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Microbiota and Mycobiota of Soy Sauce-Supplied Lactic Acid Bacteria Treated with High Pressure

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Abstract: Background: Ethyl carbamate (EC), a byproduct that naturally forms in fermented foods, can cause tumors and cell death and is classified as a probable human carcinogen (Group 2A). EC is naturally formed through the alcoholysis reaction between ethanol and carbamyl compounds. The major precursors and dominantly emerging stages of EC differ with disparate food types, including soy sauce. This work aimed to clarify the formation of EC and its influence factors throughout the soy sauce production process with or without high-pressure process (HPP) treatment. Methods: *Tetragenococcus halophilus*, *Pediococcus acidilactici*, *Zygosaccharomyces rouxii*, and *Candida versatilis* were added to soy sauce. The levels of citrulline and EC were measured, and a 16S and ITS assay investigated the microbiota. Results: L-citrulline production was found in each group after fermentation for one month. In addition, L-citrulline levels were generated the most in group D (500 MPa treated raw soy sauce with 12% saltwater and mixed fermentation bacteria, including *T. halophilus*, *P. acidilactici*, *Z. rouxii*, and *C. versatilis*) and group E (soy sauce fermentation with 12% saltwater without HPP treatment) compared to group F (soy sauce fermentation with 18% saltwater without HPP treatment). Conclusions: These results indicated that salt concentration and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*) might not be major factors for L-citrulline production.

Keywords: soy sauce; salt; high hydrostatic pressure process; lactic acid bacteria; fermentation

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1. Introduction

Soy sauce is a traditional seasoning from East and Southeast Asia. When making koji from raw ingredients, molds can release enzymes that saccharify the starch and break down the protein. Additionally, in the subsequent brine fermentation, yeast and bacteria can use components from koji. Yeast and bacteria are often a part of the soy sauce microflora, and a study has documented the microbial community in koji [1].

Ethyl carbamate (EC) is produced as a byproduct of the fermentation of group 2A carcinogens [2,3]. Soy sauce and other fermented meals and drinks are a few examples of foods containing EC [4–9]. Some biochemical reactions involving ethanol and EC precursors such as urea, citrulline, hydrocyanic acid, and carbamyl phosphate can result in the formation of EC [10]. Citrulline is the primary EC precursor among the two, and some research has shown that urea and citrulline may both be detected in soy sauce [11,12]. The ability of microorganisms to create citrulline and accumulate citrulline during the fermentation process of soy sauce is currently unreported [13].

To restrict microbial activity without triggering other chemical processes, the hydrostatic pressure process (HPP) has been applied to the production of foods such as soy sauce, sausages, and drinks [14]. Hence, it is feasible to make soy sauce without salt using HPP, which may be preferable for patients who need to limit their salt consumption [15]. After HPP, the bacterial population changed and other fermented items, such as brined green asparagus, mango pulp, and pitted sweet cherries, exhibited improved preservation [15,16]. Our recent study indicated that the microbiota in soy sauce treated with low salt (12%) with HPP was significantly different compared to the group without HPP treatment. A high abundance of *Lasiosphaeriaceae*, *Psathyrellaceae*, *Ceratobasidiaceae*, *Thelebolaceae*, *Phaffomycetaceae*, and *Olpidiaceae* were found in soy sauce treated using low salt (12%) with HPP [17].

Tetragenococcus halophilus, *Pediococcus acidilactici*, *Zygosaccharomyces rouxii*, and *Candida versatilis* are microorganisms commonly found in the fermentation process of soy sauce. It remains unclear whether these microorganisms biosynthesize EC and its precursors (urea, citrulline, hydrocyanic acid, and carbamyl phosphate). This study investigated these microorganisms' effect on EC production during soy sauce fermentation.

2. Materials and Methods

2.1. Koji Fermentation

Soybeans were cleaned and soaked (1:1.5, weight for weight) for 4 h before koji fermentation, which was carried out in accordance with our most recent work [17]. Additionally, the wheat grains were roasted for 20 min at 130 to 140 °C. Wheat grain and hydrated soybeans were mixed and autoclaved for 40 min at 121 °C to steam. The high and low carbohydrate ratios (wheat grain:soybean) for soy koji were 1:2 and 3:1, respectively. The soybean–wheat combination used to make koji also contained 10% of the *Aspergillus oryzae* starting mold. The mixture's moisture level was changed to 39.5%. The koji mixture was distributed across trays and evened out to a thickness of 2 cm. The next step was to smash each koji piece, leaving a 2.5 cm-diameter hole in the middle where mold might form.

2.2. Bacteria Culture and HPP Treatment

Tetragenococcus halophilus (BCRC12816, Bioresource Collection and Research Center, Hsinchu, Taiwan) was inoculated in a Tryptic soy medium, *Pediococcus acidilactici* (BCRC11063, Bioresource Collection and Research Center, Hsinchu, Taiwan) was inoculated in an MRS medium. *Zygosaccharomyces rouxii* (BCRC22634, Bioresource Collection and Research Center, Hsinchu, Taiwan) and *Candida versatilis* (BCRC21407, Bioresource Collection and Research Center, Hsinchu, Taiwan) were both isolated from soy sauce and were inoculated in a YPD medium. The soy koji was first mixed with an equivalent salt solution (12% or 18%) for soy sauce fermentation. Subsequently, the samples were treated with HPP (100 and 500 MPa, respectively) for 15 min at 25 °C. Finally, the 1% microbial solution (mixed *T. halophilus, P. acidilactici, Z. rouxii,* and *C. versatilis*) was added to the soy sauce and processing fermentation.

2.3. Assays for Levels of Total Aerobic Plate Counts, Yeast, and Lactic Acid Bacteria

Samples were homogenized with 90 mL sterile saline solution for 30 s on ice in a filtered stomacher bag using a stomacher (BagMixer400 VW, Interscience, Saint-Nom-la-Bretèche, France). The aerobic plate counts, yeast, and lactic acid bacteria levels were measured by a plate count agar (PCA) plate and MRS agar plate, respectively.

2.4. Measured for EC and L-Citrulline

The supernatant was freeze-dried into a powder, and about 0.6 g of the sample was weighed and added to 30 mL of methanol, followed by 1 N hydrochloric acid and ultrasonically shaken for 30–60 min, and then adjusted to the correct pH value with 1 N sodium hydroxide or 0.1 N sodium hydroxide. 7. This was re-filtered, then the supernatant was concentrated under reduced pressure, froze at −80 °C, and then freeze-dried. The quantification method for EC content is attributed to Wu et al. (2012) [18]. The RTX-WAX capillary column (30 m × 0.25 mm × 0.25 µm) and QP-2010 ultra gas chromatography/mass spectrometry (GC/MS) (Shimadzu, Suzhou, China) were used for separation. EC standard was purchased from Sigma-Aldrich, and the purity was >99%. L-citrulline accumulation assay was performed by the diacetyl monoxime method [19].

2.5. Statistical Analysis

The data were recorded as the means ± SD. The statistical significance was determined by one-way analysis of variance (ANOVA) by using the SAS general linear model procedure (SAS Inc., Cary, NC, USA), followed by ANOVA with Duncan's test. Results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Assays for Total Aerobic Plate Counts, Yeast, and Lactic Acid Bacteria

Soybeans were steamed under high pressure, mixed with wheat in 1:2 ratios, respectively, and then autoclaved to reduce the contamination of bacteria in the environment. Koji bacteria (Aspergillus oryzae) were added, and the koji bricks were placed on a sterile operating table and allowed to ferment for 14 days. Autoclaved 12% or 18% saltwater was then added to form a soy sauce mash. These microbial (total aerobic plate counts, yeast, and lactic acid bacteria) and chemical (L-citrulline and EC) indexes were measured in soy sauce mash treated without or with HPP (100 and 500 MPa) during the fermentation period.

Generally, the acidity of raw soy sauce increases with the fermentation time after one month. Therefore, it may be caused by the production of lactic acid by *T. halophilus*, produced by microorganisms through starch digestion, which ultimately reduces the pH of soy sauce. However, when the pH dropped below 5.0, *T. halophilus* could not grow, and *Z. rouxii* started alcoholic fermentation. The possible factors for the decrease in pH during the fermentation process are the self-digestion of microbial cells, accumulation of free fatty acids, amino acids, and peptides containing basal side chains resulting in the hydrolysis of soy sauce [20]. As shown in Figure 1, total aerobic plate counts in the raw soy sauce were first detected before HPP treatment at initial fermentation, and there was no difference between each group. Soy sauce was treated with or without HPP treatment. However, the total bacteria decreased by HPP treatment (500 MPa; group B). HPP treatment (500 MPa) and mixed fermentation bacteria, including *T. halophilus*, *P. acidilactici*, *Z. rouxii*, and *C. versatilis* (group D), decreased compared to the group without HPP treatments (group E and group F), from the second month to the sixth month. Moreover, the variations of yeast and lactic acid bacteria in soy sauce were similar to the total aerobic plate counts (Figures 2 and 3).

In the early stage of fermentation, organic acids are produced from the sugar metabolites in soy sauce to lower the pH. This environment is suitable for yeast growth. Saccharomyces cerevisiae undergoes alcohol fermentation in low-salt moromi to produce secondary metabolites, which are the flavor components of the final product of soy sauce [21]. Natural lactic acid bacteria and yeast will be produced during the fermentation process of the mash, which makes the raw soy sauce full of volatile substances, peptides, sugars, and other substances. During the fermentation process of soy sauce bacteria, the starch contained in the raw material is decomposed into sugars by enzymes. These sugars are metabolized by Tetragenococcus halophile into organic acids, such as lactic acid and acetic acid. In addition, the number of lactic acid bacteria increases with fermentation time. Hence, the literature points out that the lactic acid bacteria in the early stage of fermentation use glucose to produce lactic acid, which reduces the pH value and inhibits the growth of the bacteria [22]. From the comparison of Figures 1 and 3, it is known that the total number of bacteria during fermentation decreases with time, and the relative lactic acid bacteria increases with time. It is mentioned in the literature that low-salt conditions are conducive to the growth of salt-tolerant microorganisms in lactic acid bacteria in the mash [23]. According to some research, adding high-level saline (15–20 percent, w/v) was a crucial step in the fermentation of soy sauce. One study showed that the genera Weissella, Pediococcus, and Staphylococcus from koji and moromi are all tolerant to salt stress [12].

Through a reaction with ethanol aided by heat and an acidic environment, ethyl carbamate (EC) is formed from various precursors [24,25]. The primary EC precursors in alcoholic drinks and other fermented meals have been identified as urea and citrulline [26]. Pediococcus was a typical salt-tolerant species in soy sauce, and it was thought to have the capacity to degrade arginine. Citrulline in soy sauce is believed to come from *T. halophilus* strains, supposedly capable of converting arginine to citrulline [11]. It has been demonstrated that NaCl prevents various bacteria, including lactic acid bacteria, from converting ornithine from citrulline [27]. Therefore, we evaluated the productions of EC and citrulline in soy sauce during six months of fermentation with or without *T. halophilus*, *P. acidilactici*, *Z. rouxii*, and *C. versatilis* and investigated the association between EC and citrulline generation and microbiota.

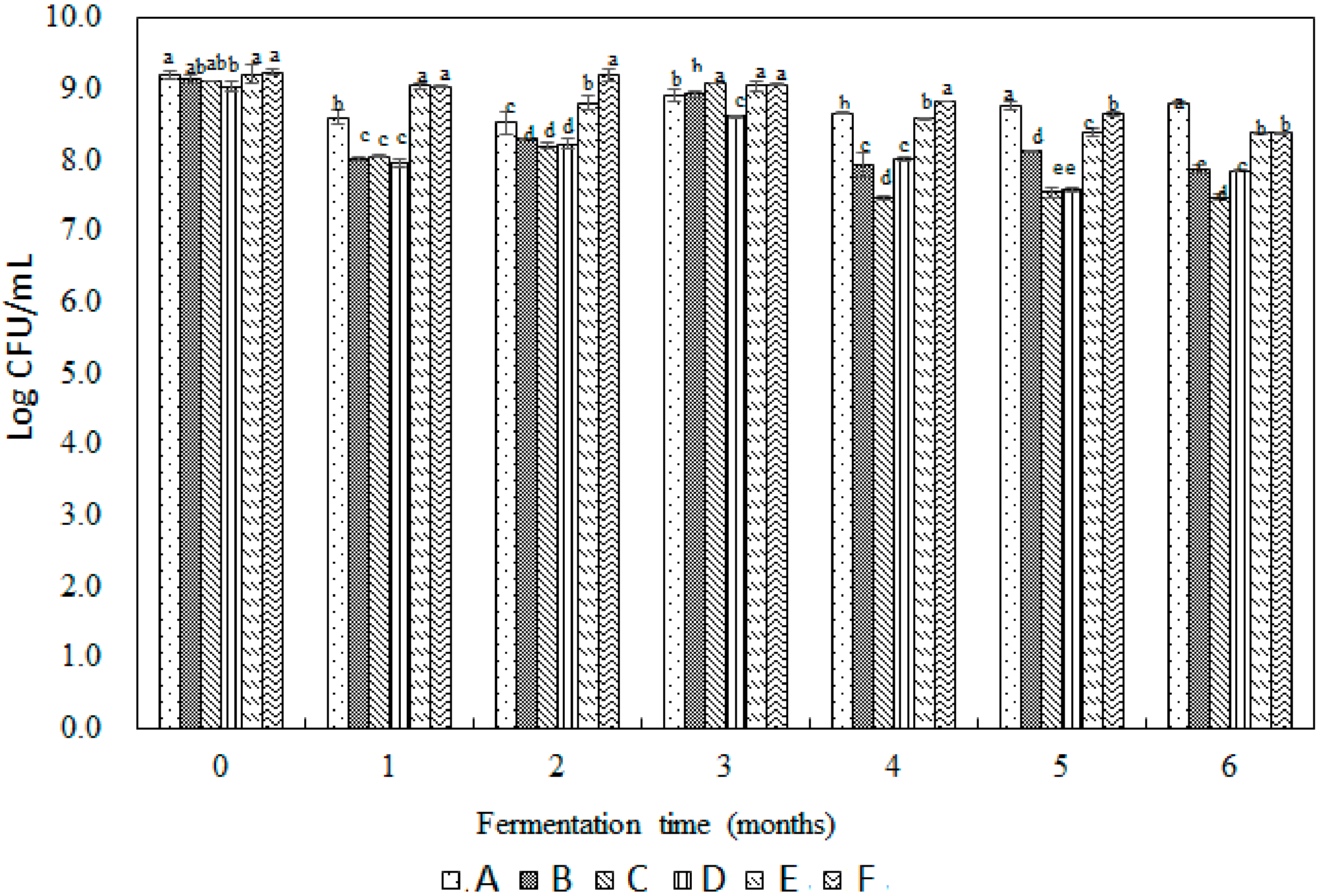

Figure 1. Effect of varying time on total aerobic plate counts (log CFU/mL) during raw soy sauce fermentation. Data are the average of triplicate experiments (Mean ± standard deviation). The different letters at the top of the columns denote the significance ($p < 0.05$). A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

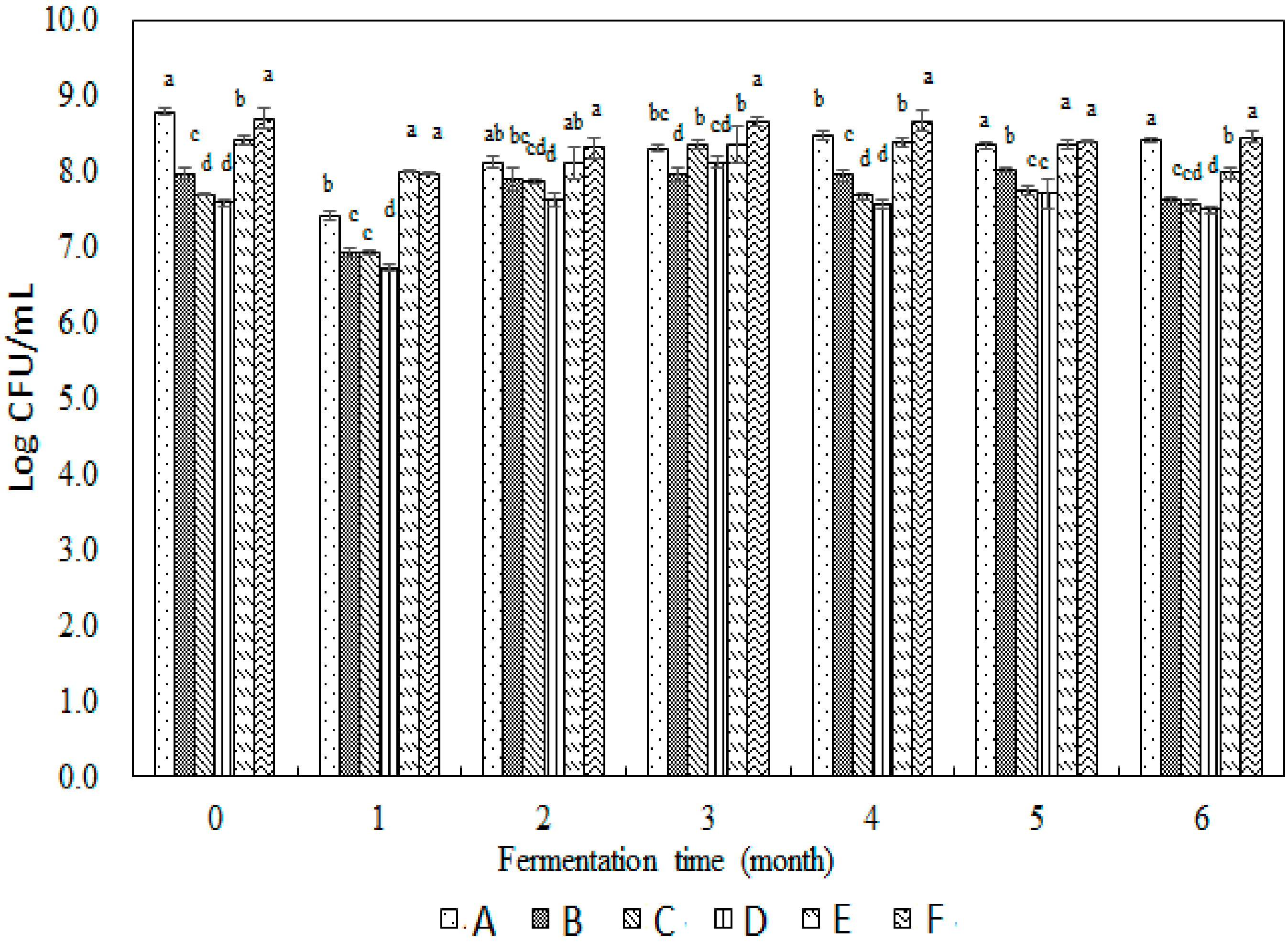

Figure 2. Effect of varying time on yeast (log CFU/mL) during raw soy sauce fermentation. Data are the average of triplicate experiments (Mean ± standard deviation). The different letters at the top of the columns denote the significance ($p < 0.05$). A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

3.2. L-Citrulline and EC Contents in Soy Sauce

As shown in Figure 4, EC generation is caused by various microbes from arginine, including lactic acid bacteria and yeast. In this study, L-citrulline production was found in each group after fermentation for one month (Table 1). In addition, the L-citrulline levels were most generated in group D (500 MPa treated raw soy sauce with 12% saltwater and mixed fermentation bacteria, including *T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*) and group E (soy sauce fermentation with 12% saltwater without HPP treatment) compared to group F (soy sauce fermentation with 18% saltwater without HPP treatment). These results indicated that salt concentration and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*) might not be major factors for L-citrulline production. Moreover, a significant difference in levels of L-citrulline in each group was not found after fermentation for six months. These results are similar to ethyl carbamate (EC) production during raw soy sauce fermentation (Table 2), indicating that not only mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*) but also koji affected L-citrulline and EC production.

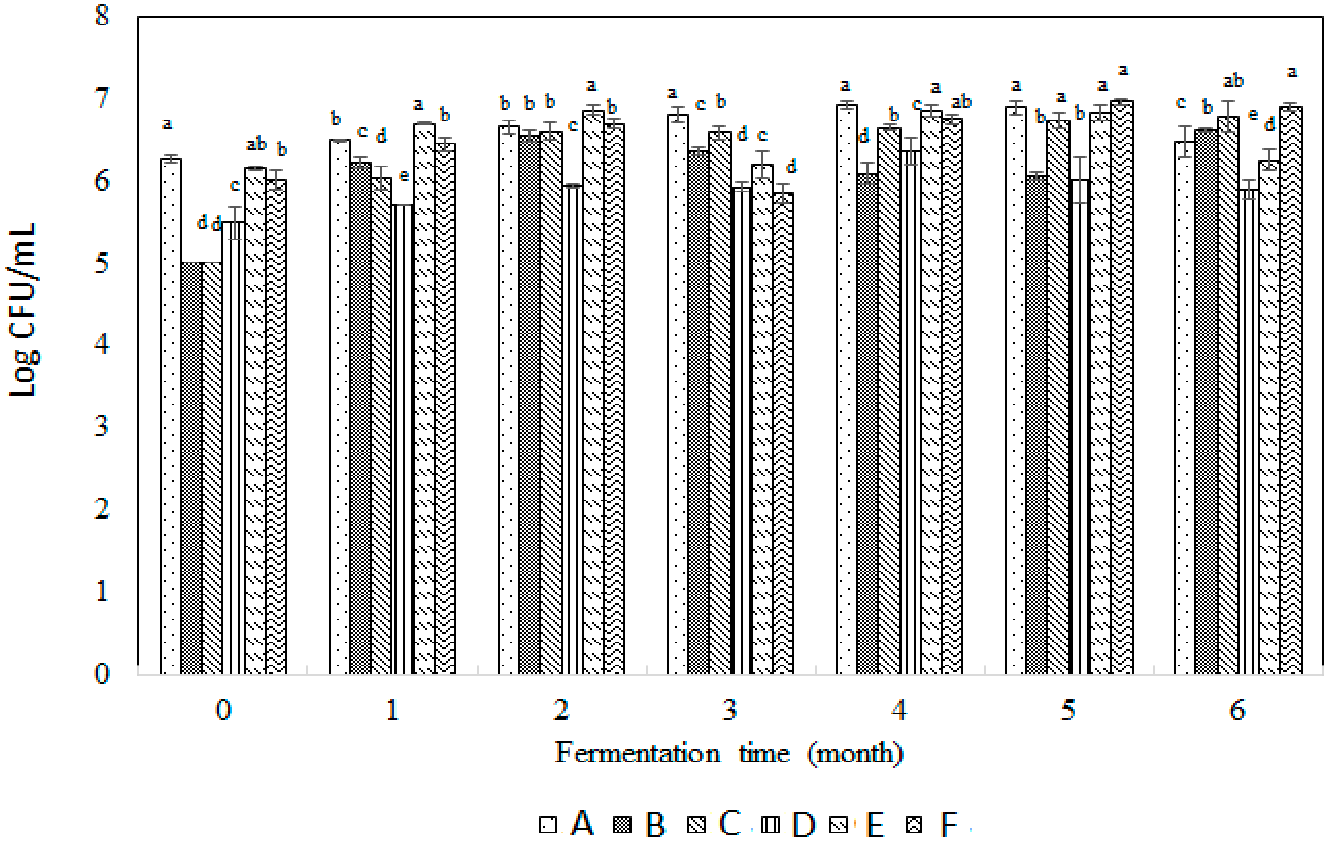

Figure 3. Effect of varying time on lactic acid bacteria (log CFU/mL) during raw soy sauce fermentation. Data are the average of triplicate experiments (Mean ± standard deviation). The different letters at the top of the columns denote the significance ($p < 0.05$). A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

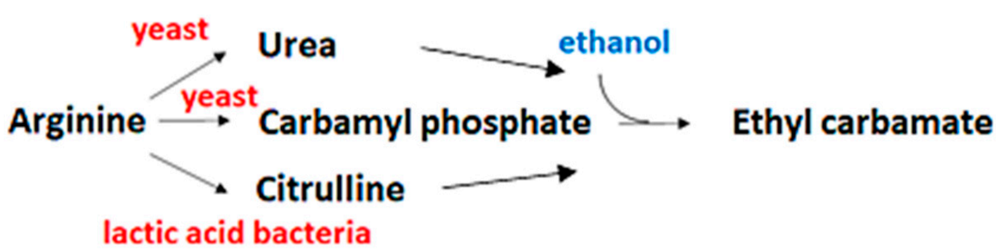

Figure 4. The synthetic pathway of EC from arginine by various microbes.

Table 1. Effect of varying time on L-citrulline contents (μg/mL) during raw soy sauce fermentation.

Time	Groups/L-Citrulline (μg/mL)					
Months	A	B	C	D	E	F
0	91 ± 24 [a]	76 ± 19 [a]	78 ± 40 [a]	85 ± 30 [a]	160 ± 99 [a]	120 ± 43 [a]
1	94 ± 6 [b]	100 ± 13 [b]	88 ± 23 [b]	153 ± 40 [a]	104 ± 20 [b]	124 ± 9 [a,b]
2	98 ± 17 [a]	89 ± 33 [a]	104 ± 19 [a]	96 ± 19 [a]	103 ± 16 [a]	104 ± 27 [a]
3	96 ± 37 [a]	98 ± 34 [a]	111 ± 14 [a]	112 ± 27 [a]	107 ± 32 [a]	83 ± 25 [a]
4	89 ± 26 [a b]	86 ± 18 [a,b]	94 ± 6 [a,b]	86 ± 8 [a,b]	72 ± 9 [b]	104 ± 15 [a]
5	81 ± 11 [a]	98 ± 7 [a]	84 ± 39 [a]	80 ± 14 [a]	104 ± 28 [a]	101 ± 14 [a]
6	92 ± 8 [c]	99 ± 17 [a,b]	123 ± 19 [a]	99 ± 18 [a,b]	104 ± 9 [a,b]	102 ± 10 [a,b]

Each value is expressed as mean ± S.D. (n = 3). [a,b,c] means in the same row with different superscript letters are significantly different ($p < 0.05$). A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

Table 2. Effect of varying time on ethyl carbamate content (mg/mL) during raw soy sauce fermentation.

Time	Groups/EC Content (mg/mL)					
Months	A	B	C	D	E	F
0	33.75 ± 1.56 [a]	3.35 ± 1.01 [c]	11.50 ± 0.56 [b]	10.93 ± 1.64 [b]	12.61 ± 5.26 [b]	6.01 ± 0.41 [c]
1	44.01 ± 4.54 [a]	10.39 ± 4.50 [b]	1.05 ± 0.08 [c]	0.16 ± 0.05 [c]	0.65 ± 0.02 [c]	3.03 ± 2.95 [c]
2	0.21 ± 0.16 [c]	0.29 ± 0.01 [c]	5.27 ± 0.25 [b]	1.47 ± 0.20 [c]	16.37 ± 3.86 [a]	2.22 ± 1.33 [c]
3	5.35 ± 0.77 [b,c]	12.25 ± 1.34 [a,b]	10.31 ± 0.60 [b]	1.88 ± 0.12 [c]	18.11 ± 8.83 [a]	1.86 ± 1.17 [c]
4	24.48 ± 7.79 [a]	4.24 ± 1.50 [b,c]	9.28 ± 0.96 [b]	9.75 ± 1.60 [b]	4.46 ± 2.20 [b,c]	0.82 ± 0.12 [c]
5	30.19 ± 24.52 [a]	1.27 ± 0.60 [b]	0.37 ± 0.31 [b]	1.16 ± 0.82 [b]	1.52 ± 0.22 [b]	0.38 ± 0.31 [b]
6	7.85 ± 0.64 [b]	13.16 ± 1.77 [a]	2.35 ± 0.92 [c]	0.40 ± 0.05 [d]	1.08 ± 035 [d]	0.69 ± 0.33 [d]

Each value is expressed as mean ± S.D. (n = 3). [a,b,c,d] means in the same row with different superscript letters are significantly different ($p < 0.05$). A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

3.3. Diversification of Fungal Diversity by ITS Assay in Soy Sauce

ITS assay measured the levels of fungi and yeast in fermented soy sauce. We found that Ascomycota (Phylum) is a major microbe in each group (Figure 5A). Moreover, the results indicated that Aspergillaceae (Family) population was reduced in group F (soy sauce fermentation with 18% saltwater without HPP treatment), suggesting that the Aspergillaceae microbe did not show salt-tolerance (Figure 5B). Similarly, the *Aspergillus* (Genus) was also suppressed, and *Zygosaccharomyces* were elevated in group F, but these results were not shown in other groups (Figure 5C). It was revealed that mixed fermentation microbes, including *Z. rouxii* and *C. versatilis,* were inhibited and may caused by HPP treatment. Alternatively, 18% saltwater is the optimal growth condition for these two microbes during fermentation.

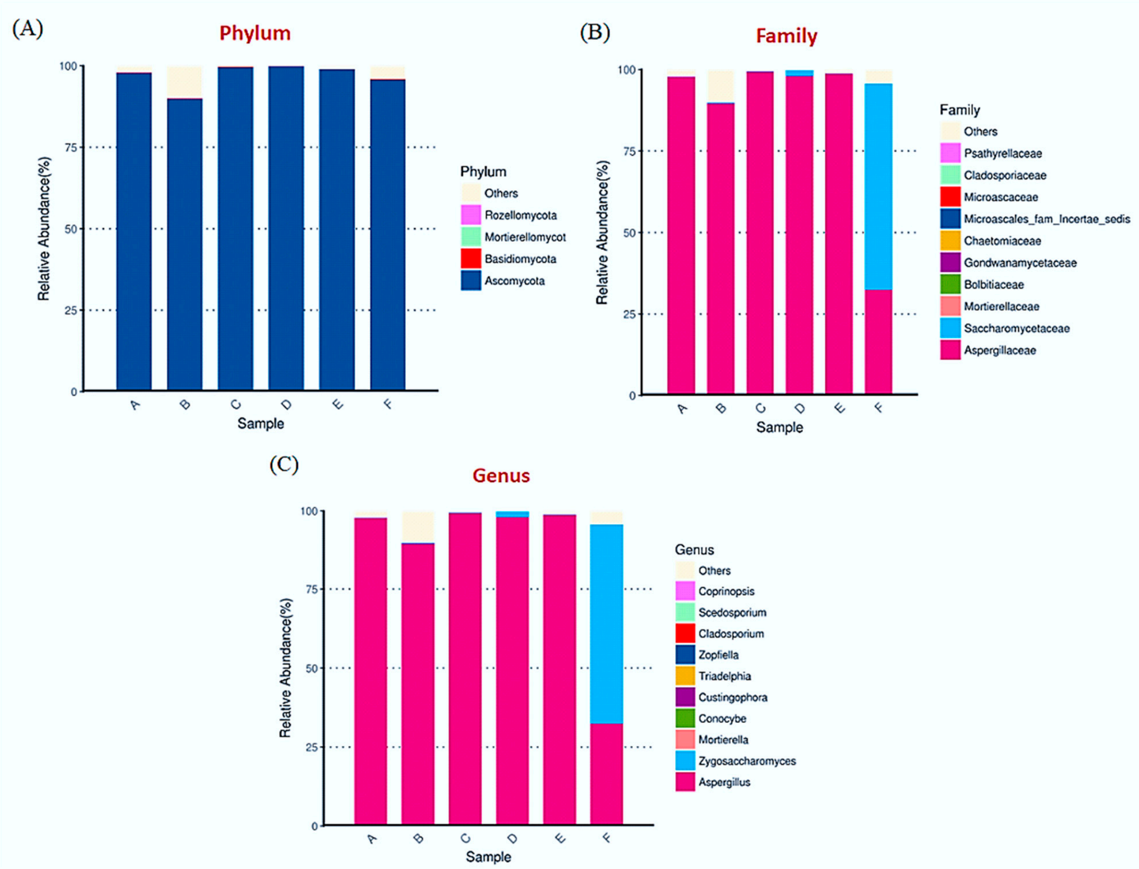

Figure 5. The diversification of fungal diversity by ITS assay on (**A**) phylum, (**B**) family, and (**C**) genus in soy sauce with or without HPP treatment after fermentation for one month. A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

3.4. Diversification of Bacterial Diversity by 16S Assay in Soy Sauce

In addition to fungi and yeast, bacterial microbiota were also analyzed in this study. We found a large proportion of Proteobacteria (Phylum) present in group C (Figure 6A), and a considerable amount of Enterobacteriaceae (Family) (Figure 6B) were found. However, there was less *Enterococcus* (Genus) present than other unknown bacteria in group C (Figure 6C). Moreover, we also found that the number of *Bacillus* were reduced by HPP treatment (500 MPa). Still, the number of lactic acid bacteria (Lactobacillaceae and *Pediococcus*) were not inhibited, indicating that lactic acid bacteria should be more resistant to HPP. A study reported that the conversion from citrulline to ornithine by *P. acidilactici* was inhibited by saline, claiming that low salt is a better controlling factor and could be developed as a strategy for regulating EC and citrulline production [12]. Lactic acid bacteria, mostly belonging to the *Weissella* and *Pediococcus* genera, are the predominant microorganisms in koji and the lactic acid fermentation process [28]. Due to the suppression of *Pediococcus* by saline brine, *Staphylococcus*, *Micrococcus*, and *Bacillus* are the predominant bacteria during the fermentation of alcohol [3]. According to a study, halophilic and mesophilic aerobic bacteria (*Bacillus* sp. and *Staphylococcus* sp.) and halophilic lactic acid bacteria were among the microbes that could metabolize arginine (*T. halophilus*). Koji strains with the ability to accumulate citrulline include *P. acidilactici* and *Weissella*. This finding indicated that the koji strains that produce citrulline were not the dominant bacteria throughout the alcoholic fermentation and were not in charge of the citrulline buildup during this time [13]. Arena observed that ethanol concentration did not impact Lactobacillus's ability to convert arginine to citrulline [29]. As a result, other variables, particularly detergents, may impact how ethanol affects the ratio of arginine to citrulline. According to some reports, citrulline formation is significantly affected by the presence of surfactants. When cetyl trimethyl ammonium bromide (CTAB) is present, Streptococcus may convert arginine into citrulline [30]. Citrulline was successfully reduced throughout the lactic acid fermentation process, which resulted in a considerable drop in citrulline and EC levels in soy sauce, demonstrating the influence of ethanol and other variables on citrulline formation by soy sauce microorganisms [31]. When soy sauce is heated, citrulline is used as an EC precursor rather than urea. Yeast extract, which is rich in amino acids, is frequently added to soy sauce to enhance its mellow aroma during manufacture. Yeast extract was not involved in the synthesis of EC because it lacked citrulline and ethanol [20]. In addition, temperature is a major controlling factor for EC and citrulline production in several fermented foods. *Weissella*, *Pediococcus*, and *Lactococcus* were the dominant bacteria resulting in ethanol accumulation. *Z. rouxii* was closely related to EC accumulation in soy sauce. Salt stress limited the utilization of citrulline and urea by *Z. rouxii* during soy sauce fermentation [32,33].

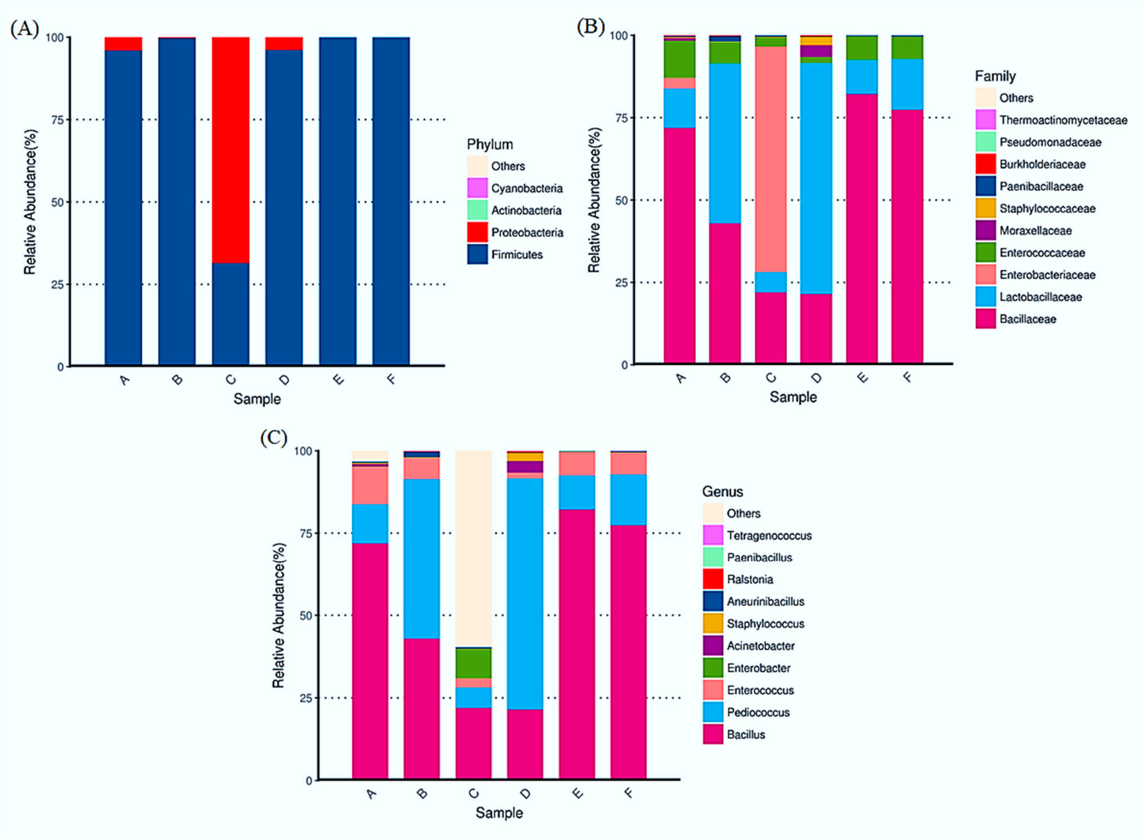

Figure 6. The diversification of bacterial diversity by 16S assay on (**A**) phylum, (**B**) family, and (**C**) genus in soy sauce with or without HPP treatment after fermentation for one month. A: 100 MPa treated raw soy sauce (12% saltwater). B: 500 MPa treated raw soy sauce (12% saltwater). C: 100 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). D: 500 MPa treated raw soy sauce (12% saltwater) and mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). E: soy sauce fermentation with 12% saltwater without HPP treatment. F: soy sauce fermentation with 18% saltwater without HPP treatment.

4. Conclusions

In this study, we found that HPP treatment (500 MPa) could suppress *Bacillus* populations and led to the elevation of lactic acid bacteria (*Pediococcus*) in soy sauce compared to groups without HPP treatment, whether adjunction of mixed fermentation bacteria (*T. halophilus*, *P. acidilactici*, *Z. rouxii*, *C. versatilis*). A large amount of *Zygosaccharmyces* was only found in the 18% saltwater group without HPP treatment. However, each group found no significant difference between citrulline and EC levels. Our results indicated that as well as lactic acid bacteria and *Bacillus*, other microbes may be involved in citrulline and EC production in soy sauce fermentation.

Author Contributions: Conceptualization, C.-Y.L. and C.-Y.H.; methodology, P.-T.C. and C.-Y.L.; software, W.-H.H.; formal analysis, W.-H.H.; investigation, W.-H.H. and S.-C.W.; resources, W.-H.H. and S.-C.W.; data curation, P.-T.C.; writing—original draft preparation, C.-Y.H.; writing—review and editing, W.-H.H. and S.-C.W.; project administration, W.-H.H. and S.-C.W. All authors have read and agreed to the published version of the manuscript.

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