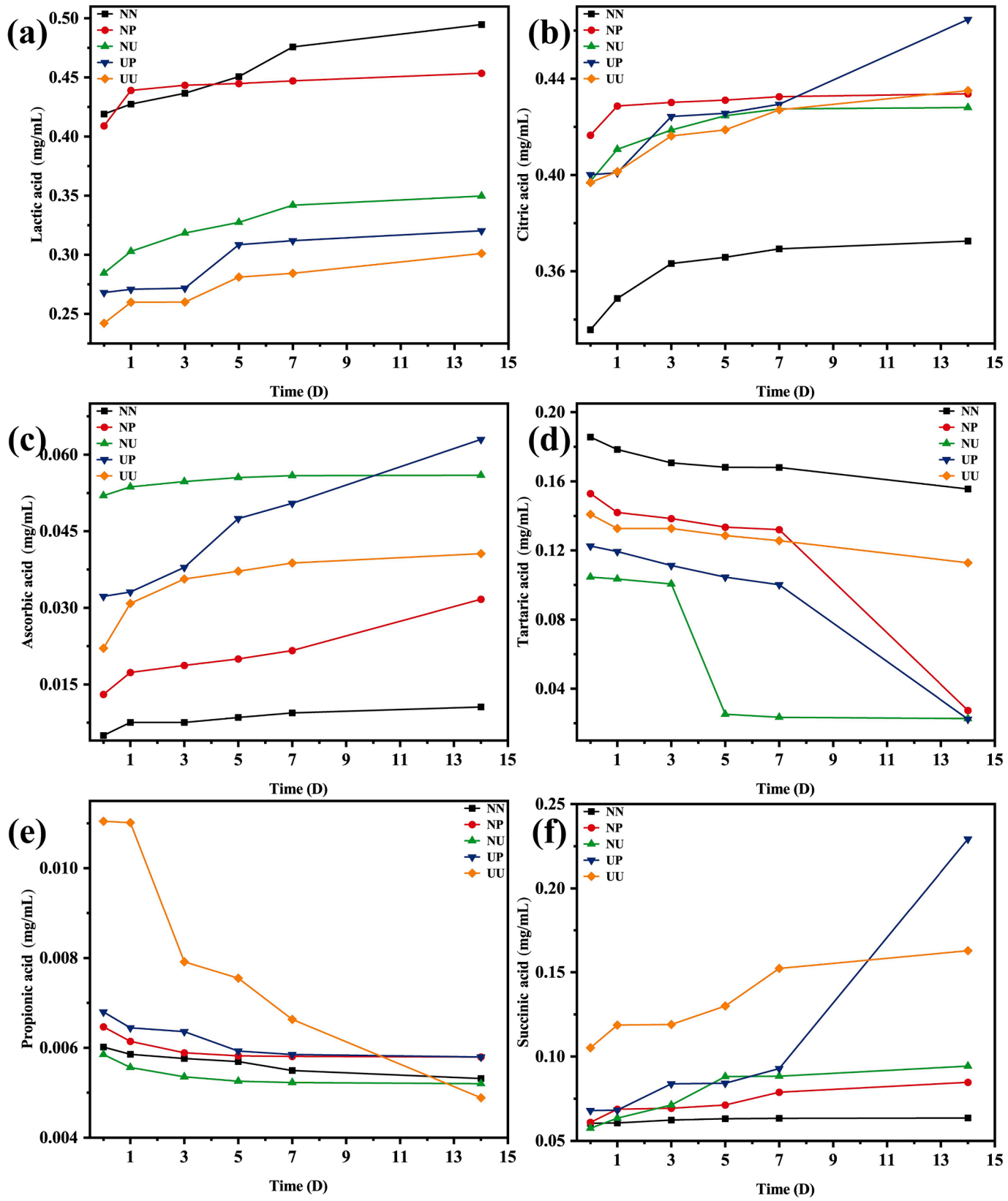

Fig. 11. Changes of organic acid content in fermented milk obtained from varied manufacturing processes during the chilled storage: (a) lactic acid; (b) citric acid; (c) ascorbic acid; (d) tartaric acid; (e) propionic acid; (f) succinic acid.

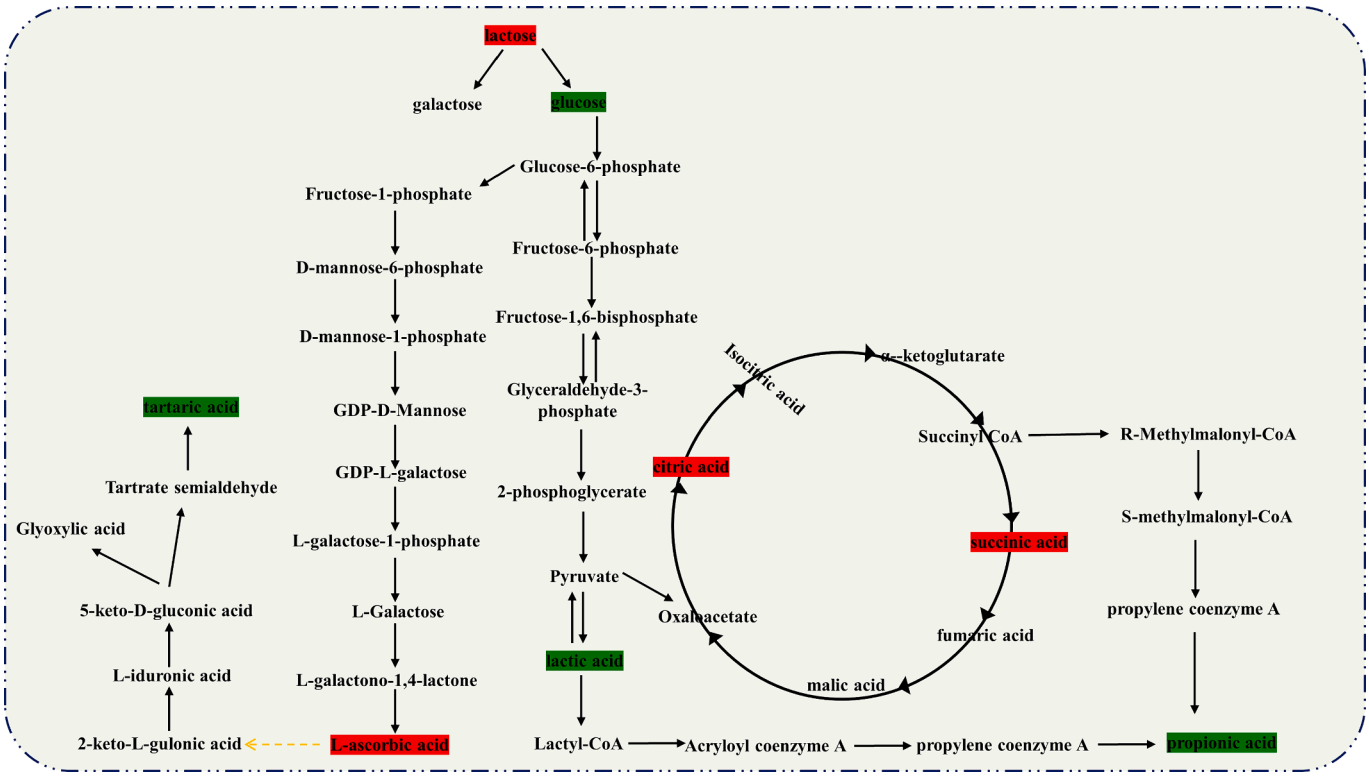

Fig. 12. Overall organic acid metabolism processes in lactic acid bacteria as influenced by manufacturing: red represents an increase in the contents of metabolites; green represents a decrease. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Methodology, Visualization, Writing – review & editing. **Changyu Zhou:** Formal analysis, Visualization, Writing – review & editing. **Jinxuan Cao:** Formal analysis, Funding acquisition, Writing – review & editing. **Yifeng Zhang:** Formal analysis, Methodology. **Zhen Wu:** Project administration, Writing – review & editing. **Daodong Pan:** Funding acquisition, Project administration, Writing – review & editing. **Zhendong Cai:** Conceptualization, Formal analysis, Funding acquisition, Writing – review & editing. **Qiang Xia:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2024.106857.

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