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Identification of Bacterial Species from Fermented Anchovy (*Engraulis encrasicolus*) Samples by MALDI-TOF MS and Determination of Some Microbiological Properties of These Samples

Nurcan ERBİL[1*], Yusuf ALAN[2]

Highlights:

- Fermented fish products are not very common in Türkiye.
- Fermented anchovy samples contain different bacterial strains.
- The total microorganisms of the Artvin sample is higher than the Trabzon sample

Keywords:

- Fermented anchovy
- MALDI-TOF MS
- Total mesophilic aerobic bacteria
- Total lactic acid bacteria
- Total yeast and mold

ABSTRACT:

Türkiye is a country surrounded by sea on three sides. Therefore, it is very rich in terms of seafood; however, unfortunately, fermented seafood is not very common in our country. Fermented anchovy (*Engraulis encrasicolus*) is traditionally produced in some villages and/or local provinces of the Black Sea Region. In this study, two traditionally fermented anchovy (*Engraulis encrasicolus*) samples obtained from Artvin and Trabzon were used. Characteristic colonies isolated from these samples were identified via MALDI-TOF MS. Moreover, total mesophilic aerobic bacteria, total lactic acid bacteria (LAB), and total yeast and mold of these samples were also determined. *Bacillus thuringiensis*, *Bacillus subtilis*, and *Paenibacillus pabuli* were identified in the Trabzon sample, whereas *Micrococcus luteus* and *Bacillus simplex* were identified in the Artvin sample by MALDI-TOF MS. Moreover, Artvin sample had the more total lactic acid bacteria (LAB), total mesophilic aerobic bacteria, and total yeast and mold than Trabzon sample. This study aims to increase awareness of fermented seafood in our country, and some microbiological properties of these fermented anchovy samples were revealed.

[1]Nurcan ERBİL (**Orcid ID:** 0000-0001-9553-2306), Ardahan University, Faculty of Health Sciences, Department of Emergency Aid and Disaster Management, Ardahan, Türkiye

[2]Yusuf ALAN (**Orcid ID:**0000-0003-0007-0212), Bitlis Eren University, Department of Medical Services and Techniques, Bitlis, Türkiye

*** Corresponding Author:** Nurcan ERBİL, e-mail: nurcanerbil@ardahan.edu.tr

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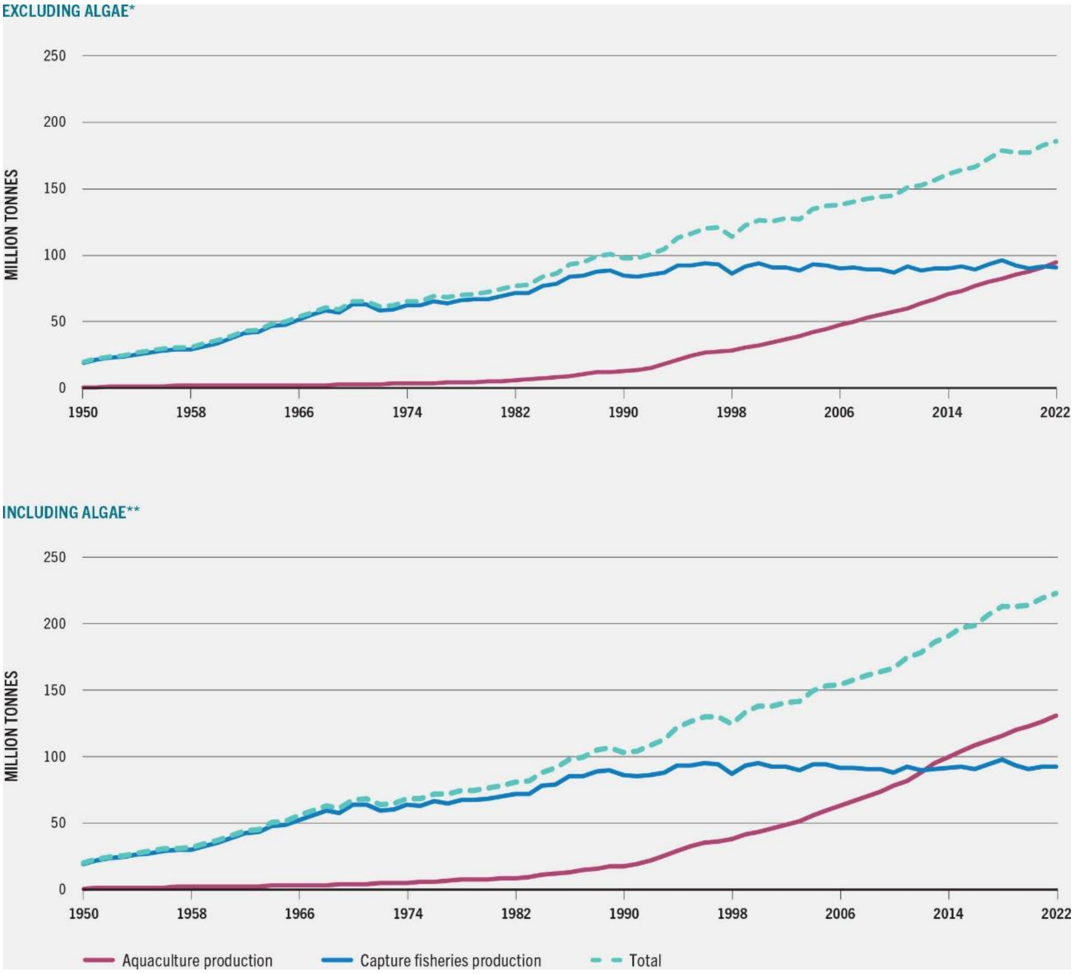
INTRODUCTION

Fish, with its rich protein content and polyunsaturated fatty acids in its structure, meets the body's basic nutrient needs, and is among the most important nutrients in maintaining a healthy life, as it has a positive effect on human physiology and metabolic functions (Kaya et al., 2004).



Şekil 1. World fisheries and aquaculture production (FAO, 2024)

Fishing activity is significant worldwide, and the consumption rate of fish and other seafood is also at a parallel level. As seen in Figure 1, global capture fisheries production has fluctuated between 86 million tons and 94 million tons per year since the late 1980s, reaching 96 million tons in 2018. Aquaculture, on the other hand, has grown significantly during the same period (FAO, 2024).

Humans have developed many different methods for preserving foods since ancient times. One of these methods is fermentation (Erkmen et al., 2020). Fermented foods are an important part of the human diet and also play an essential role in the human health (Mondal et al., 2022). In fact, there are many studies showing that fermented food products support human health and reduce the risk of many diseases (Babio et al., 2015; Larsson et al., 2008; Park & Bae, 2016).

In general, fermented foods are produced in two ways. The first is spontaneous fermentation, in which microorganisms naturally present in raw materials carry out the fermentation. The second method uses a starter culture (Rezac et al., 2018). Spontaneous fermentation is generally preferred for fish fermentation. In studies on fermented fish, bacterial species such as *Lactobacillus pentosus*, *Lactobacillus plantarum, Tetragenococcus halophilus, Enterococcus faecalis, Enterococcus hirae, Staphylococcus carnosus* and *Staphylococcus piscifermentans* etc. have been reported among the microorganisms detected in these fish samples (Tanasupawat & Daengsubha, 1983; Tanasupawat et al., 1992; Tanasupawat et al., 1991; Tanasupawat et al., 1992; Tanasupawat et al., 1998).

Türkiye is a country surrounded by sea on three sides; however, unfortunately, the production and consumption of fermented fish and seafood are not very common in our country. Fermented fish is produced traditionally and on a small scale in some local regions of the Black Sea region. This study was aimed to identify the specific bacterial species and to investigate total mesophilic aerobic bacteria, total lactic acid bacteria (LAB), and total yeast and mold counts of two fermented anchovy samples obtained from Artvin and Trabzon.

MATERIALS AND METHODS

Fermented Anchovy Samples

Two fermented anchovy samples produced by spontaneous fermentation were used in this study. The samples were traditionally produced by the local people from Kuledibi Neighborhood/Hopa/Artvin (Türkiye) and Birlik Village/Of/Trabzon (Türkiye).

In the production stage of fermented anchovies, the heads and guts of Black Sea anchovies (*Engraulis encrasicolus*) were removed and were washed. Then, salt was added to the whole anchovies and allowed to ferment spontaneously at room temperature for 6-8 weeks. Samples were taken into sterile sample containers from local people and stored at 4 °C until further analysis.

Bacterial species identification by MALDI-TOF MS

5 g of each fermented anchovy sample homogenized with 5 mL of 0.85% NaCl solution. The homogenate was centrifuged at 3500 rpm for 2 minutes. 1 mL of the resulting supernatant was taken and centrifuged again at 10000 rpm for 15 minutes. Then, the supernatant was removed and the pellet was suspended in sterile 0.85% NaCl (1 mL). The suspension was then serially diluted up to 10^{-5} and inoculated onto MRS (De Man-Rogosa-Sharpe) and M17 agar media. Plates were incubated at 37 ± 2 °C for 24-48 hours. Typical bacterial colonies were stocked for use in species identification by MALDI-TOF MS (Alan, Topalcengiz, & Dığrak, 2018).

For MALDI-TOF MS (Matrix assisted laser desorption ionization- time of flight mass spectrometry) analyses, preliminary protein isolations were made from pure cultures. The species identification process was continued by comparing the spectra obtained with the device's flex control software program (Biotyper 3.0, Microflex LT, Bruker Daltonics GmbH, Bremen, Germany) with the Maldi Biotyper Real-Time Classification (RTC) software (version.9). As a result of the analysis, data determined as yellow/green between 1.700-3.000 were accepted as reliable score values (Uysal, 2019).

Determination of the counts of total lactic acid bacteria (LAB), total mesophilic aerobic bacteria and total yeast and mold

The diluted samples were cultured using the spread cultural counting method to determine the total mesophilic aerobic bacteria count on Plate Count Agar (PCA) and to determine the total LAB count on De Man, Rogosa, and Elisabeth Sharpe (MRS) and M17 agar media. The plates were incubated at 37 °C for 24-48 hours and then colonies were counted. To determine the total yeast-mold count, cultivation was done on Potato Dextrose Agar (PDA) medium using the spread cultural counting method. The plates were incubated at 25 °C for 48-72 hours and then colonies were counted (Alan, 2019). Colony forming units were calculated as “LogCFU/mL”.

Statistical analyses

The results were calculated as the "mean ± standard deviation" by the SPSS statistical analysis package program (version 25).

RESULTS AND DISCUSSION

Fermented foods are food samples that are formed as a result of the food raw material being metabolized by different microorganisms and changing in terms of appearance, smell, taste and texture. Accordingly, it is normal to find live microorganisms in fermented food samples. In this study, the typical bacterial colonies isolated from the fermented anchovy samples were identified by MALDI-TOF MS and the results were presented in Table 1. *Bacillus subtilis, Paenibacillus pabuli,* and *Bacillus thuringiensis* were identified in Trabzon sample, whereas *Micrococcus luteus* and *Bacillus simplex* were identified in Artvin sample. The results revealed that most species isolated from two fermented anchovy samples belong to the *Bacillus* genus. It was reported that *Paenibacillus pabuli* could degrade the Aflatoxin B1 in foods (Li et al., 2021). It was also reported that *Bacillus* was isolated in an indigenous food to Japan, fermented puffer fish ovaries, "fugunoko nukazuke" (Kobayashi, Okuzumi, & Fujii, 1995). Similarly, *Bacillus* was determined as one of the major microbial population in Cambodian products prahok (fish paste), kapi (shrimp paste) and toeuk trey (fish sauce) (Chuon et al., 2014). The presence of *Bacillus* in fermented seafood was also reported in different studies. The most commonly isolated bacterial genus in Malaysian fermented anchovy sauce has been reported to be *Bacillus* (Mohd Isa et al., 2020; Zaman et al., 2010). *Bacillus* sp., which grows in fermented foods, can potentially produce biosurfactants that can inhibit the growth of undesirable pathogenic bacteria in the environment (Mohd Isa et al., 2020).

Table 1. Bacterial species isolated from fermented anchovy samples

Fermented anchovy samples	Bacterial species
T1*	ND**
T2	ND
T3	*Bacillus subtilis*
T4	*Paenibacillus pabuli*
T5	*Bacillus thuringiensis*
A1***	*Bacillus simplex*
A2	*Micrococcus luteus*
A3	ND

*T: Trabzon sample, **ND: Could not be safely identified, ***A: Artvin sample

Bacillus subtilis was identified in Trabzon sample (Tablo 1). *Bacillus subtilis* is a type of bacteria that has the ability to produce broad-spectrum antibiotics. Because of the low toxicity of these antibiotics, *Bacillus subtilis* is beneficial for many fermented foods. Thanks to the broad-spectrum antibiotics it produces, *Bacillus subtilis* has the inhibition potential against foodborne pathogens, especially *Bacillus cereus* which can produce toxins and can cause foodborne diseases (Bartoszewicz et al., 2008; Eom et al., 2005; Stein et al., 2005; Yeo et al., 2012). In a study investigating the in vitro antioxidant and antibacterial activities of protein hydrolysates prepared by fermentation of fish proteins with *Bacillus subtilis* A26, a proteolytic bacterium and it was reported that these hydrolysates had varying levels of antioxidant and antibacterial activity (Jemil et al., 2014). In another study, lentils were fermented with *Bacillus subtilis* and solid-state fermented lentils by *Bacillus subtilis* showed ACE inhibitory activity (Torino et al., 2013).

Naturally fermented food products can easily become contaminated with toxigenic microorganisms because the preparation process is carried out in open environments (Galvez et al., 2008). *Bacillus thuringiensis* is closely related to the food spoilage bacteria *Bacillus cereus* and can produce δ-enterotoxin. However, *Bacillus thuringiensis* is generally known as an insect pathogen and is generally considered safe for humans. But, some strains of *Bacillus thuringiensis* can produce diarrhoeic enterotoxin in humans (Damgaard et al., 1996). It was reperted that *Bacillus thuringiensis* was determined in *doenjang*, a fermented soybean food (Park et al., 2016).

Table 2. Microbiological properties of fermented anchovy samples

Fermented anchovy samples	**Total lactic acid bacteria (M17)**	**Total lactic acid bacteria (MRS)**	**Total mesophilic aerobic bacteria**	**Total yeast and mold**
		(LogCFU/mL)		
Trabzon sample	1.72 ± 0.08	0.00 ± 0.00	2.20 ± 0.08	1.66 ± 0.08
Artvin sample	2.26 ± 0.08	2.20 ± 0.09	2.32 ± 0.06	2.08 ± 0.12

The microbiological analysis results of fermented anchovy samples were presented in Table 2. It was determined that the total LAB, mesophilic aerobic bacteria and yeast and mold counts in the Artvin sample were higher than in the Trabzon sample. In a previous study, total mesophilic aerobic bacteria (TMAB), yeast-mold (MK), and lactic acid bacteria (LB) of fermented sea bass sausages were detected as 6.25, 3.61, and 5.31 log cfu/g, respectively. The TMAB, MK, and LB, bacterial counts of fermented fish sausages produced from trout were also determined as 6.57, 4.44, and 5.14 log cfu/g, respectively (Kılınç & Çaklı, 2021).

CONCLUSION

Turkish cuisine is one of the well-known culinary cultures in the world. However, unfortunately, fermented seafood has not found the place it deserves in Turkish culinary culture. Although Türkiye is surrounded by seas on three sides, fermented fish or fish products are not common there. Creating awareness about the production and consumption of fermented seafood and popularizing their production will further enrich Turkish culinary culture.

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Conflict of Interest

We declare that Nurcan ERBIL and Yusuf ALAN have no competing interests.

Author's Contributions

Nurcan Erbil: Investigation; Writing - review & editing; Methodology; Funding. Yusuf Alan: Investigation; Methodology.

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