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Lactic Acid Bacteria Isolation from Üçburun Peppers and Comparison of the Different Production Process for Pickled Pepper

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Abstract: In recent years, the number of conscious consumers who care about accessing safe food has increased, and this has brought about an increased interest in pickle products that do not contain preservatives and are obtained by natural fermentation. With the negative effects of food additives on health coming to the forefront, the search for new and natural methodologies in pickle production processes has begun. For this purpose, lactic acid bacteria (LAB), which is the most common bacteria in pickle fermentation and a normal microbiota member of fresh peppers, is used for natural fermentation studies in pickle production. In this context, this study aimed to sample Üçburun pepper (*Capsicum annuum* var. *annuum* L., "Golden Greek") for LAB isolation and to compare two different pickle production techniques within the scope of industrial processing. Accordingly, sampling was performed from two different sampling points for LAB isolation. The phenotypic and biochemical characteristics of the obtained isolates were determined. Kit-based identification of 10 isolates that were determined to exhibit different profiles was carried out using the API 50CH kit. To obtain additive-free pickled peppers on an industrial scale, two different pickle production processes (fermentation and acidification methods) were applied. According to the analysis results and the differences in the production stages of stock pickles, it has been seen that the pickles obtained by the acidification method are more suitable for pickle industry production.

Keywords: lactic acid bacteria; pickled pepper; fermentation; pepperoncini; fresh pepper; isolation; Üçburun

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1. Introduction

Pepper (*Capsicum* sp.), which is widely produced and consumed in general, belongs to *Capsicum* genus of the Solanaceae family, and is among the most important vegetable species grown worldwide. The origins of peppers are in Central and South America. Pepper is grown in many geographic areas of Türkiye. Türkiye has significant potential in the world's pepper production. Pepper is one of the most important components of nutrition. In addition to being consumed fresh, it is also used to prepare many foods. In addition to all this, it is also offered to the end consumer in the form of hot sauce, pickles or spices. It is known that 48.6% of total pepper production in Türkiye is used in industry. Jalapeno and Üçburun peppers are among the most widely used pepper varieties, particularly in the pickling industry [1,2]. *Capsicum annuum* varieties are numerous and cultivars often have specific cultural and market requirements [3]. In the Aegean region, Izmir, Manisa and Balikesir Provinces, Üçburun Greek pepper (peperoncini), Jalapeno type (Mexican pepper) varieties, Macedonian, Kardola, lombardi, Frankincense and Banana (Banana pepper) type varieties are produced for pickling [4].

Fermentation methods have been used since ancient times to consume fruits and vegetables when they are not abundantly fresh. The lactic acid fermentation process can provide a variety of foods with impressive aromas and flavors. It can also cause foods to

have a long shelf life and contain beneficial compounds. Fermented products are produced by the use of different micro-organisms such as yeast and mostly lactic acid bacteria found in the natural biota of many vegetable products, and most of the vegetable-based products produced show acidic properties [5,6]. However, studies on the exploitation of Üçburun pepper (*Capsicum annuum* var. *annuum* L. "Golden Greek") through lactic acid fermentation are limited, and related products in markets are also produced by the acidification method.

Today, although pickles can be produced without fermentation (with the acidification method) and with the addition of LAB or lactic acid fermentation of micro-organisms in the raw material biota, the demand for pickle products produced by fermentation is increasing day by day, as "clean label" products are preferred by consumers. Pepper pickled is fermented by (LAB) and yeasts, which are the natural biota of pepper. The fermentation process is carried out through competition between these two groups of micro-organisms.

Micro-organisms that play a role in lactic acid fermentation cause various changes in the product by producing enzymes during fermentation. These changes increase the consumption appeal of the fermented product in terms of taste, smell and appearance, as well as increase its quality and nutritional value and facilitate its digestibility in the gastrointestinal tract [7]. The increase in the consumption of additive-free food and the fact that consumers are more sensitive to these issues also increase the use of fermented products and natural food preservatives that emerge as a result of fermentation. In particular, organic acids formed as a result of the fermentation of carbohydrate sources, such as carbon dioxide, diacetyl, acetaldehyde, bacteriocin and bacteriocin-like metabolites, ethyl alcohol, ruterine and substances such as H_2O_2 produced by aerobic growth, can have an inhibitory effect on other micro-organisms [8,9]. Production of pickles by lactic acid fermentation generally occurs with the formation of lactic acid and other metabolites that occur as a result of the breakdown of some sugars with the help of micro-organisms added to the raw material or brine. In this context, lactic acid fermentation can be carried out using two different methods: natural fermentation (spontaneous) and controlled fermentation (culture-added) [10].

Although LAB are dominant in the natural biota of fermented foods, they play a role in the production and maturation stages of new products that are fermented from many foods, such as fermented meat, vegetables, milk, fruits and grains. The optimal conditions for micro-organisms to play a role in natural fermentation vary. Factors such as temperature, pH, amount of acid produced and salt concentration in the environment during fermentation affect the breakdown of sugars to be fermented, such as fructose, glucose and sucrose, by micro-organisms and the formation of products such as lactic acid in the environment as a result of this reaction [10,11].

Moreover, microbiota composition is an important factor in the quality of fermented products, and beneficial micro-organisms need to be investigated to process the target food matrix. LAB isolation from fresh raw materials and fermented product sources has been carried out by many researchers and continues to be created in this context. On the other hand, production techniques applied in the pickle industry have also been examined and scientific studies continue to contribute to the industry. Nevertheless, studies focusing on the screening of LAB strains for the fermentation of Üçburun peppers and pickled production methods are less reported. In our study, contrary to the literature, we chose to use the Üçburun pepper variety, which is widely used as a raw material in both our country's and international pickle industries. The widespread use of this pepper variety in the pickle industry, its commercial significance for our country, and the absence of studies in the literature regarding the isolation of LAB from Üçburun peppers enhance the importance of the data obtained. The objectives of this work were to examine the isolation of LAB from the fresh Üçburun pepper and to compare two different pickle production methods by industrial processing.

2. Materials and Methods

2.1. Materials

Üçburun peppers (*Capsicum annuum* var. *annuum* L. species, 3.0 to 5.0 cm length) were harvested from two different regions, where they were cultivated as part of the K.F.C. Gıda A.S. R&D Center University Industry Cooperation Project (Figure 1). The samples were stored at 4 °C until transport to the laboratory environment.

(**a**) (**b**)

Figure 1. (**a**) Üçburun pepper variety plant development area, Balıkesir (Series no: 12). (**b**) K.F.C. Gıda A.Ş. Üçburun pickle production raw material prewashing line (Production serial no: 110) Schemes follow the same formatting.

2.2. Isolation of LAB Strains from Fresh Üçburun Pepper Sources

Samples were collected from different regions and coded with the corresponding sample codes. For isolation of LAB, MRS agar, which was previously sterilized and added with 0.01% (*v*/*v*) cycloheximide after reaching 40–45 °C, was poured into sterile petri dishes under aseptic conditions [12]. A piece of pepper from three different regions of each sample was cut using sterile scissors (Figure 2). The cut pieces were homogenized for 5 min in a stomacher bag with 225 mL buffered sterile peptone water (Merck 107228, Darmstadt, Germany) to obtain a 10^{-1} dilution. Then, decimal dilutions were obtained up to 10^{-5} dilutions for each sample. In the isolation step of LAB, the spread plate method was used in parallel for each sample. Then, 100 µL was taken from the dilutions in turn and transferred to sterile MRS agar with automatic pipettes and spread with an L-shaped sterile baguette. Inoculations were performed for 24 h under aerobic and anaerobic conditions at a 37 °C incubator and also for 72 h under aerobic conditions at a 30 °C incubator. At the end of incubation, LAB counts were obtained in petri dishes where 15–300 colonies were found [13,14].

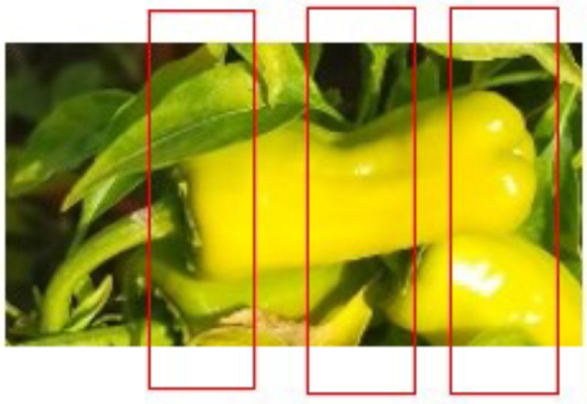


Figure 2. Areas sectioned for sampling from each fresh Üçburun peppers.

Colonies, exhibiting unique characteristics and derived from the petri dishes used for LAB enumeration, were meticulously selected. To ensure their purity, these chosen colonies were subjected to successive streak plate techniques in sterile MRS agar media. Once confirmed to be pure, the lactic acid bacterial isolates were stockpiled on slanted MRS agar at 4 °C. Subcultures were performed at four-week intervals for culture preservation. For long-term preservation, glycerol stocks were prepared at a final concentration of 25% for each isolate and stored at −80 °C [15].

2.3. Determination of Characteristics of LAB

In order to identify LAB by classical methods, the morphological and cultural characteristics of the pure cultures were determined. The strains preserved at −80 °C were reinvigorated by inoculating them with 10 mL of MRS broth. In this context, colony morphology, catalase test, oxidase test, KOH test, gram staining for size measurements, cell morphology and growth tests at different salt concentrations [0%, 6.5% and 10% NaCl (*w*/*v*)] were performed. Catalase test was performed based on the principle of observing bubbles as a result of mixing the bacterial culture with hydrogen peroxide (3%) [16].

For CO_2 production assessment, aliquots from the activated culture were introduced into MRS broth supplemented with a Durham tube, then placed in an incubator set at 30 °C for a duration of 7 days. The strain was classified as heterofermentative upon observing CO_2 emission, while it was deemed homofermentative if no CO_2 evolution was noted [17].

The isolates were cultured at two distinct temperatures employing four dissimilar growth media, namely MRS agar, BHI agar, M17 agar, and nutrient agar, to identify their growth patterns and optimal nutrient provisions. Each petri dish containing an isolate, inoculated with the four different growth media, was incubated at 30 °C for 72 h and at 37 °C for 48 h (Table 1).

Table 1. Determination of the optimum growth medium.

Incubation Temperature (°C)	Medium 1	Medium 2	Medium 3	Medium 4
37 °C	MRS Agar	BHI Agar	M17 Agar	Nutrient Agar
30 °C	MRS Agar	BHI Agar	M17 Agar	Nutrient Agar

Biochemical tests included the carbohydrate fermentation test (API 50 CH). In this context, the carbohydrate metabolism of the isolates was determined according to the API 50 CHL Medium test kit guideline (BioMérieux, Marcy l'Etolie, France) within the scope of the study [18].

2.4. Preparation of Üçburun Pepper Pickles

At this stage of the study, two different industrial-scale pickling methods were employed to obtain Üçburun pepper pickles. Pickling processes using the acidification method and the lactic acid fermentation method were conducted in 220-L barrels. Raw peppers underwent washing and sorting processes in accordance with the flowchart presented in Figure 3 for each pickling method. Approximately 70 kg of Üçburun peppers were coded and filled into 220 L barrels prepared with the formulations of P0A (acidification method pickling brine) and P0F (fermentation method pickling brine) as specified in Table 2 [19].

Table 2. Brine formulations for pickle production.

Product No	Vinegar %	Citric Acid %	Salt %	Calcium %
PA (Acidification method pickling brine)	5.56	1.00	14.00	0.30
PF (Fermentation method pickling brine)	-	-	10.00	0.30

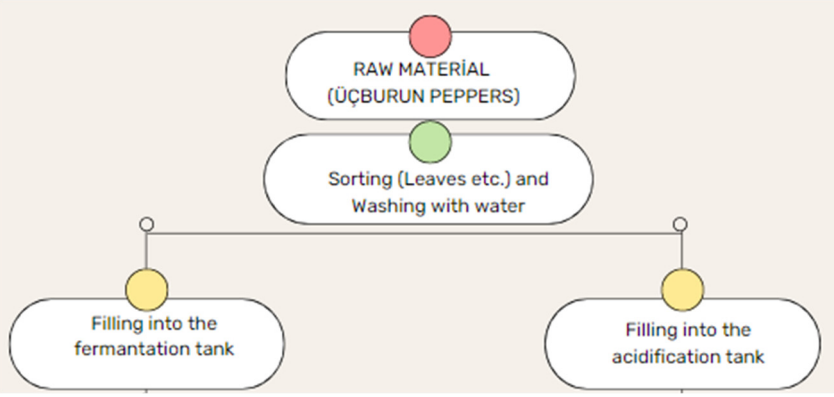


Figure 3. Flow chart of pickle production by fermentation and acidification methods.

Samples from the barrels were collected on specific days during the fermentation period for chemical and microbiological analyses of the pickling brines and pepper pickle compositions. The fermentation progress was tracked based on the obtained results.

To fill the 10- and 32-day-old semifinished products into jars, 370-cc jars were used. Each jar was filled with semifinished products and the brine formulation specified in Table 3 was added while hot by heating it to 80 °C. The jars were then sealed and pasteurized at 50–70 °C for 20 min (see Supplementary Information in Figure S1).

Table 3. Brine formulations for pickle production for P10A, P32A, P10F, P32F1 and P32F2.

Product No	Vinegar %	Citric Acid %	Salt %	Lactic Acid %	Calcium %
P10A (Acidification method of filling brine)	2.00	0.18	1.91	0.45	0.30
P32A (Acidification method of filling brine)	1.72	0.15	1.42	0.39	0.30
P10F (Fermentation method of filling brine	-	-	0.47	1.35	0.30
P32F1 (Fermentation method of filling brine)	-	-	1.50	1.50	0.30
P32F2 (Fermentation method of filling brine)	-	-	1.50	1.00	0.30

2.5. Microbiological and Chemical Analyses of Samples

Microbiological analyses were carried out on 25 g of Üçburun pepper pickles, which were homogenized with 225 mL of 0.1% peptone water by a stomacher. The suspension was serially diluted tenfold with 0.1% peptone water and spread in duplicate onto the following agar media (i) MRS (De Man Rogosa and Sharpe) agar for LAB (incubated at 37 ± 1 °C for 48 ± 2 h); (ii) PC (Plant Count) agar for total viable counts (TVC) (incubated at 30 ± 1 °C for 48 ± 2 h) and (iii) DRBC (Dichloran Rose Bengal Chloramphenicol) agar for yeast and mold (incubated at 25 °C for 5–7 days). Results were expressed as log values of colony-forming units per gram (log CFU/g) of Üçburun pepper.

Salt and acid analyses were carried out by taking into account the Titrimetric Method of the Association of Official Analytical Chemists, and the chemical composition was determined as a result of the analyses performed in duplicate [20]. For the analysis of the brine or pepper pickles, pickled peppers were cut into small pieces and thus a homogeneous product was obtained.

The pH of the brine and pepper pickle samples was measured using a pH meter. The measurements were performed on the pH meter (Thermo Orion Star A111 USA). The sample was passed through a grinder and made into a paste. The samples (100 mL) were placed in an empty flask, and the pH values of the samples were analyzed by immersing the pH meter probe in the sample [20].

Various chemical (such as salt, acid and pH) and organoleptic (flavor, texture, scent and color) properties of the products were determined during storage with the semifinished products taken on the 10th and 32nd days of fermentation (see Supplementary Information in Figure S1).

3. Results and Discussion

3.1. Microbiological Characteristics of Fresh Üçburun Pepper Samples

An assessment of the total aerobic mesophilic micro-organism, yeast, mold and LAB count was performed on the samples of Üçburun peppers. The results of this analysis, which were obtained from plates that were specifically diluted to contain 15–300 colonies, are displayed in Table 4 in units of colony forming units (CFU) per gram. As indicated in Table 2, TAMM counts varied from 1.4×10^5 to 6.1×10^5 CFU/g, yeast counts ranged from 5.1×10^5 to 7.4×10^5 CFU/g, and total LAB counts ranged from 1.1×10^3 to 3.6×10^3 CFU/g, contingent on the two distinct sampling regions. The mold growth in recent fresh Üçburun pepper samples was found to be less than 10 CFU/g.

Table 4. Microbiological analysis results of fresh Üçburun pepper variety samples (P110: K.F.C. Gıda A.Ş. Üçburun pepper variety with serial number 110; P12 Üçburun pepper variety with serial number 12).

				LAB Counts (CFU/g)		
Sample No (Serial Number)	**Total Aerobic Mesophilic Micro-Organism Counts (CFU/g)**	**Yeast Count (CFU/g)**	**Mold Count (CFU/g)**	**48 h Aerobic Incubation at 37 °C**	**48 h Anaerobic Incubation at 37 °C**	**72 h Aerobic Incubation at 30 °C**
P110	6.1×10^5	5.1×10^5	<10	1.1×10^3	2.0×10^3	1.9×10^3
P12	1.4×10^5	7.4×10^5	<10	3.6×10^3	3.4×10^3	2.5×10^3

A study by Raffaella Di Cagno et al. (2009) [21] utilized MRS agar medium to determine LAB counts in fresh red and yellow peppers. Results indicated counts ranging from 3.4 to 4.5 log CFU/g. Although MRS agar is recommended for lactobacilli in many studies, M17 agar is suggested for lactococci [22,23]. As MRS agar is not selective, other bacteria and yeasts may grow in the medium. To overcome this, the medium was treated with 0.01% (*v*/*v*) cycloheximide [12–14,21].

3.2. Identification Result of LAB from Fresh Üçburun Samples

A total of 10 LAB isolates were obtained from fresh Üçburun pepper samples taken from the sampling areas. All bacterial isolates exhibited the features typical of LAB. Following analysis, all isolates were identified as being gram-positive, catalase-negative and cytochrome oxidase-negative.

In previous research, LAB has often been cultivated on MRS and M17 media. Furthermore, brain heart infusion (BHI) is employed for the cultivation and enumeration of these bacteria. The isolates gathered in this context were incubated in four distinct media (MRS agar, BHI agar, M17 agar and nutrient agar), at 30 °C for 72 h and at 37 °C for 48 h. After the incubation period, we examined each isolate in the cabin using different media and temperatures. We then evaluated and classified the growth results according to five different growth conditions: intense growth, standard growth, medium-intensity growth, low-intensity growth and no growth.

After incubation on MRS agar medium for 72 h at 30 °C, A1 and B5J isolates displayed intense growth, while A3J exhibited intense growth after 48 h of incubation at 37 °C. On the other hand, none of the isolates exhibited intensive growth on M17 agar medium incubated at 30 °C for 72 h. However, A3J, A1E, and A11E isolates grew intensively after 48 h of incubation at 37 °C. Among the isolates incubated at 30 °C for 72 h on BHI agar medium, A1, B5J, and A3J isolates exhibited significant growth, while A1, B5J, A1E, and A11E isolates demonstrated substantial development at 37 °C for 48 h. A1E, A11E and B3 isolates exhibited significant growth at 37 °C with a 48-h incubation period in a nutrient agar medium (Table 5).

Table 5. Growth at different temperatures and optimum medium results (α: intense growth, β: standard growth, γ: medium intensity growth, ε: low intensity growth, and x: no growth).

Sample No	Isolate Code	30 °C				37 °C			
		MRS Agar	M17 Agar	BHI Agar	Nutrient Agar	MRS Agar	M17 Agar	BHI Agar	Nutrient Agar
P12	A4E	x	β	β	β	x	ε	ε	x
	A11E	x	β	β	β	x	α	α	α
	A1E	β	γ	β	β	ε	α	α	α
	A3J	β	β	α	γ	α	α	β	γ
	A1	α	γ	α	γ	ε	γ	α	γ
P110	B5J	α	γ	α	γ	β	ε	α	γ
	1B4J	x	x	ε	γ	x	ε	γ	γ
	B2	γ	ε	γ	γ	γ	γ	γ	ε
	B5	γ	ε	γ	γ	γ	γ	γ	ε
	B3	ε	ε	γ	β	ε	β	γ	α

One complete tube of active cultures was transferred to sterile 0.85% physiological saline, and 0.1 mL was then inoculated into liquid media prepared in accordance with the salt concentrations and adjusted for bacterial density equivalent to 2 Mc Farland. The tubes were subsequently incubated at 37 °C for a period of 7 days and their growth was assessed according to the turbidity criterion, with regular checks performed every two days. After the incubation period, the isolates developed on a medium with 0% and 6.5% NaCl, but not on a medium with 10% NaCl. Durham tubes were utilized to observe the gas formation of LAB isolates using glucose. All 10 isolates examined for gas formation from glucose were homofermentative (Table 6).

Table 6. Phenotypic characteristics of LAB isolated (+ positive reaction, − negative reaction).

Sample No	Isolate Code	Growth Characteristics in Medium	Gram Reaction	%3 KOH Reaction	Catalase Reaction	Oxidase Reaction	Gas from Glucose	Salt Consantration		
								0%	6.5%	10%
P12	A4E	Cream, Mat, Zoned	+	−	−	−	−	+	+	−
	A11E	Cream, Bright	+	−	−	−	−	+	+	−
	A1E	Cream, Bright	+	−	−	−	−	+	+	−
	A3J	Cream, Mat	+	−	−	−	−	+	+	−
	A1	Cream, Bright	+	−	−	−	−	+	+	−
P110	B5J	Cream, Bright	+	−	−	−	−	+	+	−
	1B4J	Cream, Mat	+	−	−	−	−	+	+	−
	B2	White, Bright	+	−	−	−	−	+	+	−
	B5	Yellow, Mat	+	−	−	−	−	+	+	−
	B3	Yellow, Bright	+	−	−	−	−	+	+	−

3.3. Carbohydrate Fermentation Profile (API 50 CH)

The API 50 CH test was applied to 10 different isolates, whose growth characteristics were examined in the optimal nutrient medium, in order to determine and identify the carbohydrate fermentation metabolism of the isolates. Following this study, after 24 h of incubation, five isolates (A3J, A1E, A1, B2 and B5J) produced acid from L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, mannitol, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose and gentiobiose, showing visually the same fermentation profile. On the other hand, the remaining five isolates (A11E, B3, 1B4J, A4E and B5) produced acid from ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, trehalose, melezitose, gentiobiose, D-tagatose and gluconate after 24 h of incubation, displaying visually the same fermentation profile.

As seen in Figure 4, according to the API 50 CH kit identification, isolates A3J, B5J, A1E and A1 showed similarity percentages ranging from 99.9% to 90.9%, indicating *Lactobacillus paracasei* ssp. *paracasei* 1; isolates B5, A4E and B3 exhibited a similarity percentage of 80.3%, suggesting *Lactococcus lactis* ssp. *lactis* 1; isolate A1 showed a similarity percentage of 84.5%, indicating *Lactobacillus brevis* 1; isolate B2 displayed a similarity percentage of 96.5%, signifying *Lactobacillus rhamnosus* 1; isolate 1B4J demonstrated a similarity percentage of 91.7%, denoting *Lactobacillus pentosus*; and isolate A11E was identified with a similarity percentage of 95.5% as *Lactobacillus paracasei* ssp. *paracasei* 3. In this context, similarities were observed among LAB isolates isolated from two different regions according to the API 50 CH test kit.

API 50CH TEST RESULT																	
İzolat No		Significant taxa	%ID	T	TESTS AGAİNST				Next taxon	%ID	T	TESTS AGAİNST				45 C
1	A11E	*Lactobacillus paracasei ssp paracasei 3*	95.5	0.73	RHA %1	GNT %20			*Lactococcus lactis ssp lactis 1*	2.7	0.66	RHA %0				
2	A3J	*Lactobacillus paracasei ssp paracasei 1*	99.9	0.88	GLY %20	TUR %80			*Lactobacillus paracasei ssp paracasei 3*	0.1	0.52	GLY %0	SOR %20	MLZ %20	GNT %20	
3	B5J	*Lactobacillus paracasei ssp paracasei 1*	90.9	0.41	GLY %20	DXYL %0	LAC %99	TUR %80	*Lactobacillus paracasei ssp paracasei 2*	6.3	0.33	GLY %16	DXYL %0	MDG %83	TUR %100	
4	B5	*Lactococcus lactis ssp lactis 1*	80.3	0.65	RHA %0				*Lactobacillus pentosus*	9.6	0.46	GLY/ RAF %75	RHA %25	SOR %100	MEL %100	
5	A4E	*Lactococcus lactis ssp lactis 1*	80.3	0.65	RHA %0				*Lactobacillus pentosus*	9.6	0.46	GLY/ RAF %75	RHA %25	SOR %100	MEL %100	
6	A1	*Lactobacillus paracasei ssp paracasei 1*	99.9	0.88	GLY %20	TUR %80			*Lactobacillus paracasei ssp paracasei 3*	0.1	0.52	GLY %0	SOR %20	MLZ %20	GNT %20	
	A1	*Lactobacillus brevis 1*	84.5	0.37	RHA %0	SOR %14	MLZ %14	TAG %14	*Lactobacillus plantarum 1*	9.1	0.2	DXYL %2	MEL %94	INU %0	TAG %7	
7	B2	*Lactobacillus rhamnosus*	96.5	0.70	SBE %92	MDG %85	TUR %92									.+/-
	B2	*Lactobacilus paracasei ssp paracasei 1*	3.4	0.56	GLY %20	RHA %1	INO %6	TUR %80	*Lactobacillus plantarum 1*	0.1	0.13	GLY %1	INO %0	MEL %94	TAG %7	
8	B3	*Lactococcus lactis ssp lactis 1*	80.3	0.65	RHA %0				*Lactobacillus pentosus*	9.6	0.46	GLY/ RAF %75	RHA %25	SOR %100	MEL %100	
9	IB4J	*Lactobacillus pentosus*	91.7	0.51	SOR %100	MEL %100	MLZ %25	RAF %75	*Lactobacillus brevis 1*	3.9	0.5	GLY %0	MLZ %14	GNT %85		
10	A1E	*Lactobacillus paracasei ssp paracasei 1*	99.9	0.88	GLY %20	TUR %80			*Lactobacillus paracasei ssp paracasei 3*	0.1	0.52	GLY %0	SOR %20	MLZ %20	GNT %20	

Figure 4. API 50 CH API Identification Software (API Lab Plus Program, BioMérieux, API version v1) Results.

A study by Bello et al. (2013) [24] conducted a study in which four LAB species from three different genera, *Streptococcus pyogenes*, *Enterococcus faecalis*, *L. casei* and *L. fermentii*, were isolated from fresh peppers and tomatoes. Di Cagno et al. (2009) [21] identified *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and *Weissella cofusa* strains from fresh red and yellow peppers using 16S rRNA gene sequencing.

3.4. Processing of Üçburun Peppers Pickles

Stock pickle barrels created through industrial-scale acidification and fermentation methods were monitored for 40 days using chemical analyses (% acidity, % salt and pH analyses). The pepper (content) and brine acid analyses of the P0F barrels, where the fermentation method was applied, were calculated based on total lactic acid type, while the content and brine acid analyses of the P0A barrel, where the acidification method was applied, were calculated based on total acidity type.

In the P0F barrel, the content acid values reached 0.81% in terms of lactic acid type on the 13th day of fermentation, whereas in the P0A barrel, due to the effect of the acidification method, the content, total acidity value reached 0.90% on the 4th day. Unlike the acidification method, in the fermentation method, both content and brine acid levels increased due to LAB development. With the fermentation occurring in the P0F barrel, acidity reached 0.72% in terms of lactic acid type on the 13th day. During this process, the highest content acidity value in the P0F barrel reached 1.08%, while in the P0A barrel, the

highest content acidity value reached 1.14%. The analysis results showed variations in acidity levels at specific time intervals (Figure 5). This is thought to be due to the lactic acid produced depending on the pepper size in the samples taken at different time intervals. On the 32nd day of the fermentation barrel P0F, a separation was created based on the size of the pickled peppers, and it was determined that the amount of acid produced depending on the pepper size changed. On the other hand, in the acidification method, the acid level content, depending on the pepper size, did not change.

	0	3	4	13	14	17	19	20	24	25	26	32	40
P0A Pepper Acid %		0.60	0.90	1.02	1.20	1.14	1.08	1.20	1.14	1.14	1.14	1.20	0.90
P0F Pepper Acid %		0.63	0.54	0.81	0.72	0.9	0.9	0.9	1.08	0.99	0.9	0.63	0.72
P0A Pepper pH		4.24	3.78	3.48	3.50	3.52	3.39	3.40	3.47	3.34	3.17	3.32	3.23
P0F Pepper pH		4.63	4.79	3.9	3.89	3.76	3.64	3.65	3.64	3.62	3.4	3.57	3.48

Figure 5. The titration acidity (%) and pH values of the P0A and P0F treatments throughout the fermentation duration.

When the pH analysis results of the P0A and P0F barrels were examined, it was observed that the pH values of the pepper pickles in the P0F barrel decreased during the fermentation period. However, the difference between the two methods is also reflected in the pH values. The brine pH values in the P0F barrel, with the occurrence of fermentation, reduced the lactic acid pH value to 3.4 on the 26th day. According to the content pH analysis results for the P0A barrel, the pH value decreased to approximately 3.50 on the 13th day, while in the P0F barrel, it was observed to decrease on the 26th day (Figures 5 and 6). The observed increase in content and brine acidity in the analysis results for the P0F application and the parallel decrease in pH suggest that no undesirable group of microorganisms formed in the environment during fermentation and that lactic acid fermentation proceeded in a controlled manner.

The pH level is the primary determinant in discerning the various fermentation phases of pickled vegetables. According to Zhang et al. (2023) [25], the pH values of fermented chili pickles decreased significantly during the first 7 days of fermentation and then remained constant. By the 30th day of fermentation, the pH ranged from 3.71 to 3.96, indicating complete fermentation. The pH values of the pickles produced by both fermentation methods were found to be consistent with those reported in the literature.

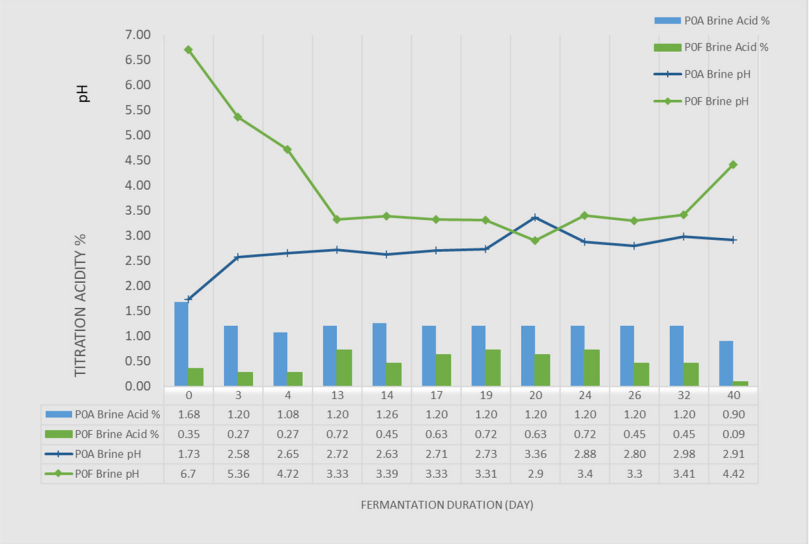


Figure 6. Brine titration acid (%) and pH values of P0A and P0F treatments during the fermentation duration.

Organic acids are essential to the composition of pickled vegetables, as they give them their distinctive sour taste. They not only influence the overall flavor profile but also contribute to the sensory experience of enjoying these preserved culinary delights.

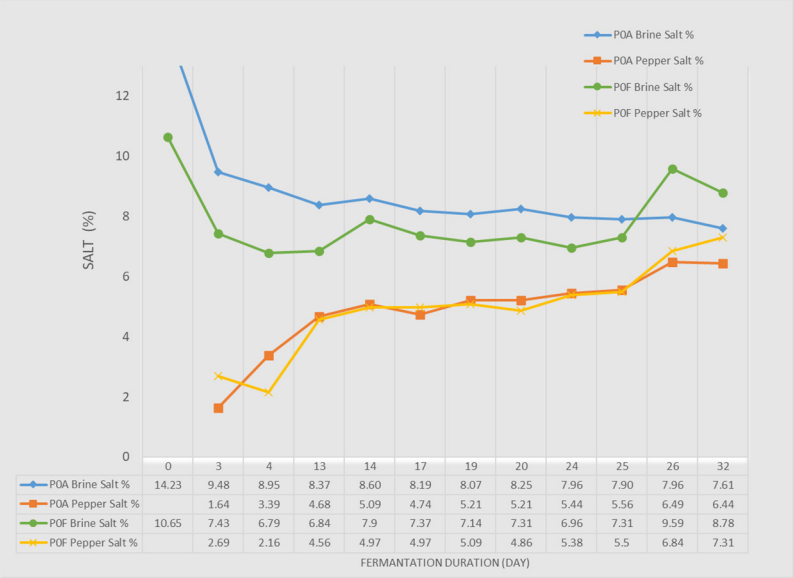
In their analysis, Zhang et al. examined chili peppers that were fermented with aged brine and fresh brine for 30 days, measuring a total of eight organic acid values during fermentation. The analysis revealed that the organic acid content of the peppers fermented with aged brine was higher (ranging from 1 to 0.8) than that of the peppers fermented with fresh brine. The authors attributed this difference to the higher concentration of LAB in the aged brine. Furthermore, our study found that the organic acid content increased with fermentation time, which is consistent with previous research. In addition, Zhang et al. found that fermentation reached its highest level in 14 days and then decreased. In our study, it was observed that the acid values of pickles obtained by the fermentation method reached their highest level on the 24th day and then decreased. The findings are similar to the literature, and this is explained by the fact that lactic acid bacteria can be used as a carbon source in the conversion of organic acids to other flavor substances in pickles [25]. Based on these findings, the acidity levels of pickles produced through the fermentation method align with those reported in the literature.

The brine salinity equivalence of the P0A barrel containing 14% salt and the P0F barrel containing 10% salt began to equalize in 26 and 24 days, respectively. However, since salt and brine were occasionally added to the barrels, deviations occurred in the analysis results during the time intervals when brine was added. The highest salt value reached in the content on the 32nd day was observed in the P0F barrel, with a value of 7.31% (Figure 7).

Even though the gradual fermentation process can take several months, the high salt content effectively preserves the pickles for up to twelve months in large storage tanks before bottling [26].

Pepper pickles taken from the P0F and P0A storage barrels (Figure 8) on days 10 and 32 were pickled, sorted, washed and packed in brines suitable for consumption and final product jars. When the acid analysis results of the P10A-coded pickles were analyzed, it was found that the acid values were in the range of 0.42–0.90%, while the acid values of the P10F-coded pickles produced by fermentation were in the range of 0.54–0.90%. The results of the 32-day semifinished pickles and the total acid analyses of the final products obtained after composition equivalence were in the range of 0.72–0.99%. When the results of the salt analyses were analyzed, it was found that the products coded P10A were saltier than the products coded P10F because more salt was added to the brine. The salt values of

the products coded P32A, P32F1 and P32F2 during their shelf life were between 2.93% and 4.1% (see Supplementary Information Figures S2–S4).



	0	3	4	13	14	17	19	20	24	25	26	32
P0A Brine Salt %	14.23	9.48	8.95	8.37	8.60	8.19	8.07	8.25	7.96	7.90	7.96	7.61
P0A Pepper Salt %		1.64	3.39	4.68	5.09	4.74	5.21	5.21	5.44	5.56	6.49	6.44
P0F Brine Salt %	10.65	7.43	6.79	6.84	7.9	7.37	7.14	7.31	6.96	7.31	9.59	8.78
P0F Pepper Salt %		2.69	2.16	4.56	4.97	4.97	5.09	4.86	5.38	5.5	6.84	7.31

Figure 7. Pepper and brine salt (%) values of P0A and P0F treatments during the fermentation period.

Figure 8. Pickled Üçburun pepper production time-dependent pepper raw material and pickle pictures.

In a study conducted by Çetinyokuş (1991) [27], the brine titration acidity of commercial pickles collected from different provinces was investigated and found to be 0.50–3.70% in pepper. The salt content values of pickled peppers were determined to be 1.73–10.24% [27]. Akbaş (2006) [5] determined the titration acidity values of pepper pickles to be 0.68–1.44% at storage periods of 0, 2 and 4 months at 4 °C and 20 ± 2 °C. Salt content changes in pickles were determined to be 5.01–6.19% [5].

Sensory analysis was carried out throughout the shelf life of the products, looking at changes in taste, odor, texture and color. Tasting tests were carried out with at least 5 panelists at intervals throughout the shelf life of all samples obtained. It was found that

the fastest changing difference of all parameters during shelf life was the color criterion (see Supplementary Information in Figure S2). The final jars of P10A and P10F products started to differ in terms of the taste criterion at month 11. In terms of taste, odor and color parameters, the P10A application maintained the highest score until the 9th month, and as a result of this evaluation, it was considered appropriate to end the shelf life at approximately the 9th month. When the products were evaluated in terms of structural parameters, it was found that the jars with the P10A application were stable for up to 14 months. It was observed that the fastest-changing difference in terms of all parameters during the shelf life of the final product jars of P32A, P32F1 and P32F2, obtained with the semifinished products taken on the 32nd day of storage tanks, was the taste and color criterion. Compared to the other treatments in terms of taste, odor and color parameters, the P32A application maintained the highest score until the 11th day of storage (see Supplementary Information in Figures S3 and S4).

In addition, microbiological analyses were conducted on samples taken from pasteurized pickle jars coded P10A, P10F, P32A, P32F1 and P32F2 using various methods. Micro-organisms were detected in the range of 3.0×10^1 to 8.5×10^1 CFU/g in the TVC analyses of P10A, P10F, P32F1 and P32F2 products on the 4th day. No yeast or lactic acid bacteria were detected in the yeast analyses of the P10A, P10F and P32A samples. However, yeast in the range of 2.5×10^4–2.9×10^4 CFU/g was detected in the 1st- and 10th-month yeast analyses of P32F1 and P32F2 samples. Additionally, no mold was detected in the shelf-life analyses of the samples. Furthermore, the 10th month lactic acid bacteria count analyses of P32F1 and P32F2 samples detected lactic acid bacteria within the range of 5.0×10^3–1.3×10^4 CFU/g (see Supplementary Information Table S1). It was found that this increase in micro-organisms in the jars was due to the increase in biota during the fermentation process of the products and that the pasteurization recipe used was not sufficient to eliminate the micro-organisms in the product. On the other hand, it was found that the initial acid content of the P32A-coded jars obtained by the acidification method was higher than that of the jars obtained by the fermentation method. In this context, it can be said that the pasteurization prescription applied is sufficient for acidification.

Although there are studies on pickles created from various pepper varieties in the literature, there is no research on the fermentation of the Üçburun pepper variety. However, many countries have conducted research on the fermentation process of vegetables and fruits. Pickled Üçburun peppers, which are produced by different companies, are typically created using additives and an acidification method.

4. Conclusions

In summary, this study investigated the LAB present in the biota of Üçburun pepper and compared two different production methods in terms of their suitability for industrial production. LAB with similar characteristics were isolated from the microbial biota of fresh Üçburun peppers from two different regions.

According to the results obtained from our study on additive-free pickle production from Üçburun peppers, it has been determined that the acidification method is more advantageous in an industrial sense compared to the fermentation method. However, the fermentation method is one of the fundamental practices for the widespread application of clean label pickles in today's industry. In this context, in an advanced study, by including culture transfer studies for fermentation methods, increasing the number of experimental setups, conducting metagenomic studies to identify natural microbiota members to be converted into pickles, and subsequently determining the microbiota members in the pickle production stages, after identifying the most suitable cultures in terms of ratios, numbers and types, creating a starter culture, it is considered appropriate for the fermentation method, which was considered advantageous in this study, to replace the acidification method. Bringing the fermentation method to a level where it can compete on an industrial scale is believed to significantly enhance the study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10040196/s1.

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