

Enumeration and Identification of Dominant Microflora during the Fermentation of Shalgam

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ABSTRACT

The aim of this study is to isolate and identify lactic acid bacteria and yeast strains of dominant microflora developed during the fermentation of shalgam produced by the traditional method consisting of two stages. Isolated strains were identified by morphological, physiological and biochemical characterizations using the commercially available system API 50 CH and API 20C AUX. The number of LAB and yeast increased during fermentation. The most dominant LAB and yeast during the first and second fermentation stages were *Lactobacillus plantarum* and *Saccharomyces cerevisiae*, respectively. Moreover, low populations of *Lactobacillus pentosus* and *Candida krusei* were present during fermentation. Future studies should focus on the microbiological and technological properties of shalgam in details to improve quality and stability properties of this product. Potential use of starter cultures in shalgam production should be studied, and shalgam producers should be encouraged to use these starter cultures for industrial production.

Keywords: Shalgam, Fermentation, Lactic acid bacteria, Yeast

Şalgam Fermantasyonundaki Baskın Mikrofloranın Sayımı ve Tanımlanması

ÖZ

Bu çalışmanın amacı, iki aşamadan oluşan ve geleneksel üretim yöntemi kullanılan şalgam fermantasyonu sırasında gelişen laktik asit bakterisi ve maya türlerini izole etmek ve tanımlamaktır. İzole edilen türler morfolojik, fizyolojik ve biyokimyasal olarak uygun ticari kitlerin (API 50 CH and API 20C AUX) kullanımıyla tanımlanmıştır. Fermantasyon sırasında laktik asit bakterisi ve maya sayısı artmıştır. Birinci ve ikinci fermantasyon sırasında en baskın laktik asit bakterisi ve maya türünün sırasıyla *Lactobacillus plantarum* ve *Saccharomyces cerevisiae* olduğu bulunmuş ve bunun yanı sıra düşük miktarda *Lactobacillus pentosus* ve *Candida krusei* olduğu saptanmıştır. Araştırmanın sonraki aşamalarında, kalite ve stabilite bakımından daha iyi bir ürün elde etmek için şalgamın mikrobiyolojik ve teknolojik özelliklerinin detaylı olarak belirlenmesi önerilmiştir. Böylece, araştırmacılar starter kültür kullanarak şalgam üretme imkânını düşünecekler ve üreticiler endüstriyel kullanım için bu starter kültürlerden yararlanacaklardır.

Anahtar Kelimeler: Şalgam, Fermantasyon, Laktik asit bakterisi, Maya

INTRODUCTION

Since old years, foods and drinks have been produced by fermentation in most countries. Mainly fermented drinks produced in Turkey are beer, wine, boza, kefir, ayran and shalgam. Especially shalgam is getting more and more popular in Turkey. Shalgam is defined as traditional Turkish fermented drink [1, 2]. Shalgam is a red colored, cloudy and sour drink obtained by lactic acid fermentation. Lactic acid bacteria play an important role during shalgam fermentation [3-5].

According to traditional production methods, ingredients used in shalgam production are bulgur flour, water, black carrot, salt, sourdough and turnip [6]. Today, the most common technology for shalgam industry is based on traditional production methods. Traditional shalgam production consists of two stages. In first stage of shalgam production, bulgur flour, sourdough, salt and water are mixed and left for fermentation at 25°C. Fermentation times vary from 3 to 5 days. First stage of shalgam production is called as first fermentation. First fermentation occurs thanks to microbial flora in sourdough. The main microbial flora in sourdough are *Lactobacillus sanfranzisko*, *L. brevis*, *L. plantarum*, *L. fructivorans* and *L. fermentum*, mayalardan; *Turulopsis holmii*, *Saccharomyces cerevisiae*, *Pichia saitoi* and *Candida krusei*. In second stage of shalgam production (2nd fermentation), salt, sliced black carrot and turnip are added to extracts from first fermentation. Mixtures are again left for fermentation, at 25°C. Fermentation times are about seven days. After fermentation, shalgam is stored at 4°C [1, 7-12].

Shalgam is produced around especially Adana in Turkey, and it is mostly consumed together kebabs and the other meat meals. Today, shalgam is sold in several super markets. The number of research about shalgam is quite few. The microbiology of shalgam is complex and not known in details. The fermentation of shalgam is carried out spontaneously and contains mixed cultures of mainly lactic acid bacteria and yeasts. Shalgam producers do not inoculate the fermentation tanks with selected strains of lactic acid bacteria or yeasts, because there are no commercial cultures available for shalgam fermentation. Actually shalgam production with selected strains can be more convenient to obtain a standard product. Furthermore there is not enough information about microbial flora of shalgam. So it is necessary to do detailed researches about microbial flora of shalgam. Thus, commercial selected strains or starter culture will be used industrially on shalgam production. Use of starter culture will cause quality and standard production [13-18].

In this study, it was aimed to isolate and identify lactic acid bacteria and yeasts during first and second fermentation in traditional shalgam production.

MATERIALS and METHODS

Shalgam Production

Shalgam were produced in a small scale industry in Adana region of Turkey. Shalgam production by traditional method was carried out according to Erten et al. [1]. Traditional method consisted of two stages. At stage one (first or dough fermentation), bulgur flour (30 g/L), rock-salt (2 g/L), sourdough (2 g/L) made with the incubation of baker's yeast at 30°C for 24 h and adequate drinkable water were mixed and kneaded for the formation of dough. The dough obtained was fermented in a tank at 25°C for 3 days. After this time, about 20 L of water was added into fermented mixture, blended well and extracted for 15 min. The extraction was carried out four times. The extracts obtained from the first fermentation were combined to perform the second (carrot or main) fermentation with sorted and chopped black carrots (150 g/L), rock-salt (10 g/L), sliced turnip (10 g/L) in a 100 L of closed stainless steel tank. If necessary, adequate water was added to fill the tank. Fermentation was carried out at 25°C and followed daily by measuring pH. Shalgam was produced in triplicate.

Enumeration and Isolation of LAB

During the first (dough) fermentation, daily samples of 25 g of dough were dispersed aseptically into 225 mL of sterile physiological saline (8.5 g/L) and mixed thoroughly. During the second (carrot) fermentation, shalgam samples (200 mL) were taken from the centre of 100 L of fermentation vessel. Samples were serially diluted (10^{-1} to 10^{-8}) in sterile physiological saline and spreadinoculated (0.1 mL) onto plates of de Man, Ragosa, Sharpe (MRS) Agar (Merck AG, Darmstadt, Germany), supplemented with 50 mg/L cycloheximide to prevent the growth of yeasts and moulds. Plates were incubated at 30°C for 3-5 days in jars made anaerobic with GasPaks (Anaerocult A, Merck AG, Darmstadt, Germany) for colony development. The LAB colonies were counted on their morphology from the plates and various colony types were selected and purified by restreaking three times on MRS agar (without cycloheximide) to obtain pure cultures. Gram-positive and catalase-negative isolates were maintained as liquid cultures in MRS broth with 200 g/L sterilized glycerol at -20°C for subsequent identification [19-22].

Identification of Isolates of LAB

A total of 10 isolates were identified by morphological, physiological and biochemical characterisation. Isolates were tested for Gram reaction, catalase formation, cell morphology, CO₂ production from glucose, ability to grow at 10°C and 45°C and at pH 4.4 and 9.6, tolerance to 65 g/L and 180 g/L salt, hydrolysis of arginine, nitrate reduction, acetoin formation and MR-VP test. Subsequently, API 50CH galleries and API CHL medium

(Bio-Mérieux, France) were used according to the manufacturer's instructions for determining the utilization of 49 carbohydrates of isolated strains. APILAB PLUS database identification software (BioMérieux, France) was used to interpret the results [23-26]. All results obtained by API were found between good identification/excellent identification.

Enumeration and Isolation of Yeast

During the first (dough) fermentation, daily samples of 25 g of dough were dispersed aseptically into 225 mL of sterile physiological saline (8.5 g/L) and mixed thoroughly. During the second (carrot) fermentation, shalgam samples (200 mL) were taken from the centre of 100 L of fermentation vessel. Samples were serially diluted (10^{-1} to 10^{-8}) in sterile physiological saline and spread inoculated (0.1 mL) onto plates of PDA with cultural cultivation method and incubated at 25°C, for 72 hours [23]. The yeast colonies were counted on their morphology from the plates and various colony types were selected and purified by restreaking three times on malt extract agar to obtain pure cultures. Isolates were maintained as liquid cultures in nutrient broth with 200 g/L sterilized glycerol at -20°C for subsequent identification [23, 27-29].

Identification of Isolates of Yeast

A total of 10 isolates were identified by morphological, physiological and biochemical characterisation. Primary classification of colonies from the PDA plates was based on colony characteristics (pigmentation and shape), mode of vegetative reproduction, formation of hyphae or pseudohyphae and ascospore production. The methods described by Harrigan and McCance [23] were followed. Identification of the yeast isolates to species level was done using the API 20C AUX (BioMérieux, Marcy l'Etoile, France) system of carbohydrate assimilation profiles. All results obtained by API were found between good identification/excellent identification.

Analysis of pH

pH was determined using a pH meter (Inolab WTW, Weilheim, Germany) [30, 31].

RESULTS and DISCUSSION

In this study, shalgam fermentations were carried out using traditional method which consists of first (dough) fermentation and second (carrot or main) fermentation and growth of LAB and yeasts were examined.

Enumeration and Identification of LAB Species and Yeast during First (Dough) Fermentation

Figure 1 shows the growth of lactic acid bacteria and yeast during the first fermentation, which was carried out for 4 days by mixing bulgur flour, salt, sourdough and

adequate drinkable water to form dough for the production of shalgam.

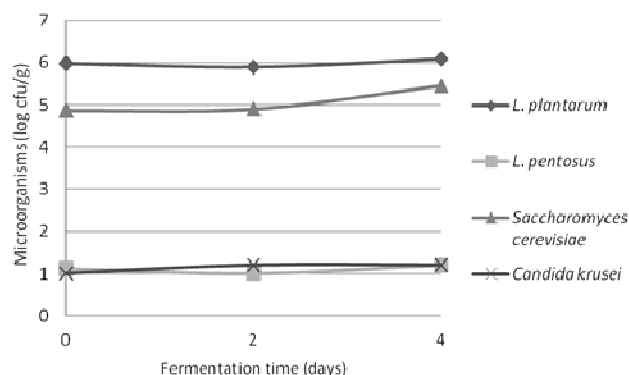


Figure 1. The growth of lactic acid bacteria and yeast (log cfu/g) during the first fermentation.

At the beginning of first fermentation of shalgam, total viable LAB count was 7.10 log cfu/g and total viable yeast count was 5.86 log cfu/g. As can be seen from our results, count of yeast (5.86-6.66 log cfu/L) was lower, relative to the LAB. Also, at the end of the fermentation (eight day), both the number of lactic acid bacteria (7.30 log cfu/g) and yeast (6.66 log cfu/g) reached highest value.

L. plantarum ranging populations from 5.90 to 6.10 log cfu/g were the dominant LAB in first fermentation of shalgam at the beginning (Fig. 1). Also *L. pentosus* was identified but its counts were highly low (1.00-1.20 log cfu/g). *L. plantarum* and *L. pentosus* slightly declined to 5.90 and 1.00 log cfu/g, respectively on the 2nd day. But *L. plantarum* and *L. pentosus* continued to proliferate giving maximum populations of 6.10 log cfu/g and 1.20 log cfu/g, respectively on the 4th day.

The dominant yeast in first fermentation of shalgam were *Saccharomyces cerevisiae*. Whereas counts of *Candida krusei* (1.00-1.20 log cfu/g) was lower than *Saccharomyces cerevisiae*. The number of *Saccharomyces cerevisiae* were 4.86 log cfu/g in first fermentation of shalgam at the beginning, then on second and fourth days its number increased (respectively 4.90 and 5.46 log cfu/g). Number of *Candida krusei* increased on the 2nd day of fermentation and then its proliferation was stationary on the 4th day of fermentation.

Although shalgam is a very popular beverage in such metropolitan cities as Istanbul, Ankara and Izmir Metropolises in Turkey, there are limited studies about shalgam. Especially, there are a few studies about microflora that play an important role in shalgam fermentation. Microflora in shalgam fermentation is very important because this microflora is responsible for shalgam fermentation. It is generally accepted that the main fermentation agents of shalgam are LAB which are responsible for the acidification process by converting sugars into mainly lactic acid and other end compounds,

giving shalgam its typical taste and flavour [32]. In addition to lactic acid bacteria, yeasts improve during shalgam fermentation. Yeasts have low effect on acidification, whereas they are highly effective on aroma of shalgam. It was thought that there is a symbiotic association between yeast and lactic acid bacteria involved in shalgam fermentation [2].

Populations of LAB and yeast at the beginning of first fermentation were lower than at the end of the first fermentation. Their high populations at the end of the first fermentation were probably due to the raw materials, especially sourdough flora. Similar high levels were reported by Aydar [15], Gunes [17], and Utus [33], who found the total viable LAB levels ranged from 6.97 to 8.14 log cfu/g at 0 day to 7.1-8.90 log cfu/g at the end of first fermentation.

Our results in which the main species isolated were *L. plantarum* confirm the previous study of Tanguer [32] who isolated the dominating species as *L. plantarum*, *L. paracasei* subsp. *paracasei* and *L. brevis* during first fermentation of shalgam produced by traditional methods. There weren't any study about identification of yeasts during first fermentation in shalgam production, so this study is very important. As it is known, both yeast and lactic acid bacteria play an important role. It is not enough to identify only lactic acid bacteria in shalgam for microflora occurred during shalgam fermentation. In addition to lactic acid bacteria, yeast effect on flavour of shalgam. So identification of yeast is necessary.

Enumeration and Identification of LAB Species and Yeasts during Second (carrot) Fermentation

During the second fermentation, occurrence and growth of LAB species were examined in shalgam. Population dynamics of total viable LAB are given in Figure 2.

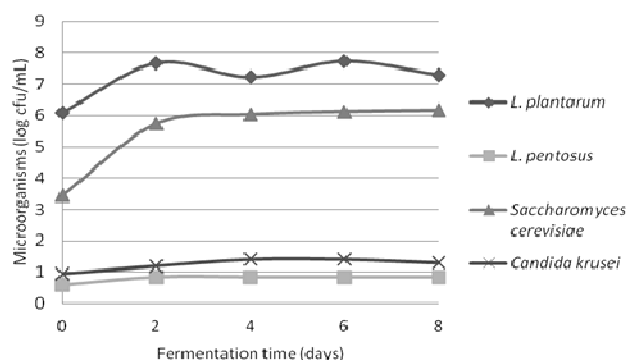


Figure 2. The growth of lactic acid bacteria and yeast (log cfu/mL) during the second fermentation

At the beginning of second shalgam fermentation, total viable LAB count was 6.70 log cfu/mL and total viable yeast count was 4.40 log cfu/mL. On the 6th day of the fermentation, both the number of lactic acid bacteria (8.56

log cfu/mL) and yeast (7.53 log cfu/mL) reached a maximum and then slight decline (respectively, 8.13 log cfu/mL and 7.46 log cfu/mL) was observed at the end of the fermentation.

L. plantarum ranging populations from 6.10 to 7.72 log cfu/mL were the dominant LAB in second fermentation of shalgam likely first fermentation. Whereas counts of *L. pentosus* were highly low (0.60-0.84 log cfu/mL).

Counts of yeast (5.86-6.66 log cfu/mL) in second fermentation were lower than the LAB. The dominant yeast in second fermentation of shalgam were *Saccharomyces cerevisiae* like as in first fermentation. The number of *Saccharomyces cerevisiae* were 3.45 log cfu/g in first fermentation of shalgam at the beginning and than its counts increased day by day. At the end of the fermentation, *Saccharomyces cerevisiae* reached a maximum (6.16 log cfu/mL). Whereas it was found that counts of *Candida krusei* (0.95-1.41 log cfu/mL) was lower than *Saccharomyces cerevisiae*. Also number of *Candida krusei* (1.30 log cfu/mL) increased at the end of the fermentation.

Second fermentation is naturally carried out like as the first fermentation, so microflora in second fermentation is mostly similar to first fermentation. As can be seen from our results, species of identified lactic acid bacteria and yeast are the similar or the same. Moreover, numbers of lactic acid bacteria and yeast can change during first fermentation or second fermentation [1].

According to our result, counts of lactic acid bacteria and yeast during second fermentation were more than during first fermentation. Also, in our study, the amount of lactic acid bacteria and yeast increased towards the end of second fermentation. Similar growth patterns were reported in previous studies by Gunes [17] and Utus [33] who found the initial total LAB counts between 7.31 and 7.95 log cfu/mL and the last counts in the range of 6.76-7.66 log cfu/mL at the end of fermentation.

The results of this study in which the main species isolated were *L. plantarum* confirm the previous study of Arıcı [7] who isolated the dominating species as *L. plantarum* and *L. paracasei* subsp. *paracasei* in bottled shalgam beverages sold at the market. *L. plantarum* subsp. *arabinosus* was also isolated from shalgam fermentation [2].

However, in this study, only two species of lactic acid bacteria (*L. plantarum* and *L. pentosus*) and yeast (*Saccharomyces cerevisiae* and *Candida krusei*) were identified. Another different species weren't identified. It was considered that the death of another different species during shalgam fermentations was due to their relative sensitivity to acidic and salty conditions.

Lactobacillus and yeast were isolated from fermenting plant material [34, 35]. These species were also found in some shalgam fermentations in present study in accordance with the data given by Erginkaya and Hammes [2].

pH Levels during the First (dough) and Second Fermentation of Shalgam

Development of pH during first and the second fermentation of shalgams is given in Table 1. For shalgam, pH value decreased from 3.93 at day 0 of first fermentation to 3.90 after 4 days of fermentation. In second fermentation, pH levels were 4.16 in shalgam at day 0. At the end of fermentation, pH values were measured as approximately 3.26.

Table 1. pH levels during the first (dough) and second fermentation of shalgam

Fermentation time (day)	pH1	pH2
0	3.93	4.16
2	3.86	3.33
4	3.90	3.40
6	-	3.36
8	-	3.26

The decrease in pH and increase in lactic acid followed the same trend as reported for other natural fermented foods. The high levels of lactic acid might be attributed to the predominance of LAB. A slight decrease in lactic acid concentration was observed in the late stages of fermentation. Namely, decrease in pH or increase in acidity is an indicator of good fermentation [36]. Also in our study, it was observed increase in pH at the end of the fermentation. Similar results for pH (4.01-5.01) were reported at the end of fermentation in previous investigations using traditional method for shalgam production [17, 33, 37]. Drop in pH during fermentation are good parameters to monitor the development of microorganisms. According to our pH results, at the end of the fermentation, pH decreased especially as depending rising in count of lactic acid bacteria. pH levels in this study are in good agreement with the previous study of Canbas and Deryaoglu [37].

CONCLUSION

As a result, this study has shown that the microflora involved in production of shalgam comprises a combination of LAB and yeasts. The dominant species isolated during the fermentations made at small scale production plants in industry were *L. plantarum* and *Saccharomyces cerevisiae*. In addition to these species, *L. pentosus* and *Candida krusei* was identified in lower proportion.

In conclusion, future directions of research should be performed on microbiological and technological properties of shalgam in details to obtain better product in terms of

quality and stability. Thus, researchers should think about possibility of shalgam production by using starter culture and producers should utilize from this starter culture for industrial usage. Also, shalgam fermentation can be controlled and thus standard products can be obtained and quality of shalgam may be improved. Thanks to industrial production of shalgam by starter culture, shalgam can be recognized at international level and it can be produced throughout the world.

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