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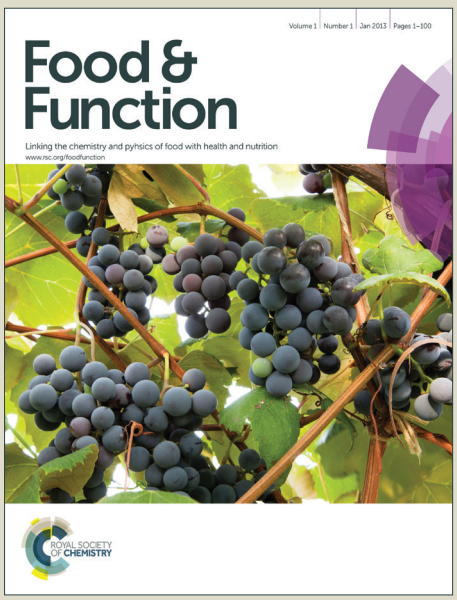

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Microbial diversity and flavor formation in onion fermentation

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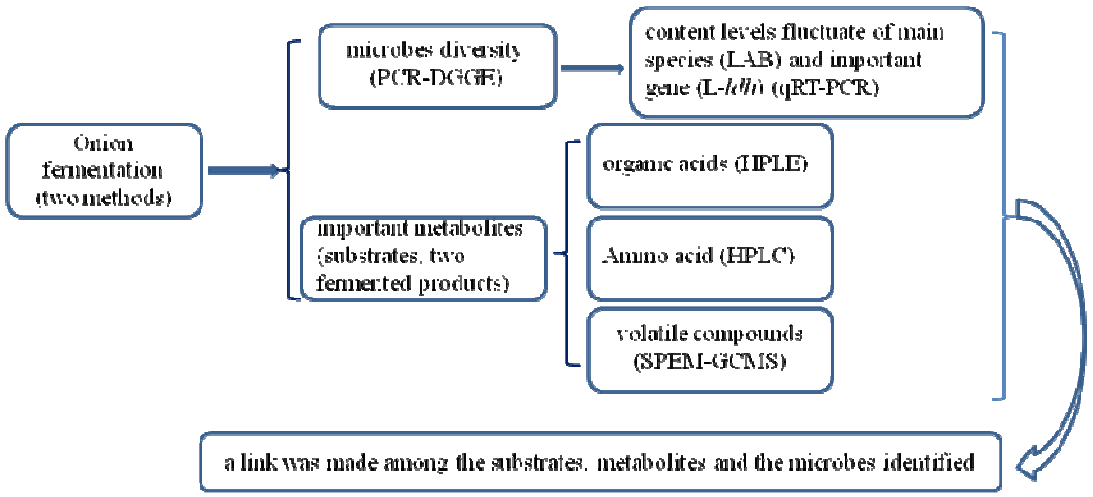
Content



The fermentation of onions is actually not well. We focused on the analysis of microbial diversity and flavor formation in onion fermentation with indigenous flora (without starters).In this work, two different methods were used to prove the relationship between the microbes and the production of flavor. A link was made among the substrates, metabolites and the microbes identified. It would be a great benefit for the further modification of the fermentation process to produce onion juice with more attractive flavors and tastes.

Abstract

Fermented onion products are popular in many countries. We conducted fermentation with and without salt to identify the microorganisms involved in the fermentation and unique taste of onion. The results of PCR-DGGE revealed that lactic acid bacteria (*Lactobacillus zymae*, *L. malefermentans*, *L. plantarum*), acetic acid bacteria (*Acetobacter pasteurianus*,*A. orientalis*), citric acid bacteria (*Citrobacter* sp.,*C. freundii*), and yeasts (*Candida humilis*, *Kazachstania exiqua*, *Saccharomyces bulderi*) were the dominant microorganisms involved in onion fermentation. Organic acid analysis indicated that lactic acid and acetic acid increased significantly after the fermentation. After fermentation, the type of amino acids had no significant changes, but the total concentration of amino acids significantly decreased after the fermentation with salt. The increase in esters, alcohols, and aldehydes after the fermentation was responsible for the unique flavor of fermented onion. The fermentation with salt inhibited the accumulation of organic acids and limited the conversion of proteins into amino acids but maintained the unique odor of onion by limiting the degradation of sulfur-containing compounds.

Keywords: Onion fermentation; microbial community; flavor; PCR-DGGE; HPLC; HS-SPME-GC-MS

1. Introduction

Onion (*Allium cepa L.*), easily recognized in vegetables for its flavor and odor, is a commonly used food ingredient in cooking. Onion also has several biological activities, including antithromotic, antiplatelet, and antiasthmatic[1, 2, 3, 4].Onion contains many flavonoids, which can significantly prevent cancer, heart disease, and ageing[5]. Alkyl sulfides in onion also prevent the initiation of carcinogenesis[6]. Even the by-products of onion show a rich content of dietary fiber. Thus, regulatory authorities and consumer groups have developed alternative means to convert onion waste into food-grade products [7].

Onion is usually consumed in three different ways: bulbs for the fresh market, dehydrated onions for food processing, and green salad onions for fresh consumption[5]. But now, people are growing more interesting in the onions that have low pungency. Fermentation is known to contribute unique flavor to vegetables[8-10]. Fermentation may be the best choice, for it can both decrease the pungency of the onion and increase its sweetness. Roberts and Kidd [11] produced sour onion by using either brine from sauerkraut or slices of cabbage to ferment onions. Fermentation of onion by-products also produces ethanol and vinegar [12, 13].

Microorganisms are mainly responsible for fermentation. During vegetable fermentation, lactic acid bacteria (LAB) are the major microflora (mainly *lactobacilli* and *pediococci*) and are a part of starter cultures that produce desirable acid and flavor compounds [14, 15]. The abilities of LAB to acidify raw material rapidly by producing organic acids and to produce aromatic compounds, bacteriocin, and several enzymes are important in fermentation [8]. Yeasts and acetic acid bacteria are also usually present during the fermentation process [16, 17].

Understanding and controlling the fermentation process are necessary to enhance the quality of fermented food. In this study, we used two fermentation processes (fermentation without salt and fermentation with salt) to enhance the flavor of fermented onion. The effects of these two fermentation processes were compared. The diversity of microbes involved in onion juice fermentation was investigated by the culture-independent method denaturing gradient gel electrophoresis (DGGE), the population changes in LAB were quantified by quantitative reverse transcription polymerase chain

reaction (qRT-PCR), important metabolites (e.g., organic acids and amino acids) were analyzed by high-performance liquid chromatography (HPLC), and volatile compounds were analyzed by headspace (HS)–solid phase microextraction (SPME)–gas chromatography mass spectrometry (GCMS). In this way, a link was made among the substrates, metabolites and the microbes identified. It would be a great benefit for the further modification of the fermentation process to produce onion juice with more attractive flavors and tastes.

2. Materials and methods

2.1. Sampling

Fermented onion samples were collected from a factory in Jiangmen City, Guangdong Province, in April 2013. It was usually fermented spontaneously, and based on an empirical process. In order to see whether salt could influence the flavor of final product, onion fermentation was conducted in two ways in this work. And each had three independent fermentations. Approximately 250 kg onions were trimmed of their outer leaves, washed, cut into small pieces (approximately 0.5×0.5 cm), and then placed in fermented pools (3270 mm×1385 mm×740 mm) motionless without salt for96 hours or with 1% (w/w) salt for108 hours. During the fermentation processes, temperature and pH were measured by on-line temperature and pH meters (Sartorius PB-10), respectively. The samples were collected in sterile bottles from the pools at the same depth each time. The samples were stored at −20 °C and then transported to a laboratory for further study. For qRT-PCR analysis, RNA was isolated as soon as the samples were collected.

2.2. DNA and RNA extraction

Total DNA and RNA were extracted directly from the fermented onion juice. Microbial cells were collected by centrifugation at 12,000 ×g for 2 min. The cell pellet was washed with TENP (20 mM EDTA, 50 mM Tris, 1% PVP, 100 mM NaCl, pH 10.0) and phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). DNA and RNA were extracted and purified using a Soil DNA kit (OMEGA, American) and an RNAiso^{TM} Plus (TaKaRa, Japan) kit according to the manufacturers' instructions, respectively. The DNA and RNA concentration and

quality were assessed with a UV-vis spectrophotometer (NanoDrop ND-2000, USA). Meanwhile, RNA degradation and contamination was monitored on 1.2% agarose gels. The genomic DNA of the isolates was stored at −20 °C for further analysis, whereas the RNA was used immediately in the following steps.

2.3. Nested PCR –DGGE analysis

Fragments of rDNA were amplified by the two-step nested PCR protocol described by Marzia[18]. First, nearly complete 16S and 18S rDNA were amplified with primer sets 27F/1492R and NS1/FR1, respectively (Table 1). Second, the PCR product of the first step was used as the template, the V3 region of the 16S rDNA was amplified by the primer pair GC-338F/518R, and the region of fungal 18S rDNA was amplified by the primer pair GC-NS3/YM951r (Table 1).PCR amplification was performed in 50 μL reaction mixtures containing 25 μL of *Premix* ExTaq (Takara, China), 2 μL of each primer (20 μM) and 3 μL of template DNA. Touch-down PCR program was carried out with an initial denaturation step of 98 °Cfor 5 min, followed by 20 cycles of denaturation at 98 °C for 45 s, annealing temperature starting at 65 °C for 45 s and decreasing by 0.5°C/cycle, and 72°C for 1 min for extension. This step was followed by 15 cycles of 98 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min before holding at 16 °C. The PCR samples were subjected to DGGE analysis on an 8% polyacrylamide gel with a denaturing gradient of 20% to 60% urea and formamide for bacteria and 20% to 40% for fungi. Electrophoresis was carried out in 1× TAE (Tris–Acetate–EDTA) buffer at 60 °C and at a constant voltage of 160 V for 4.5 h with a Bio-Rad DCode system. Subsequently, the gels were subjected to silver staining. Bands from the gels were excised using a sterile blade. The slices were washed by ddH_2O and then incubated overnight at 4 °C in TE buffer to make the DNA run out of the gel. The eluted DNA was reamplified with the GC-clamp primers described above and was run on another DGGE gel with a narrower gradient range to confirm their identity. A denaturing gradient of 20% to 35% urea and formamide was used for bands 1, 2, 3, 4, 5, 6, 7, 35% to 50% urea and formamide was used for bands 8, 9, 10, 11, 15, 35% to 50% urea and formamide was used for bands 12, 13, 14, 20% to 30% for bands 1', 2', and 30% to 40% for 3', 4', 5', 6'. The eluted DNA was amplified with the same primer pairs without the GC clamp, and the products were purified with a gel extraction kit (OMEG,

American). The purified fragments were inserted into the pMD18-T vector (TaKaRa, Japan) and sequenced by Invitrogen (Shanghai, China). Finally, BLAST tool was used to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

2.4. cDNA synthesis and qPCR assays

qRT-PCR was performed to evaluate the quantity of *Lactobacillus plantarum* and the expression levels of the L-lactate dehydrogenase gene (*ldhL*) during the fermentation. Approximately 1μg of RNA was taken for cDNA was synthesis with a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). The remaining genomic DNA contamination was eliminated, and reverse transcriptase reactions were carried out according to the manufacturer's instructions.

qRT-PCR amplification was performed in 96-well plates on an ABI Prism 7500 sequence detection system by using the double-stranded DNA intercalating fluorescent agent SYBR green for product detection. Each well contained 10 μL of 1×SYBR Green Master Mix (Applied Biosystems), 200 nM of each primer, and 2 μL of cDNA template. Primer pairs lac1/lac2 and ldh1/ldh2 were used to amplify a 16S rRNA fragment of *L. plantarum* and an *ldh* gene fragment (Table 1), respectively. PCR amplification was initiated at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. All sample and primer combinations were assessed in triplicate.

The generation of quantitative data by qRT-PCR was based on the number of cycles required for amplification-associated fluorescence to reach the detection threshold (C_T). For each reaction, C_T values were obtained. The relative expression level of the *ldhL* gene during onion fermentation was calculated using the comparative $2^{-\Delta\Delta CT}$ method [19], with the 16S rRNA of *L. plantarum* as an internal control. The PCR amplification products were also purified and sequenced.

2.5. Organic Acid Analyses

Approximately 2 L of the samples was homogenized and centrifuged at 12,000 ×g for 10 min to eliminate onion particles and microorganisms in order to determine the organic acids in the supernatant. The supernatant was diluted with double distilled water 10 times, filtered through a polyamide membrane filter with a pore size of 0.22 μm, and then

stored at 4 °C until use. All samples were prepared in triplicate of 3 independent fermentations. Subsequently, the solutions were determined with a Waters 1525 series liquid chromatograph machine equipped with a UV-visible diode array detector and a Waters RP-C_{18} column (250 mm × 4.6, i.d., 5 μm). The conditions for HPLC analysis were as follows: column temperature, 25 °C; injection volume, 10 μL; and detection wavelength, 210 nm. The flow rate for each gradient elution was as follows: 0.5 mL/min for 0 min to 10 min and 1.0 mL/min for 10 min to 20 min with a mobile phase containing 0.5% acetonitrile and 99.5% of 0.1% H_3PO_4 (pH adjusted to 2.20 with 0.02 M KH_2PO_4).

2.6. Amino Acid Analysis

Free amino acids were determined by precolumn derivatization with phenylisothiocyanate and reversed-phase HPLC. The samples and standards were prepared as previously described[20, 21]. Prior to derivation, both the standard (SIGMA) and samples were added with L-norleucine as an internal standard and dried in a frozen dry machine. They were neutralized by adding a 2:2:1 mixture of methanol:water:TEA (v/v), mixed well with a vortex stirrer, and then redried by a frozen dry machine. Derivatization was performed by adding a derivatizing reagent (methanol:water:TEA:PITC=7:1:1:1) to the samples and standards. The samples were incubated at room temperature for 20 min to ensure that their reaction with PITC produced phenylthiocarbamyl amino acids. Finally, the samples were completely dried in a frozen dry machine and then stored at −20 °C until analysis. All samples were prepared in triplicate of 3 independent fermentations. Prior to analysis, the dried samples were dissolved in 500 μL (250 μL for salt fermented samples) of Buffer A (20 mM CH_3COONa, pH=7.2, 0.5% THF), vortexed, filtered through a 0.22 μm membrane, and then analyzed immediately.

The liquid chromatograph used was the same as that above, and derivatization residues were separated by a gradient resulting from mixing eluents A (20 mM CH_3COONa, pH=7.2, 0.5% THF) and B (20 mM CH_3COONa, pH=7.2: CH_3OH: ACN=1:2:2). The gradient conditions were 5% to 48% for 39 min, 100% B for 40 min to 45 min, and 5% B for 46 min. The flow rate was 1 mL/min, the column temperature was 38 °C, and PIT-AA was detected at 254 nm.

2.7. Analysis of Volatile Compounds

Volatile compounds were extracted by HS–SPME[22]. Approximately 7 mL of the fermented onion juice was placed in a 20 mL HS vial and pre-equilibrated at 50 °C for 15 min. Then, 65 and 75 μm PDMS fibers were housed on the handle, pushed into the vial septum, and then subjected at 50 °C for 30 min. Volatile compounds were concentrated on the fibers. The SPME device was directly inserted into the injection port of GC-MS.

Chromatographic identification was carried out by an Agilent 7980A (Agilent Technologies, Palo Alto, CA, USA) gas chromatographer equipped with a 5975C mass selective detector. The sample was separately analyzed on an Agilent HP-5MS column (30m × 0.25 mm i.d., 0.25 μm film thickness). The column carrier gas was pure helium with a constant flow rate of 1 mL/min. The detector and injector were held at 280 and 250 °C. Oven temperature was held at 35 °C for 2 min, raised to 120 °C at 5 °C/min and held for 5 min, increased to 150 °C at 3 °C/min and held for another 2 min, and finally raised to 220 °C at 10 °C/min and held for 5 min. A splitless injection mode was used. The mass spectrometer was operated in electron impact mode at 70 eV scanning in the range of 34 m/z to 348 m/z, and the ion source temperature was set to 230 °C. Repeatability was evaluated by analyzing the samples in triplicate.

2.7 GenBank accession numbers

The sequences reported in this paper have been deposited in the GenBank database under the following accession numbers: KF684038 to KF684052 for bacterial DGGE bands, KF684053 to KF684058 for fungal DGGE bands.

3. Results

3.1. pH and temperature changes in the onion fermentation

The initial pH during the fermentation without salt was approximately 4.5, increased to 5.3 on the first 24 hours, and then kept decreasing to approximately 3.4 in the following fermentation process. The complex environment and the microbial ecology may be the he reason for the increase in pH initially. Trend of pH during the fermentation after a day with salt was accordant with that during the fermentation wthout salta day later (Fig. 1A).The temperatures in both fermentation processes kept increasing from 23.0 °C to 28.5 °C, and the ambient temperature was slightly higher than

169 that of the sample (Fig. 1B).

170 **3.2. Microbial diversity in onion fermentation**

171 The DGGE profiles suggested that the bacterial and fungal communities remained relatively stable during onion
172 fermentation with and without salt (Fig. 2). In the bacterial community, bands 1, 2, 6, 9, 10, and 11 were present in all
173 samples. Their abundance was low at the beginning of the fermentation and increased toward the end. Bands 3, 4, 13,
174 and 14 were also present throughout the fermentation, but they became lighter toward the end. Band 12 disappeared
175 toward the end of fermentation(Fig. 2A). Band 15 visibly increased from 60 h during the fermentation without salt but
176 became lighter during the fermentation with salt (Fig. 2A).In the fungal community, band 1′ was present throughout the
177 fermentation process. Bands 2′, 3′, and 4′ not present at the beginning were detected at the end of the fermentation.
178 Bands 5′ and 6′ were present throughout the fermentation process without salt but were absent at the end of the
179 fermentation with salt (Fig. 2B).

180 Sequencing analysis suggested that LAB (*Lactobacillus zymae*, *Lactobacillus malefermentans*, and *Lactobacillus*
181 *plantarum*), acetic acid bacteria (AAB, *Acetobacter pasteurianus*, *Acetobacter orientalis*), *Citrobacter* sp. (*C. freundii*),
182 and fungi (*Candida humilis*, *Kazachstania exiqua*, and *Saccharomyces bulderi*) were the predominant microrganisms
183 during fermentation (Table 2). The density of *L. malefermentans*, *L. plantarum*, *A. pasteurianus*, *A. orientalis*, *K. exiqua*,
184 and *S. bulderi* increased toward the end of the fermentation, suggesting that these bacteria significantly influenced
185 fermentation. *Myroides odoratus* was detected at the beginning of the two fermentation processes and disappeared from
186 48 h (fermentation with salt) and 72 h (fermentation without salt) because the biological activity of LAB to produce a
187 range of metabolites can suppress the growth and survival of undesirable microflora in foodstuff [23]. Fungal DNA bands
188 5′ and 6′ closely related to *Allium cepa* were detected in the fungal community by mismatch.

189 **3.3. qRT-PCR analysis**

190 LAB dominates the fermentation of many vegetable. *L. plantarum* was the predominant LAB in vegetable fermentation,
191 and it was also reported to play a dominant role in the fermentation of many varieties of vegetables (xiong T et al, 2012,

Oguntoyinbo FA and Dodd CE, 2010). The PCR-DGGE analysis showed that *L. plantarum* contributed most to the onion fermentation. Enumerating the content levels fluctuate of this species during the fermentation was of great interest for evaluation of the importance of this bacterium in onion fermentation.

Normalizing qRT-PCR data based on the volume of the fermented onion juice indicated that the population of *L. plantarum* slowly increased at the beginning (hours 0 to 48) of the fermentation without salt. In the following 72 hours, this population increased and reached its peak on 84 h. Subsequently, the population declined (Fig. 3A). However, the rate of increase in *L. plantarum* population was faster during the fermentation with salt than during the spontaneous fermentation. However, this rate rapidly decreased on 48 h to 72 h of the fermentation and then increased again at the end of the process (Fig. 3A).

The expression of *ldhL* was down-regulated throughout the fermentation process. During the fermentation without salt, the expression of the *ldhL* gene declined to its lowest level on 60 h until the end. During the fermentation with salt, the expression of the *ldhL* gene decreased from 0 h to 48 h and then increased in the following day. The expression then declined to its lowest level on 96 h.

3.4. Organic acid analysis

Representative chromatograms from fermented onion juice samples were presented in Fig. S1, and the results are summarized in Table 3. Six organic acids were detected in the samples. Oxalic acid, formic acid, citric acid, and succinic acid were the major organic acids in initial fermentational materials. After the fermentation, formic acid disappeared. The concentrations of lactic acid were 12.40±0.12 and 5.90±0.40 g/L, and those of acetic acid were 4.30±0.20 and 1.30±0.04 g/L in the fermentation without and with salt, respectively. These acids were not detected in the initial materials. In the fermentation without salt, the concentrations of oxalic acid and citric acid were almost unchanged after the fermentation, but these concentrations were lower than those in the initial materials in the fermentation with salt. The total concentration of organic acids in the salt-fermented onion juice (26.70±1.21 g/L) was similar to that in the initial materials (26.70±1.66 g/L) but lower than that in the samples fermented without salt

(52.60±0.36 g/L) (Table 3).

3.5. Analysis of free amino acids

Seventeen types of free amino acids (except Pro) were detected in the initial and fermented onion samples (Fig. S2, Table 4). Compared with the 9.43±0.85g/L of free amino acids in the initial materials, the total amount of free amino acids increased to 11.92±0.85 g/L in the samples fermented without salt, and the total amount of amino acids decreased to 2.07±0.12 g/L in the samples fermented with salt (Table 4). After fermentation, Ile and Leu disappeared in the samples fermented without salt, and four free amino acids (His, Met, Cys, and Phe) disappeared in the samples fermented with salt (Table 4). All free amino acids in the samples fermented with salt had lower concentrations than those in the samples fermented without salt. The concentrations of Asp, Glu, Gly, His, Thr, Cys, Phe, and Trp were higher than those in the initial materials, and the concentrations of Ser, Arg, Ala, Tyr, and Lys were lower than those in the initial materials in the samples fermented without salt (Table 4).

3.6. Analysis of volatile compounds

Sixty-eight volatile compounds, including esters (17), alcohols (6), algehydes (5), ketones (5), sulfur-containing compounds (7), heterocyclic compounds (17), alkanes (3), phenols (2), benzenes (3), and others (3), were applied to analyze the flavor of fermented onion. In addition, 24, 30, and 39 volatile compounds were detected in the initial fermentation material, in the samples fermented without salt, and in the samples fermented with salt, respectively (Table 5). The result of HS–SPME–GCMS analysis showed that the unique flavor of fresh onion is mainly produced by the following compounds: esters (8.10%), alcohols (7.07%), algehydes (0.86%), ketones (0.17%), sulfur-containing compounds (24.00%), alkanes (0.47%), heterocyclic compounds (48.35%), phenol (7.93%), and others (3.06%) (Table 5). After the fermentation process, the major compounds in the samples fermented without salt were heterocyclic compounds (58.30%), alcohols (19.10%), and esters (17.14%), whereas those in the samples fermented with salt were heterocyclic compounds (37.69%), sulfur-containing compounds (17.51%), alcohols (16.18%), esters (13.78%), and oxime-, methoxy-phenyl- (11.75%) (Table5). Compared with that of the initial fermentation materials, the concentration

238 of esters, alcohols, ketones, and aldehydes significantly increased, whereas that of phenolic compounds considerably
239 decreased in both fermentation processes. The sulfur-containing compounds in the samples fermented without salt
240 decreased from 24.00% to 1.85%, and oxime-, methoxy-phenyl- disappeared in the samples fermented without salt and
241 increased from 0.63% to 11.75% in the samples fermented with salt (Table 5).
242

243 ## 4. Discussion

244 Onion juice as a fermented food (usually fermented with or without salt and microorganisms) is mainly responsible for
245 the unique flavor of onion by converting proteins, polysaccharides, and lipids into organic acids, free amino acids, and
246 volatile compounds [24, 25].
247 In this study, LAB (*L. zymae*, *L. malefermentans*, *L. plantarum*, and uncultured *Lactobacillaceae* bacterium), AAB (*A.*
248 *pasteurianus* and *A. orientalis*), *Citrobacter* sp. (*C. freundii*), and yeast species (*C. humilis*, *K. exiqua*, *S. bulderi*) were
249 identified as the predominant microorganisms in the fermentation process (Table 2). This finding suggested that these
250 bacteria and fungi contributed to the unique flavor of onion, just like in other naturally fermented vegetables that
251 produce lactic acid, acetic acid, and other compounds in the fermentation process[14, 23, 26, 27]. These results were
252 supported by the production of acetic acid and lactic acid (Table 3), and the conversion of many volatile flavor
253 compounds (Table 5) in both fermentation processes. LAB are crucial in the spontaneous fermentation of vegetables,
254 milk, and meat, and are responsible for the production of lactic acid [14, 28]. L-lactate dehydrogenase significantly
255 influences the formation of lactic acid. The results of qRT-PCR demonstrated that the LAB *ldhL* gene was kept at high
256 regulation levels, suggesting the activity of lactate dehydrogenase (Fig. 3) and confirming the major effect of LAB in
257 onion fermentation. Yeasts can produce aromatic compounds, antioxidants, and enzymes, as well as improve the growth
258 of LAB, during fermentation [16, 29]. AAB produce acetic acid during growth and are generally isolated from fermented
259 food, fruits, and flowers[30, 31]. During fermentation, LAB produce lactic acid, acetic acid, and alcohol from sugars, yeasts
260 produce ethanol from sugars, and AAB convert ethanol into acetic acid[17]. This study is the first to detect *Citrobacter* sp.

in fermented vegetables. Lactic acid and acetic acid were found to be the main products of carbohydrate catabolism by LAB and AAB, respectively. Lactic acid and acetic acid can inhibit the undesirable growth of pathogens and other microorganisms because of their acidity [32]. Thus, the microbial community is dominated mainly by lactic acid- and acetic acid-producing bacteria and acid-tolerant yeasts (Table 2).

Amino acids and volatile compounds are mainly responsible for the unique flavor of fermented onion. Amino acids act as important precursors for volatile flavor compounds or as free amino acids that contribute flavor[33].Although the type of amino acids had little difference between the initial and fermented samples, the concentration of each amino acid had significant changes, especially in the samples fermented with salt, in which the total concentration of amino acids was only 2.07±0.12 g/L compared with the 9.43±0.851g/L of the initial materials (Table 4). These cases can be attributed to the following reasons. First, salt inhibited the protease activity of some microorganisms, and the initial amino acids were consumed for their growth. Second, the amino acids were used as precursors to produce volatile flavor compounds. The increase in amino acid content fermentation without salt is mainly because that the LAB has a proteolytic system that allows degradation of proteins to increase the amino acids levels.

The special odor of onion is mainly due to sulfur-containing compounds that involve dimethyl trisulfide, propenyl propyl disulfide, dispropyl disulfide, propenyl methyl disulfide, methyl propyl trisulfide, and dipropyl trisulfide [5], which account for 24.00% of all volatile compounds in fresh onion (Table 5). After fermentation without salt, the concentration of sulfur-containing compounds decreased to 1.85%, causing the disappearance of the unique onion odor. However, the sulfur-containing compounds remained at 17.51% in the samples fermented with salt. These results suggested that salt can maintain the unique odor of onion. The amount of esters significantly increased in both fermented onion juice and unfermented onion. Only two types of esters were found in raw onion; however, 10 and 12 types of esters were found in the samples fermented without and with salt, respectively. Most ester compounds have sweet and fruity aromas [34]. The increase in esters makes fermented onion more enjoyable. Compounds involving esters, alcohols, ketones, phenols, and aldehydes principally contribute to the flavor of onion juice [35]. The increase in these

compounds by fermentation makes fermented onion favorable.

5. Conclusion

The quality of fermented onion depends on the microbial community and abundance of LAB and AAB groups. The effect of fermentation parameters, such as pH, temperature, and technological performance, is responsible for the activity of microorganisms and is important for the quality of fermented onion juice. After comparing onion fermentation without and with salt, we found that salt inhibits the accumulation of organic acids (Table 3) and limits the conversion of protein into amino acids (Table 4) but maintains the unique onion odor by limiting the degradation of sulfur-containing compounds (Table 5). The maintenance of LAB, AAB, *Citrobacter*, and yeast communities is important to form the unique flavor in fermented onion, and the timely addition of salt during fermentation facilitates the production of high-quality fermented onion.

6. Acknowledgements

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7. Conflict of interest

The authors declare no competing financial interest.

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Tables

Table 1 PCR primers used in this study.

Primers	Sequence (5'→3')	Reference
27F	AGAGTTTGATCCTGGCTCAG	[36]
1492R	GGCTACCTTGTTACGACTT	
GC-338F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG	[37]
	CACGGGGGGACTCCTACGGGAGGCAGCAG	
518R	ATTACCGCGGCTGCTGG	
NS1	GTAGTCATATGCTTGTCTC	[38]
FR1	AICCATTCAATCGGTAIT	
GC-NS3	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC	[49]
	GGGGGGGC AAGTCTGGTGCCAGCAGCC	
YM951r	TTGGCAAATGCTTTCGC	
lac1	AGCAGTAGGGAATCTTCCA	[40]
lac2	ATTYCACCGCTACACATC	
ldh1	TGATCCTCGTTCCGTTGATG	[41]
ldh2	CCGATGGTTGCAGTTGAGTAAG	

Table 2 Identification of DGGE bands

Band no.[a]	Closest related (accession no.)	Identity [b] (%)
1	*Cronobacter sakazakii* (JQ246831.1)	93
2	*Citrobacter* sp. (KF019680.1)	100
3	*Citrobacter* sp. (KF019680.1)	98
4	Uncultured bacteria (DQ816596.1)	97
5	*Lactobacillus zymae*(KC625331.1）	100
6	*Lactobacillus malefermentans* (AB680994.1)	96
7	*Acinetobacter ursingii* (KC178577.1)	100
8	Uncultured *lactobacillaceae* bacterium (JQ809314.1)	98
9	*Acetobacter pasteurianus*(NR_102925.1)	100

10	*Lactobacillus plantarum*(CP00033.1)	100
11	*Acetobacter orientalis*(JQ080257.1)	100
12	*Myroides odoratus* (JQ407801.1)	100
13	Uncultured *acetobacteria* (AB797138.1)	100
14	*Citrobacter* sp. (KF019680.1)	99
15	*Citrobacter freundii*(KF418613.1)	100
1′	*Candida humilis* (AY046243.1)	99
2′	*Kazachstania exiqua*(AB628064.1)	99
3′	*Saccharomyces bulderi* (AY046241.1)	99
4′	*Candida humilis* (AY046243.1)	99
5′	*Allium cepa* (JQ283941.1)	90
6′	*Allium cepa* (JQ283941.1)	100

[a] Bands were numbered according to Fig. 2.

[b] Identity represents the % identity shared with the sequences in the GenBank databases.

Table 3 Concentration of organic acids in fermented onion juice samples.

Organic acid	Concentration [a] (g/L)		
	Initial fermentation	Fermentation without salt	Fermentation with salt
Oxalic acid	0.60±0.01	0.60±0.02	0.30±0.02
Formic acid	4.20±0.10	–	–
Lactic acid	–	12.40±0.12	5.90±0.40
Acetic acid	–	4.30±0.20	1.30±0.04

Citric acid	1.20±0.10	1.60±0.05	0.70±0.02
Succinic acid	20.70±1.10	33.70±0.70	18.50±1.20
Total acid	26.70±1.21	52.60±0.36	26.70±1.66

[a] Samples were obtained at 96 h in fermentation without salt and 108 h in fermentation with salt; –: not detected;

Results were presented as means of the area percentage in triplicate.

Table 4 Concentration of amino acids in fermented onion juice samples.

Amino acids	Concentration [a] (g/L)		
	Initial fermentation	Fermentation without salt	Fermentation with salt
Asp	0.18±0.01	0.26±0.01	0.05±0.00
Glu	0.45±0.03	1.46±0.20	0.05±0.00
Ser	0.23±0.01	0.15±0.00	0.03±0.00
Gly	4.31±0.03	5.09±0.37	1.60±0.09
His	0.20±0.00	0.63±0.03	—
Arg	0.18±0.01	0.11±0.01	0.06±0.00
Thr	0.08±0.00	0.10±0.01	0.03±0.00
Ala	0.75±0.04	0.14±0.02	0.01±0.00
Pro	—	—	—
Tyr	0.62±0.03	0.42±0.02	0.05±0.00
Val	0.13±0.01	0.14±0.01	0.01±0.00
Met	0.11±0.01	0.13±0.01	—
Cys	0.19±0.01	0.31±0.02	—

Ile	0.83±0.00	—	0.03±0.00
Leu	0.17±0.01	—	0.11±0.01
Phe	0.11±0.01	0.34±0.02	—
Trp	0.20±0.01	2.54±0.12	0.02±0.00
Lys	0.69±0.03	0.10±0.01	0.02±0.00
Total	9.43±0.24	11.92±0.85	2.07±0.12

[a] Samples were obtained at 96 h in fermentation without salt and 108 h in fermentation with salt; –: not detected;

Results were presented as means of the area percentage in triplicate.

Table 5 Relative areas of flavor compounds in fermented onion juice samples.

Compounds	RT [b] (min)	Area Pct. [a](%)		
		Initial	Without salt	With salt
Esters				
Pentanoic acid, 4-methyl-, ethyl ester	24.11	—	—	0.16±0.005
Octanoic acid, ethyl seter	24.13	—	0.24±0.013	2.10±0.109
Acetic acid, 2-phenylethyl ester	26.58	—	—	0.34±0.01
Nonanoic acid, ethyl este	28.17	—	—	0.41±0.015
Decanoic acid, ethyl ester	32.24	—	—	0.55±0.021
Tetradecanic acid, ethyl ester	46.19	—	0.19±0.050	0.57±0.023
Undecanoic acid, ethyl ester	46.19	—	0.11±0.009	—
1,1-Dodecanediol, diacetate	46.57	—	0.10±0.003	—
9-Hexadecenoic acid, methyl ester, (Z)-	47.93	2.07±0.201	0.15±0.006	—

Pentadecanoic acid, 14-methyl-, methyl ester	48.22	—	—	0.13±0.007
Ethyl 9-hexadecenoate	48.83	—	7.46±0.573	3.07±0.025
Hexadecanoic acid, ethyl ester	49.09	—	1.34 ±0.033	1.73 ±0.101
9,12-Octadecadienoic acid, methyl ester	50.42	6.03 ±0.45	—	—
11-Hexadecenoic acid, 15-methyl-, methyl ester	50.51	—	—	0.11
Linoleic acid ethyl ester	51.47	—	3.17 ±0.031	2.38 ±0.150
9-Octadecenoic acid, ethyl ester	51.56	—	3.74±0.157	2.22 ±0.086
E-11-Hexadecenoic acid, ethyl ester	51.68	—	0.64 ±0.059	—
Alcohols				
Silanediol, dimethyl-	1.63	7.07 ±0.324	—	—
1-Pentanol, 2-methyl-	5.00	—	18.04±0.876	8.56 ±0.339
1-Hexanol, 2-ethyl-	13.15	—	—	1.82 ±0.092
Phenylethyl Alcohol	18.77	—	0.19±0.009	—
2-Tetradecanol	28.32	—	0.87±0.077	—
2-Undecanol	28.32	—	—	5.80±0.103
Aldehydes				
2-Pentenal, 2-methyl-	4.24	0.32 ±0.009	—	—
Benzaldehyde	9.61		2.78±0.057	0.21 ±0.020
Decanal	24.42	—	—	0.87 ±0.043
Benzaldehyde, 3,5-dimethyl-	24.63	—	—	0.20 ±0.008
Hexadecanal	46.56	0.54±0.006	—	—
Ketones				
2-Tridecanone	28.02	0.17 ±0.015	—	—

2-Undecanone	28.03	—	—	0.50±0.032
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	35.04	—	—	0.19 ±0.007
2-Dodecanone	36.31	—	—	0.61 ±0.071
Sulfur-containing compounds				
Disulfide, methyl propyl	8.11	—	1.85 ±0.076	2.00±0.083
Dimethyl trisulfide	9.83	18.95±0, 987	—	—
Disulfide, dipropyl	18.13	—	—	14.01 ±0.613
Tetrasulfide, dimethyl	24.46	3.76 ±0.045	—	—
Trisulfide, dipropyl	29.06	1.21 ±0.11	—	—
Methanethioamide, N,N-dimethyl-	33.13	—	—	1.50±0.113
Cyclic octaatomic sulfur	49.59	0.08 ±0.007	—	—
Alkanes				
Silane, trichlorooctadecyl-	36.46	0.32±0.008	—	—
Hexadecane	36.47	—	0.41±0.019	—
Tetradecane	44.10	0.14	—	—
Heterocyclic compounds				
Cyclotrisiloxane, hexamethyl-	4.61	—	0.53 ±0.009	0.63 ±0.034
Thiophene, 2,4-dimethyl-	7.26	—	0.37 ±0.006	—
3,4-Dimethylthiophene	7.46	2.33	0.40±0.008	2.81 ±0.097
Cyclotetrasiloxane, octamethyl-	11.76	0.20	2.91±0.073	—
Azetidine, 1-methyl-	14.51	—	—	0.11 ±0.009
2-Ethyl[1,3]dithiane	19.65	—	—	0.56 ±0.004

Cyclopentasiloxane, decamethyl-	22.25	1.49±0.023	9.11±0.321	3.19±0.132
Thiophene, 2-methoxy-5-methyl-	25.41	0.92 ±0.007	—	0.32 ±0.013
1,2,4-Trithiolane, 3,5-diethyl-	29.05	—	1.15±0.017	20.75±0.743
Cyclohexasiloxane, dodecamethyl-	29.57	25.56±1.782	30.10±2.240	3.18±0.091
1,2-Dithiolane	30.36	0.19 ±0.006	—	—
3(2H)-Furanone, 5-methyl-2-octyl-	34.03	—	1.75 ±0.026	3.08 ±0.317
2-Amino-1,4-naphthalenedione	35.04	1.01±0.033	—	—
N-[2-[4-morpholinyl]ethyl]-				
Cycloheptasiloxane, tetradecamethyl-	36.71	11.40±0.874	10.93±0.975	2.07±0.075
3(2H)-Furanone, 2-hexyl-5-methyl-	42.34	—	0.26±0.005	—
Cyclononasiloxane, octadecamethyl-	46.87	0.71 ±0.008	0.65±0.043	0.71±0.009
Morpholine, 4-octadecyl-	47.60	4.56±0.176	—	—
2-Hydrazino-2-imidazoline	47.63	—	0.13±0.008	—
Phenol				
Phenol, 2,5-bis(1,1-dimethylethyl)-	36.98	1.08±0.097	0.18±0.004	0.24±0.004
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	41.96	6.85±0.098	—	0.27±0.008
Benzenes				
Benzene, 1-methoxy-4-(1-propenyl)-	27.64	—	—	0.30 ±0.003
Others				
Oxime-, methoxy-phenyl-	8.53	0.63±0.047	—	11.75±0.998
1-Pentanamine, N-pentyl-	43.26	2.43±0.077	—	—
Pregnane-3,20-dione, 11-[(trimethylsilyl)oxy]-,	48.99	—	0.24±0.003	—
bis(O-methyloxime), (5.beta.,11.beta.)-				

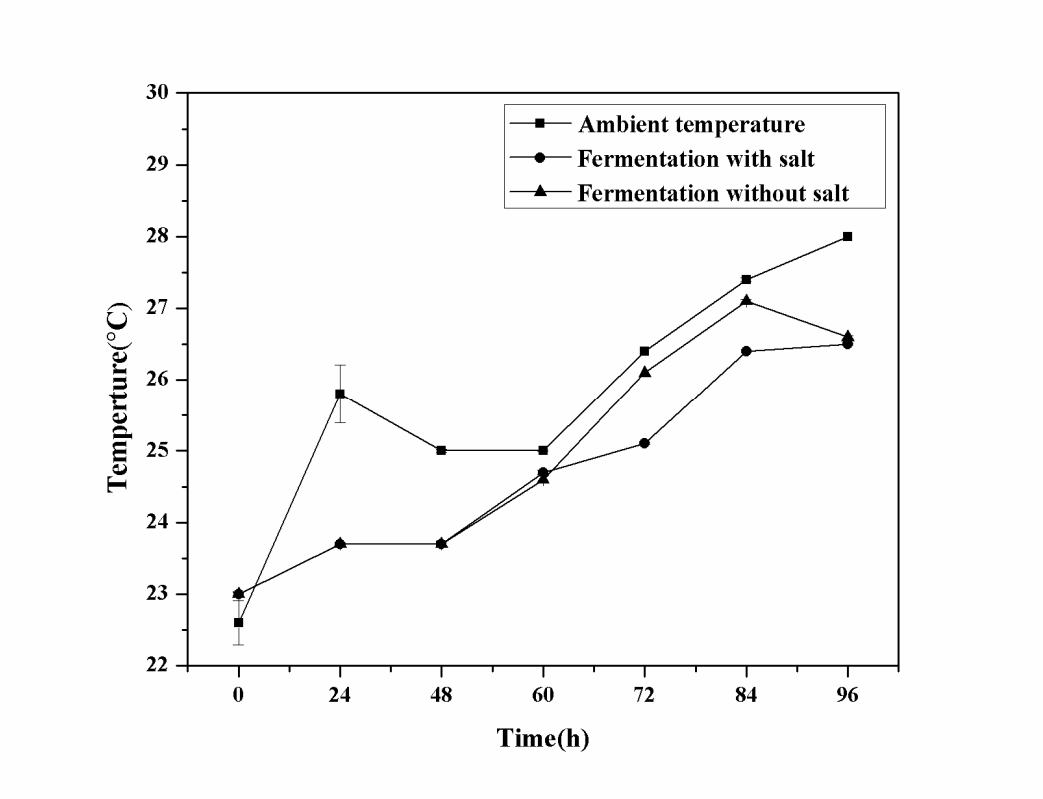
[a] Samples were obtained at 96 h in fermentation without salt and 108 h in fermentation with salt.

[b] RT: retention time on Agilent HP-5MS column in GC-MS.

–: not detected.

Results were presented as means of the area percentage in triplicate.

Figure 1 pH and temperature changes during fermentation with or without salt.



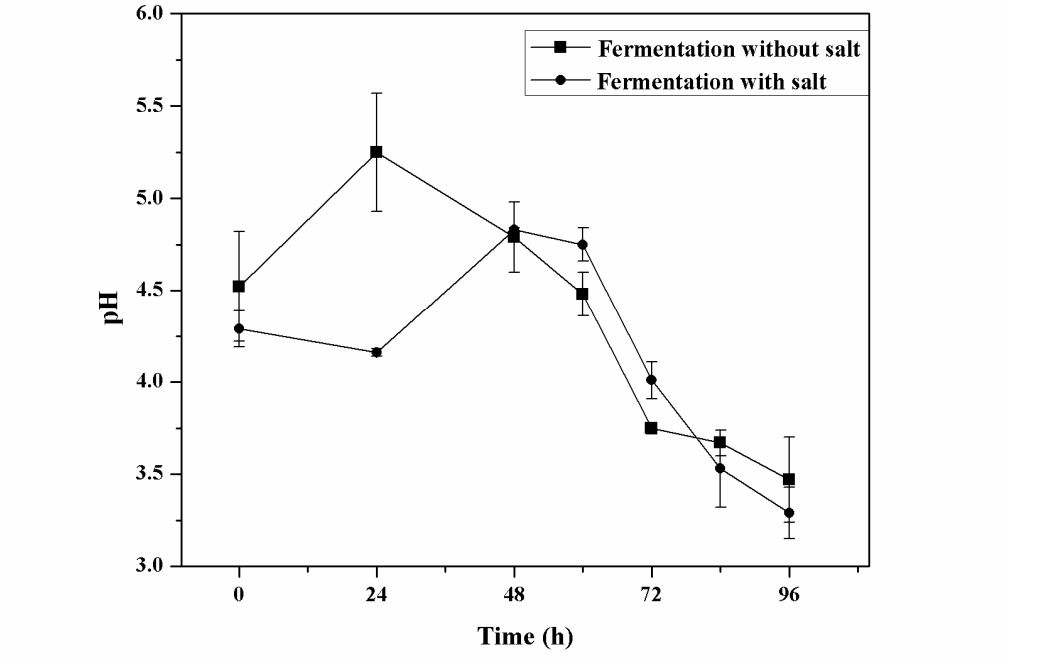


Figure 2 DGGE profiles of bacterial 16S rDNA (A) and fungal 18S rDNA (B). A, a 20-60% denaturing gradient gel was used to analysis the bacterial diversity; B, a 30-50% denaturing gradient gel was used to investigate the fungal diversity. Bands of interest were excised, re-amplified and sequenced.

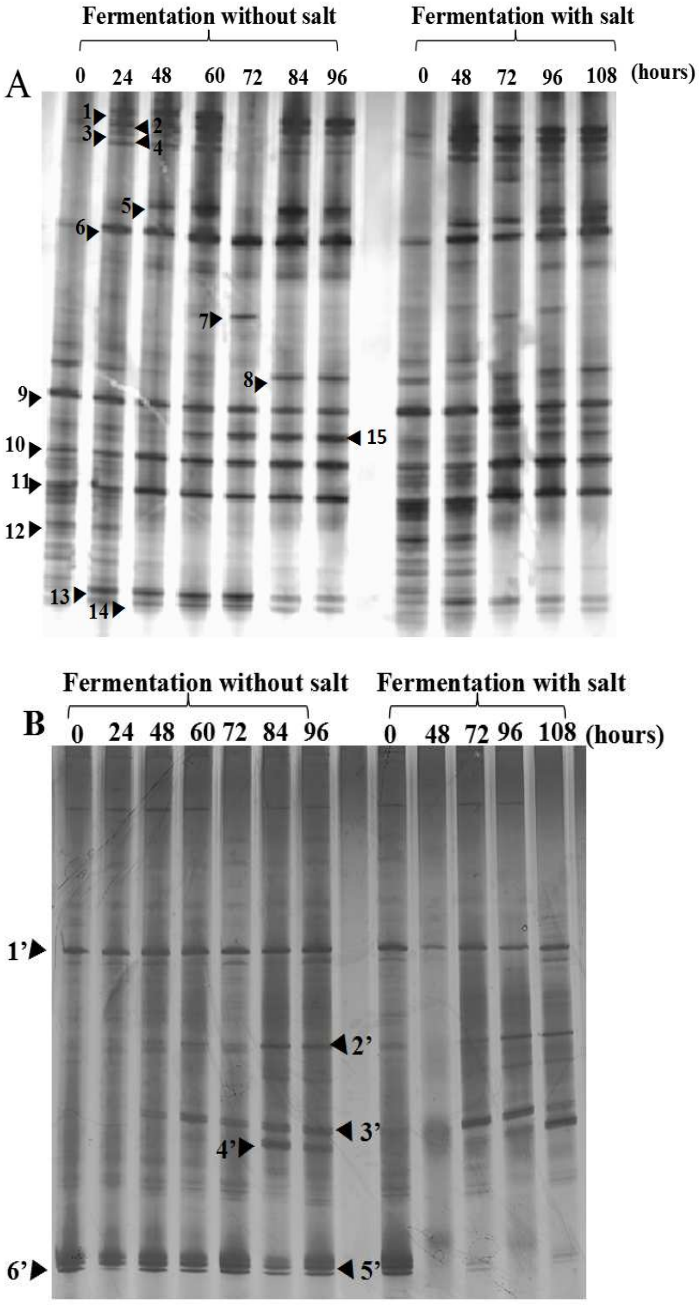
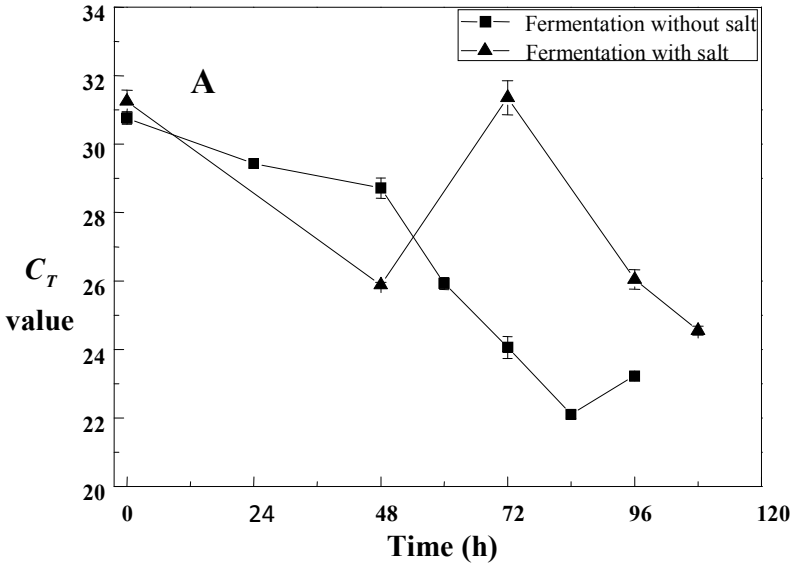
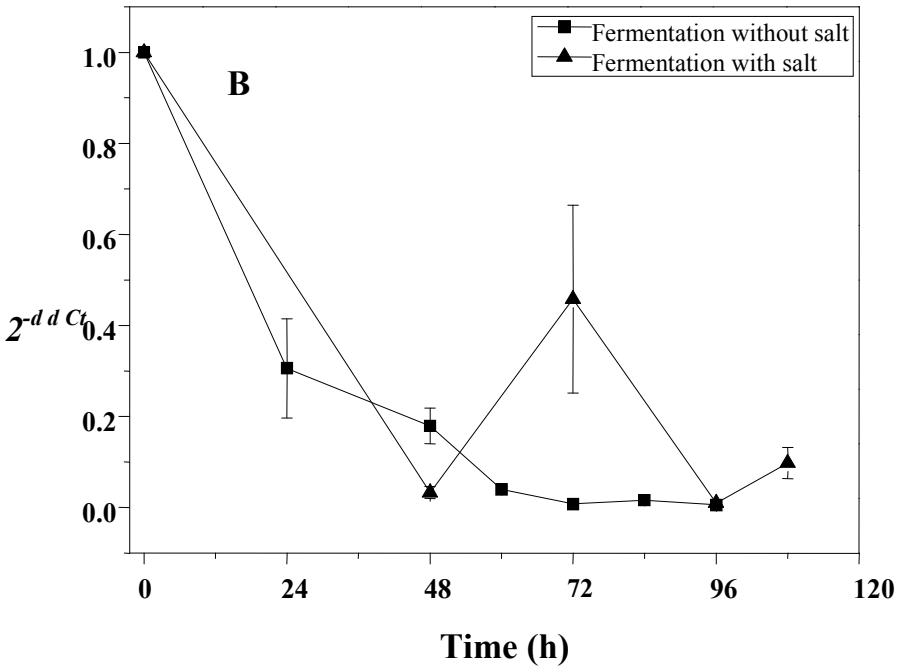


Figure 3 C_T of *L. plantarum* population changes during fermentation with or without salt by the 16S RNA gene (A) and the relative expression level of the *ldhL* gene during onion fermentation (B).

The population of *L. plantarum* was negative correlation with the C_T value. The higher the C_T value was, the less of the population of *L. plantarum*.





Highlights

1. Fermentation without and with saltwere used to enhance the flavor of fermented onion
2. Diversity of microbes during the fermentation was investigated by DGGE and RT-PCR
3. Organic acids, amino acids, volatile compounds were compared between the two ways
4. Timely addition of salt facilitates the production of high-quality fermented onion