Characterization of the *Lactobacillus plantarum* A3 with novel fermentation properties

Fengxuan Lang

Ningbo University

Jianzhu Wen

Ningbo University

Zhen Wu (✉ woodsen@163.com)

Ningbo University <https://orcid.org/0000-0002-3607-6440>

Daodong Pan

Ningbo University

Xiaoqun Zeng

Ningbo University

Zhendong Cai

Ningbo University

Yuxing Guo

Nanjing Normal University

Xiaotao Liu

Ningbo University

Research article

Keywords: L. plantarum, flavor profiles, volatile organic compounds, sensory profiles, fatty acid

Posted Date: December 18th, 2019

DOI: <https://doi.org/10.21203/rs.2.19186/v1>

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Abstract

The use of probiotic starters in the milk fermentation could provide the dairy products with unique flavors, textures and some health benefits. In this research, *L. plantarum* A3 was identified as a probiotic with good fermentation characters during the texture and flavor formation of yoghurt. The hardness, consistency, viscosity, and viscosity coefficient of the yoghurt were enhanced when fermented with *L. plantarum* as the higher activity of β -galactosidase and lactate dehydrogenase in the mixed culture condition. The contents of amino acids and volatile organic compounds (VOCs) were found accumulated in *L. plantarum* fermented yoghurt besides the butanoic acid, acetone. Sensory profiling evaluation with electronic tongue system proved the novel taste of *L. plantarum* fermented yoghurt were significant different with the former plain yoghurt. Indeed, yoghurt fermentation with *L. plantarum* A3 indeed has a good quality and nutrition properties compared with the traditional strains and the relationship between the traditional fermentation strains and probiotics still need further investigation to meet the nutrition requirements of varies consumers.

Introduction

Fermented food has been used in human diet for thousands of years and the use of microbial starters could provide the products with unique flavors, textures and some health benefits, including lowering the risk of high blood pressure, diabetes, obesity, high cholesterol, diarrhea, thrombosis [1]. Meanwhile, fermented products can also provide consumers with good source of free amino acids and vitamins which is amplified in the fermentation process [2, 3]. It has recently been shown that *Lactobacillus helveticus* fermented milk can be used in sports beverage, which could promote cardiovascular improvement during the proper exercise process [4].

Lactic acid bacteria (LAB) fermentation is the simplest and safest way of food preservation and the lactic acid produced by LAB can extend the shelf life of food and provide beneficial effects to humans by regulating the micro-ecological balance of the gastrointestinal tract [5]. Fermented milk products have been consumed in world for perhaps 4000 years and many of the products are derived from the spontaneous LAB fermentation of milk that during the milk preservation process in the local traditional methods [6].

Besides yoghurt products, fermented vegetable products, like pickles and sauerkraut are also popular in the world. As the important member of LAB, *L. plantarum* is always found in fermented plant-based foods. As a very flexible and versatile microorganism, the interest towards *L. plantarum* has increased in recent years, especially in relation to its probiotic potential [7] and its possible application in different fermented foods and beverages [8].

As an increasing commercial interest in the addition of probiotic bacteria to fermented dairy products, a wide variety of probiotic LAB strains are commercially available and co-fermented in the dairy products. The fact is that bacterial co-culture does affect the production of characteristic volatile flavor compounds

during the fermentation process, and the success of new functional foods depends not only on the enhanced nutritional value, but also the ideal sensory quality attributes that meet consumer needs [9]. Furthermore, the quality of the fermented milk is subject to co-culture ingredients and selection of probiotics, the degrees of kinetic acidification parameters were affected by the addition of prebiotics and probiotics in the yoghurt co-culture system [10]. Some studies also found the concentration of acetic acid and free amino acids in organic acids in probiotic cheese was higher, and there was a significant difference in proteolysis of three batches of cheddar cheese co-cultured with different probiotics [11].

Different combinations of LAB starters provide the fermented dairy products with potential nutrition and health benefits. However, either beneficial or undesirable changes may also generate in the composition of the bacterial flora during the fermentation process of dairy products. The aim of this work was to analyze the interactions among LAB starters with the probiotic *L. plantarum* A3, the texture and nutrition characteristics of the yoghurts fermented with this novel strain were also investigated in order to get the adequate combinations of strains for the development of probiotic dairy products.

Materials And Methods

Strains and culture conditions

Lactobacillus strains isolated from traditional Sichuan pickle were identified by the 16s rRNA methods and further culture in MRS broth. The other two traditional strains *S. thermophilus* and *L. bulgaricus* were also stored and cultured in our laboratory.

Symbiotic growth characteristics of *L. plantarum*

Exploring the effects of *L. plantarum* under symbiotic culture, *L. plantarum* and *L. bulgaricus* and *S. thermophilus* were cultured in 37°C MRS medium under the same conditions with different strain inoculation ratio. Growth and acid production of the strains in the symbiotic growth media were measured at 600 nm every 2 hours and the curves were plotted in 24 hours' culture time.

Fermented milk preparation

1. *bulgaricus* and *S. thermophilus* combined with *L. plantarum* in a ratio of 1:1:0, 1:1:1, 1:1:2, 1:1:3 were screened as the mixed starters and inoculated with fresh pasteurized milk at a concentration of 3% (v/v) for 7 h at 42 ° C, and then ripening at 4 ° C for 24 h before further use.

Texture, acidity, water holding capacity analysis

The firmness, consistency, cohesiveness and index of viscosity of the yoghurt were determined by using a texture analyzer (TA, UK), and set the cylindrical probe: 70mm height, 65mm drop, 3mm/s descent speed, 500 PPS speed, 5g contact force; return distance 70mm, return speed 10mm/s. The measuring cup and probe should be cleaned after each test. Acidity of the yoghurt was performed with the acid-base titration

method: Two drops of phenolphthalein were added to a 10 g sample and then titrated with 0.1 mol/L NaOH. The data were recorded and the acidity was calculated according to the following formula.

$$X_2 = C_2 \times (V_2 - V_0) \times 100 / m_2 \times 0.1$$

X_2 : acidity of the sample in degrees ($^{\circ}\text{T}$);

C_2 : concentration of the sodium hydroxide standard solution;

V_2 : the volume of the sodium hydroxide standard solution;

V_0 : the volume of the sodium hydroxide standard solution used in the blank experiment;

100:100g sample; m_2 : the weight of the sample.

GC-MS analysis of volatile compounds

The fermented milk was sealed in a 5ml to 20mL headspace vial (CNW Technologies, Germany) with a Teflon/silicone septum in an aluminum cap. 10 μL of 10 ng/ μL 2-methyl-3-heptanone was added as an internal standard. Extraction of Volatile Compounds by Solid Phase Micro extraction (SPME) with 75 μm Carboxy/Polydimethylsiloxane (CAR/PDMS) Fibers from Supelco Inc (Bellefonte, PA, USA). The constant flow rate of the carrier gas (helium) was 3 mL/min, with an inlet temperature of 210 $^{\circ}\text{C}$ and split less injection mode. The initial temperature of the column oven was 40 $^{\circ}\text{C}$, which was maintained for 3 min, increased to 140 $^{\circ}\text{C}$ at 4 $^{\circ}\text{C}/\text{min}$, held for 1 min, and then increased to 200 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}/\text{min}$ for 20 min. The signal acquisition of the mass spectra was in full scan mode, ionization method EI, electron bombardment energy of 70 eV, interface temperature of 220 $^{\circ}\text{C}$, ion source temperature of 230 $^{\circ}\text{C}$, quadrupole rod temperature of 150 $^{\circ}\text{C}$, scan mass range of m/z 40 to 600, and scan frequency of 3.6 scans/s.

Fatty acid composition analysis

The fermented milk sample were mixed with methanol/ CH_2Cl_2 (1:3) for 10 min at room temperature and oil components were ultrasonic extracted after centrifugation procedure (1800 rpm, 10 min). The supernatant was blow-dried with nitrogen and dissolved in methanolic KOH (6%) followed by 4N HCl. After $\text{BF}_3\text{-MeOH}$ treatment, fatty acid compositions were extracted with n-hexane for 3 times and the extraction solution was transferred to 2ml sample bottle, and blow-dried with nitrogen for further analysis.

The fatty acid composition was analyzed using a TG-5MS 30m $\times$ 0.25mm $\times$ 0.25 μm column, the heating procedure as follows: starting temperature at 80 $^{\circ}\text{C}$, maintained for one minute, then the temperature rises to 200 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}/\text{min}$, 225 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}/\text{min}$, and 250 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C}/\text{min}$ for 5min. Flow rate is 1.2 mL/min, with helium as the carrier gas. Scanning range 30-400, injection volume 1 μL . The content was calculated by external standard method and internal standard method, and all the samples were done in triplicate.

Amino acid analysis

The homogenized fermented milk samples were hydrolyzed with 6 N HCl solution in a drying oven at 110 ° C for 22 hours. The next day, the hydrolyzed sample was filtered, dried and dissolved with 1 ml citric acid buffer solution (pH 2.2). Finally, the amino acid profiles of the different samples were determined by L-8900 amino acid automatic analyzer (Physics and Chemistry Test Center, Jiangsu Province, China).

Sensory profiling evaluation

The Alpha Astree II potentiometric electronic tongue system (Isenso, New York, NY) with an Ag/AgCl electrode was adopted for the sensory profiling analysis. The prepared fermented milk samples were homogenized and poured into an electronic tongue special cup and data were collected at room temperature. For all the samples, five replicates were carried out and three stable data were collected for principal components analysis (PCA) and discriminant factor analysis.

β -galactosidase and lactate dehydrogenase analysis

Cells were suspended in PBS buffer and lysozyme was added and bathed at 37 ° C for 1 h. After that, the crude enzyme solution was dissolved in 0.4 M NaCl solution before the enzyme activity analysis. The activity of β -galactosidase and lactate dehydrogenase under symbiotic culture conditions were assayed according to the assay kit and the method of Vasiljevic [21].

Data Analysis

All statistical analyses were performed using SPSS (SPSS Inc./IBM Corp., Chicago, IL) and the plots were drawn with Origin 8.5 (Origin Lab, Northampton, MA) software. The differences between the mean values of the groups were analyzed using a one-way ANOVA with Duncan's multiple range test and P-values < 0.05 were considered statistically significant.

Results

Symbiotic growth of *L. plantarum*

The growth characteristics of the complex strains (*L. plantarum*, *L. bulgaricus* and *S. thermophilus*) in the 24 hours culture time were obtained, which is shown in Figure 1. When the proportion of *L. plantarum* increasing in the culture media, the cell density increases significantly with the culture time, and the pH also found sharp decrease with the time. It demonstrates that *L. plantarum* can promote the increase of cell density and the acid production capacity of the culture strains under symbiotic condition.

Texture changes of the yoghurt with different starters

As shown in Figure 2, *L. plantarum* A3 has a good performance on the physical quality of fermented milk. Along with the increased proportion of *L. plantarum* A3, the hardness, consistency, viscosity, and viscosity

coefficient were great changed with the ratio of *L. plantarum* A3. In terms of acidity and water holding capacity, *L. plantarum* A3 also showed a relatively positive effect in the texture of the yoghurt.

GC-MS analysis

Fermented milk contains a complex mixture of volatile organic compounds (VOCs); however, only small parts of the VOCs have the essential effects on the flavor of the yoghurt. In the present study, we used SPME-GC-MS to identify the VOCs present in fermented milk with different starters. It shows that milk fermented with *L. plantarum* A3 was significant different from *L. plantarum* A3 free groups, meanwhile, the volatile flavor components in the *L. plantarum* A3 group were relatively close to each other (Figure 3). As shown in Figure 3B, ethylbenzene, benzene, nonane, 2,5-dihydroxybenzaldehyde and styrene are the main positive volatile flavor components formed in *L. plantarum* A3 fermented group, while the main volatile flavor components of fermented milk without *L. plantarum* A3 are 2,3-pentanedione, silanediol, dimethyl-, 2-pentanone, acetone (Figure 3C).

Amino acid composition analysis

The amino acid content of the fermented milk in different groups was analyzed with the PCA methods. As shown in Figure 4A, *L. plantarum* A3 fermented yoghurt groups are relatively closer, compared with control group. It can be seen that yoghurt with *L. plantarum* A3 strain indeed promote the amino acid components formation during the milk fermentation processing, and Ser, Gly and Thr are the main positive amino acids in both PC1 and PC2 during the *L. plantarum* fermentation procedure (Figure 4B-4D), which play an important role in the nutrition formation of the fermented milk products.

Fatty acid composition analysis

The changes of fatty acid composition in yoghurt fermented by different proportions of *L. plantarum* A3 were analyzed by GC-MS. It can be seen from the PCA analysis in Figure 5A that all the groups have the similar principal components, indicating that the addition of *L. plantarum* A3 has little effect on the composition changes of the fermented milk fatty acid. Meanwhile, the first main components of all the fermented samples are C22:2, C18:3n6, C18:3n3, C11, C8:0. The second principal component contains C17:1, C18:2n6, C17:1, C16:1 (Figure 5B). However, the concentration of these kinds of fatty acid is significant different in the *L. plantarum* A3 fermented groups, and *L. plantarum* can promote the increase of the w-3 fatty acid during the yoghurt formation (Table 1).

Electronic tongue analysis

The results of the electronic tongue analysis (Figure 6) are similar with the data of volatile flavor and amino acid in the yoghurt samples. Results in electronic tongue analysis shows PC1 contributed 47.11% of the total variance and PC2 contributed 18.09%, for a cumulative contribution of 65.2%, which represent most of the information in the whole sample. Samples with linear discriminant analysis (LDA) shows the similar trends with the PCA analysis. The discriminant index for the PCA and LDA are 98.47% and 99.96%, respectively. All the data collected by electronic tongue node indicating that samples with

different starters can be easily identified and the separability between different fermented milk samples was clear and specific.

β -galactosidase and lactate dehydrogenase analysis

The activity of β -galactosidase and lactate dehydrogenase in culture media supernatant with different ratio of *L. plantarum*, *L. bulgaricus* and *S. thermophilus* were shown in Figure 7. The activity of β -galactosidase decreased gradually when the proportion of *L. plantarum* A3 increased, and the activity of lactate dehydrogenase shown similar trend, which the 1:1:1 group had the highest enzyme activity compared with other groups.

Discussion

As a common strain in Sichuan pickle product, *L. plantarum* A3 also reveals great potential in probiotic yogurt in terms of bacterial population, texture and flavor [12]. Meanwhile, probiotic yogurt has many health benefits and can maintain a sufficient amount in the human gastrointestinal tract, such as milk fermented with probiotic strains such as Bifidobacteria and Lactobacilli [13, 14]. Milk fermented with *L. plantarum* A3 in this research revealed a novel texture characteristic in terms of hardness, acidity and water holding capacity when combined with the traditional *L. bulgaricus* and *S. thermophilus* strains.

Most probiotics belong to lactic acid-producing bacteria and exist in forms of yoghurt, fermented pickles or other fermented foods [15]. Probiotics species of Lactobacilli and Bifidobacteria exist in more than 90% of probiotic products and are popular in healthy consumers [16, 17]. In this research, *L. plantarum* has a good lactic acid producing ability, which is essential for the quality of the milk fermentation. In the glucose metabolism pathway, pyruvate is an important intermediate metabolite of glucose, which is dehydrogenated by the action of lactate dehydrogenase to produce lactic acid in lactic acid bacteria. Milk fermented with *L. plantarum* A3 group have a higher lactate dehydrogenase activity than the control group, and as the proportion of *L. plantarum* A3 increased, the enzymatic activity of β -galactosidase was also found enhanced. Lactose in milk can be converted to lactic acid during fermentation, and β -galactosidase is capable of hydrolyzing lactose in the most abundant lactose milk to form lactic acid and its by-products [18, 19]. Since strains under symbiotic culture conditions can provide a lower pH environment which may affect the activity of β -galactosidase in the culture media. While, the activity of lactate dehydrogenase in *L. plantarum* A3 group was higher than the control group, indicating that the reason why acetic acid concentration was higher in *L. plantarum* A3 groups. Therefore, the strains ratio of 1:1:2 is better for the symbiotic milk fermentation in this research.

In general, probiotic strains are screened based on their safety, nutritional value and health-promoting properties, as well as the fermentation properties that may affect the texture and appearance of the probiotic yogurt [20]. Yoghurt fermentation with *L. plantarum* A3 indeed has a good quality and nutrition properties compared with the traditional strains. There was a significant difference between the *L. plantarum* A3 and the *L. plantarum* A3-free yoghurt on the content of free amino acids depends on the PCA analysis, meanwhile, the main free amino acid components of the B1, B2 and B3 groups, probably

due to the proportion of *L. plantarum* A3 in the yoghurt. Some studies have shown that the addition of *Lactobacillus* and *Bifidobacteria* can increase the concentrations of free amino acids (FAAs) in all probiotic cheeses [11, 22]. In terms of volatile flavor, there was a big difference in the main flavors between the B group and the A group in the PCA principal component analysis, which indicated that the addition of *L. plantarum* A3 can enhanced the flavor of the yoghurt. A very important factor affecting taste is the large number of volatile compounds that are present in the fermented yoghurt products from degradation of casein and side chain modifications. These reactions can produce keto acids, ammonia, amines, aldehydes, acids and alcohols, which are important contributors to the flavor profile of yogurt [23].

L. plantarum A3 doesn't show a significant difference with the *L. plantarum* free starter, however, it can be seen that unsaturated fatty acids account for a large proportion of yoghurt, wherein the first main component and the second main component mainly contain unsaturated fatty acids. In this point, yoghurt products are a good resource of the unsaturated fatty acid. According to the recent study, several non-nutritional components such as sphingolipids in yoghurt, conjugated linoleic acid (CLA) and butyric acid can act as anticancer agents [24]. Some studies have shown that fermented dairy products contain higher levels of CLA than non-fermented milk [25]. We also found that the with *L. plantarum* A3 can enhance the level of omega-3 fatty acids profile in the fermented milk, newly discovery revealed that the fatty acid composition involved in the inflammatory response of the cell and n-3 PUFAs have the potential anti-inflammatory effects which may be useful as therapeutic agents in disorders with an inflammatory component [26]. This maybe also the reason why yoghurt consumption can modulate the immune system and prevent the inflammatory process in a mouse model [27].

Conclusions

As probiotics and pre-biological industries thrive, people are more likely to pursuit food with the high quality and nutrition requirements. Nowadays, probiotics and prebiotics play an undeniable role in improving the function of milk products, enhancing sensory properties and extending shelf life in a combination of symbiotic yoghurt. *L. plantarum* A3, a novel vegetable original strain in the lactic acid fermentation, showed its special milk fermentation and probiotic characteristics, which can enhance the texture and flavor of the yoghurt, improve the nutrition value through increasing the contents of amino acids, volatile compounds and free fatty acids. Moreover, the relationship between the traditional fermentation strains and probiotics still need further investigation to meet consumer expectations of quality and nutrition requirements.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (31601487), the Natural Science Foundation of Zhejiang Province (LY19C200005), the Research Foundation (Science & Engineering) of Ningbo University (XYL19015) and the K. C. Wong Magna Fund in Ningbo University.

Authors' contributions

FXL and JZW analyzed and interpreted the GC-MS and amino acid profiles data regarding the texture of the fermented yoghurt. ZDC, DDP and XQZ performed the Sensory profiling evaluation of the yoghurt products. YXG and XTL analyzed the β -galactosidase and lactate dehydrogenase activity of the fermented lactobacillus, ZW and FXL performed the fatty acid analysis, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

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Tables

Table 1. Differences of the partial unsaturated n-3 or n-6 fatty acids in different fermented milk samples (mg/kg)

Fatty acid	Groups				
	control	1:1	1:1:1	1:1:1	1:1:1
C18:2n6c	818.49	977.62	1006.24	894.29	853.06
C18:3n3	74.59	82.24	87.9	80.87	80.36
C18:3n6	7.79	9.34	10.66	9.06	8.86
C20:3n3	0.92	0.92	1.12	1.01	1
C20:3n6	42.44	43.17	47.44	42.14	46.01
C20:4n6	53.68	49.4	56.34	52.07	57.39
C20:5n3	6.12	6.25	7.67	6.73	7.91

Figures

Figure 2

Fermentation growth kinetics of strains in symbiotic culture conditions with *L. plantarum* A3. OD values (A) and pH changes (B) of the strains in different inoculation ratios during the 24 hours culture time.

Figure 4

Texture, water holding capacity and acidity evaluation of the yoghurt fermented with different strains. The ratio of *L. bulgaricus*: *S. thermophilus*: *L. plantarum* A3 were 1:1:0, 1:1:1, 1:1:2, 1:1:3, respectively.

Figure 6

. Volatile flavor profiles of the fermented milk with different proportion of *L. plantarum* A3. wherein A1, A2 and A3 belong to the 1:1:0 group; B1-1, B1-2 and B1-3 belong to the 1:1:1 group; B2-1, B2-2, and B2-3 belong to the 1:1:2 group; B3-1, B3-2, and B3-3 belong to the 1:1:3 group. (A) PCA analysis of volatile flavors between different groups; (B) PCA analysis of the volatile flavors with different ratio of *L. plantarum* A3; (C) PCA analysis of the fermented milk without *L. plantarum* A3 starters, (D) Volatile flavor components present in the Figure 3A-3C.

Figure 8

The changes of free amino acids in different fermented milk groups. (A) PCA analysis of the amino acids in the different fermented milk group. (B) Amino acid composition in the 1:1:1 group with PCA analysis; (C) Amino acid composition in the 1:1:2 group; (D) Amino acid composition in the 1:1:3 group.

Figure 10

PCA analysis of the changes of the fatty acids in different samples. (A) Analysis of fatty acid PCA in different groups, where control is unfermented milk, A is 1:1:0 group, B group is 1:1:1 group, C is 1:1:1 group, D is 1: 1:3 group. (B) Total fatty acid composition with the PCA analysis.

Figure 11

Electronic tongue analysis of the fermented milk with and without *L. plantarum* A3 strain. Principal component analysis (A) and linear discriminant analysis (B) of the main taste compounds in the control (unfermented) and fermented milk groups (1:1:0, 1:1:1, 1:1:2, 1: 1:3). DI means discriminant index.

Figure 13

The activities of β -galactosidase (A) and lactate dehydrogenase (B) under co-culture conditions.