



بهینه سازی کیفیت گوشت تخمیری گاو به وسیله باکتری های اسید لاکتیک در تخمیر بسته

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چکیده

سابقه و هدف: باکتری های اسیدلاکتیک در ایجاد جنبه های حسی مطلوب در گوشت تخمیری ضروری می باشند. هدف از این پژوهش، تقویت کیفیت گوشت گاو با بهینه سازی شرایط تولید اسید توسط لاکتوباسیلوس ساکنی و لاکتوباسیلوس پلانتاورم در تخمیر بسته بوده است.

مواد و روش ها: در مطالعه مقطعی توصیفی حاضر دو سویه باکتریایی لاکتوباسیلوس ساکنی زیرگونه ساکنی PTCC171 و لاکتوباسیلوس پلانتاورم PTCC1058 در محیط کشت MRS تکثیر و با روش مولکولی تأیید شدند. با استفاده از نرم افزار تاگوچی تعداد ۱۶ آزمایش برای بهینه سازی سه عامل دما، تلقیح باکتری و افزودن گلوکز طراحی شد. نتایج بر اساس تولید اسید بررسی و با برنامه ANOVA مقایسه شد. شمارش کلی باکتری ها، باکتری های اسید لاکتیک، انتروباکتریاسه و کپک/مخمر در محصول، با روش های استاندارد میکروبی انجام شد.

یافته ها: بیشترین تولید اسید به وسیله لاکتوباسیلوس ساکنی در دمای ۳۶ °C، ۱۰٪ گلوکز و تلقیح ۸۰۰۰ واحد تشکیل دهنده کلنی و به وسیله لاکتوباسیلوس پلانتاورم در دمای ۳۷ °C، ۱۰٪ گلوکز و تلقیح ۹۰۰۰ واحد تشکیل دهنده کلنی تشخیص داده شد. منبع کربن، مؤثرترین عامل بر کاهش pH به وسیله هر دو باکتری بود. باکتری های اسیدلاکتیک افزایش ۴ برابری را پس از اتمام تخمیر نشان دادند و ۶۰٪ آن ها پس از حرارت دهی زنده ماندند. باکتری های انتروباکتریاسه در محصول یافت نشدند و سایر پاتوژن ها نیز کاهش شدید داشتند. هر دو باکتری به طور هم زمان، موجب ۹/۶٪ کاهش بیشتر در pH شدند.

نتیجه گیری: سویه های لاکتوباسیلوس مورد استفاده شرایط مشابهی برای تخمیر گوشت گاو داشتند و هنگام استفاده هم زمان، فعالیت هم افزایی در تولید اسید نشان دادند.

واژگان کلیدی: گوشت تخمیری گاو، باکتری های اسید لاکتیک، بهینه سازی.

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Optimization of fermented cow meat quality by lactic acid bacteria in batch fermentation

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Abstract

Background & Objectives: Lactic acid bacteria are essential factors for obtaining optimum sensory aspects in fermented meat. The aim of this study was the enhancement of cow meat quality by optimization of acid production using *Lactobacillus sakei* and *Lactobacillus plantrum* in batch fermentation.

Materials & Methods: In this cross-sectional descriptive study, *L. sakei* subsp. *sakei* PTCC1712 and *L. plantrum* PTCC1058 were grown in MRS medium and confirmed by molecular identification. Using *Taguchi* software (16 version), trials were designed to optimize three factors including temperature, bacterial inoculation, and glucose supplementation. The results were analyzed based on detection of acid production and compared by *ANOVA* program. Total product bacteria, lactic acid bacteria, Enterobacteriaceae, and yeasts/molds were counted by standard microbial methods.

Results: Maximum acid production for *L. sakei* was detected at 36 °C, 10% glucose and 8000 CFU.g⁻¹ inoculated bacteria; and for *L. plantarum* was detected at 37 °C, 10% glucose, and 9000 CFU. g⁻¹ inoculated bacteria. The best factor affecting pH decline was a carbon source for both bacterial strains. Lactic acid bacteria showed a fourfold increase after fermentation and maintained 60% of their viability following heating stage. No Enterobacteriaceae was found in the product, and other pathogens showed a great decrease. Using both strains simultaneously, 6.9% improvement in acid production was observed.

Conclusion: Both *Lactobacillus* strains had similar conditions for cow meat fermentation and showed synergistic activity for acid production when used simultaneously.

Keywords: Fermented cow meat, Lactic acid bacteria, Optimization.

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Introduction

Fermentation is one of the oldest technologies which had been used for food preservation for a long time (1). For centuries, fermentation was used to maintain and improve quality and taste of whole grains, fruits, vegetables, legumes, and meat probably because of its simplicity, and economic value.

On the other hand, fermented foods are rich sources of microorganisms, some of which have probiotic properties. Addition of probiotics to yogurt and other fermented dairy products have been increased in the past, and today it is growing for non-dairy foods as well as other applications such as therapeutic purposes (2, 3).

Fermented meat is one of “functional foods” and a new approach to achieve healthier status by reducing disease risk as well as antimicrobial effects (4). Lactic acid bacteria (LABs) as the starter in fermented meat products play an important role in preventing corruption and creating flavor and favorable texture (5).

Lactobacillus sakei, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus carvatus*, and *Lactobacillus xylosus* have been reported as the most important meat fermenting bacteria. Other bacteria such as Gram-positive, coagulase-negative *Staphylococcus* spp. and *Kocuria* spp. are proposed as technologically fundamental bacterial strains in meat fermentation (6).

One of the most important lactic acid bacteria which are used as a starter culture in fermented meat is *L. sakei*. The bacterium has the ability for flexibly adaptation to the environment and

meat components (7).

It is probable that the phenotypic changes in colony morphology and cell shape during colonization of *L. sakei* in the gastrointestinal tract is an advantage for selective growth in this environment (8).

Furthermore, *L. sakei* produces sakacin, a class II bacteriocin with strong antimicrobial activity against a wide range of foodborne pathogenic microorganisms, especially members of *Enterobacteriaceae* (9).

Another important bacterium in fermented meat is *L. plantarum*. Various strains of *L. plantarum* are able to ferment sugars to lactic acid in L and D isomeric forms. Also, this bacterium has shown strong inhibitory effect on pathogenic bacteria such as *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* (10, 11).

The aim of this study was optimization of acid production by *L. plantarum* PTCC1058, and *L. sakei* subsp. *sakei* PTCC1712 according to bacterial cell count, temperature and the carbon source to enhance fermented meat quality.

Materials and methods

Strains, media and growth conditions

L. sakei subsp. *sakei* PTCC1712, and *L. plantarum* PTCC1058 were obtained from the microbial collection of Iranian Scientific and Industrial Researchers Organization.

According to the organization instructions, the bacteria were reactivated in MRS Broth (Scharlu, Spain), at 37°C for 24 hours. Bacterial strains were frozen and stored at -70°C in MRS broth, supplemented with 20% glycerol. Fresh cow meat was purchased during one stage, wheeled out in sterile meat grinder

wheel, and stored at -20°C.

Molecular confirmative identification of bacteria

Total DNA was extracted by Sinaclone extraction kit (DN8115C). The bacterial strains were identified by amplification of *16S rDNA* fragment (12) using universal primers (Rw01: 5'-AACTGGAGGAAGGTGGGGAT-3', and Dg74: 5'-AGGAGGTGATCCAACCGCA-3'). The amplification protocol was performed in 4 steps as follows: step 1: 95 °C 60 S; step 2: 94 °C 60 s, 58 °C 50 s, 72 °C 60 s (repeated 6 times); step 3: 94 °C 60 s, 56.5 °C 60 s, 72 °C 60 s (repeated 37 times), and step 4: 72 °C 5 min. The polymerase chain reaction (PCR) mixture included 1X PCR buffer, 1.5 mM MgCl₂, 0.5 mM dNTP, 0.5 µM of each primer, 1 unit Taq DNA polymerase, and 1 µg of extracted DNA in a total volume of 50 µl. PCR products were detected by agarose gel electrophoresis. The amplified final products were sequenced by the ABI3730XL system (Bioneer Company, Korea), and aligned with the current sequences in BLAST database.

Lactic acid production according to growth stages

Initial 10000 cells of each *Lactobacillus* sp. was separately added to 1 gr of meat samples, supplemented with 20% (w/w) glucose. Samples were incubated at 37 °C. To assess maximum acid production according to growth stages, the pH value, as well as bacterial growth, was measured every 6 hours. Bacterial growth was detected by measurement of light absorbance at a wavelength of 620 nm by supernatant of meat samples following centrifugation at 500 rpm for 10 minutes (13).

Acid production optimization

Three factors including temperature (35-39 °C), bacterial count (8000-12000 CFU. g⁻¹), and the amount of additive glucose (5-40%) were optimized individually as well as a fractional factorial design using Taguchi statistical method (*Qualitek 4* software) for experiment design (14, 15).

Acid production by bacteria in fermenting cow meat was considered for detection of best conditions. A total number of 16 trials were designated. All treatments were performed in triplicate to reduce experimental error.

Fermented sausage production

Fermenting sausages containing beef (84%), fat (20%), ice (9.3%), soy (2.8%), salt (0.93%), powdered sugar (1.5%), sodium nitrite (0.012%), black pepper (0.37%), garlic (0.37%), and starch (2%) was prepared. Glucose was added according to the best amount which obtained the results of Taguchi designed experiments. Above-mentioned materials were mixed in a sterile meat grinder; then the bacteria were inoculated according to the best count obtained as the results of experiments designed by Taguchi method. After wrapping up, sausages were fermented for 3 days at the best temperature obtained in designated Taguchi experiments and dried out for 3 h at 60 °C (16, 17).

Simultaneous optimization of two bacterial strains

According to optimization experiments results, both bacterial strains were simultaneously inoculated into meat samples at different rates. pH values were recorded following fermentation process.

Microbial analysis of the product

Serial dilutions of sausage samples in different production stages were prepared using Ringer solution. Then total bacteria in plate count agar (PCA-Quelab, USA), lactic acid bacteria in MRS agar (Scharlau, Spain), coliforms in eosin methylene blue agar (EMB, Scharlau, Spain), and *Staphylococcus aureus* in mannitol salt agar (MSA, Scharlau, Spain) were counted in the pour pale inoculated media. The samples were taken initially before filling in a wrapper, after fermentation and after the heating stage. The cultivated media were incubated at 37 °C for 24 hours. Sabouraud dextrose agar (SDA, Scharlau, Spain) medium was used for counting molds and yeasts in above-mentioned samples, and the inoculated media were incubated at 28 °C for 96 hours (18).

Statistical analysis

The results of optimization experiments were analyzed using *ANOVA* test. Determination of phylogenetic relationships between identified

fragments and relative sequences, and drawing the phylogenetic trees were carried out using *MEGA5* software (19).

Results

Confirmative identification of bacteria

The bacterial strains were identified as short gram-positive rods in Gram staining. Alignment of the sequences of 370 bp bands

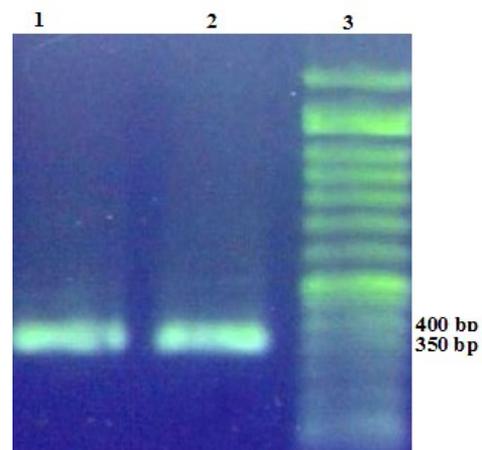


Figure 1. The 370 bp band obtained in amplification of 16SrDNA sequence by universal primers (RW01 and DG74). Lane 1: *Lactobacillus sakei*, lane 2: *Lactobacillus plantarum*, lane 3: 50 bp DNA marker.

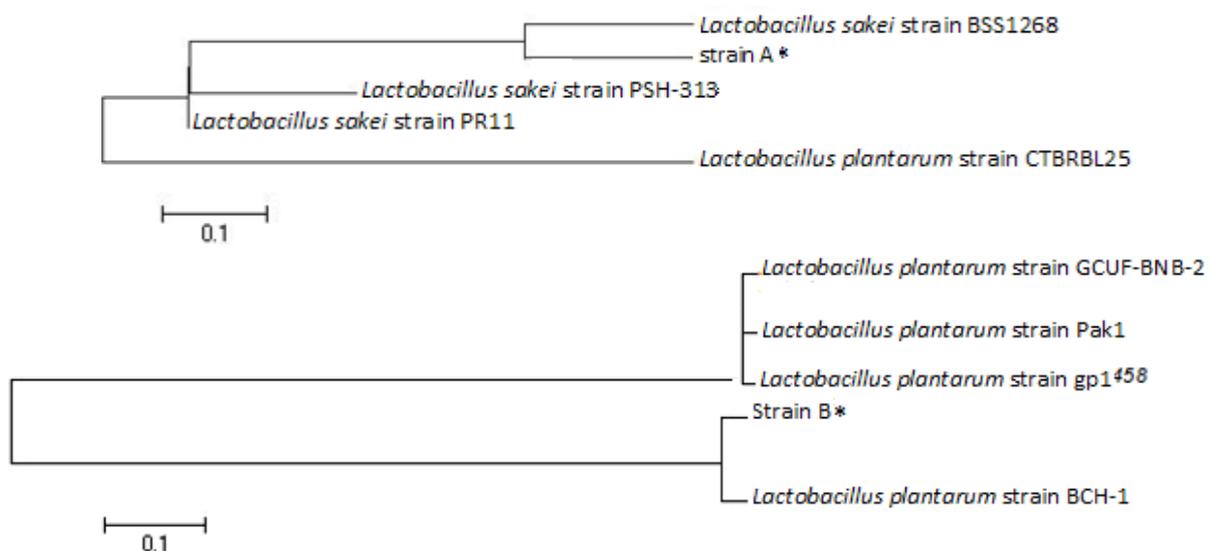


Figure 2. Phylogenetic tree of the isolated bacteria shows relationship to *Lactobacillus sakei* (Strain A*) and *Lactobacillus plantarum* (Strain B*).

(Figure 1) showed both *L. sakei* and *L. plantarum* strains in gene bank, with 99% similarity for both strains. Phylogenetic trees of both strains are shown in Figure 2.

Bacterial growth curve

As shown in Figure 3, maximum acid production for *L. sakei* occurred after 30 hours growth in meat which is approximately at the start point of the stationary phase of growth. Maximum acid production for *L. plantarum* was detected after 36 hours in meat which is at the end of the stationary phase of growth (Figure 4).

The best individual conditions affecting acid production

Maximum acid production by *L. sakei* and *L. plantarum* occurred at 37 °C, and 36 °C, respectively (Figure 5). Moreover, both bacterial strains showed the most decrease in pH when 11000 CFU. g⁻¹ bacterial cells were inoculated to each gram of meat, and 10% (w/w) glucose was added before starting fermentation process (Figures 6 and 7).

Optimization of acid production by experiments designed by Qualitek 4 software

The results of designed 16 trials (Table 1) showed that maximum acid production for *L. sakei* occurs at the temperature of 36 °C, supplementation of 10% glucose, and 8000 CFU. g⁻¹ bacterial inoculation (trial 1). Also, *L. plantarum* showed maximum acid production at the temperature of 37 °C, supplementation of 10% glucose, and 9000 CFU. g⁻¹ bacterial inoculation (trial 5).

Analysis of variances using ANOVA test indicated that carbon source has the most

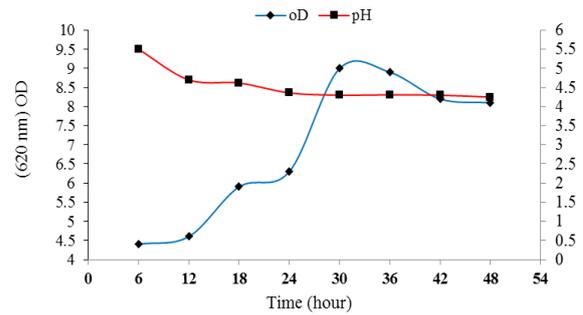


Figure 3. Acid production (detected by pH decline) according to growth stages of *Lactobacillus sakei*.

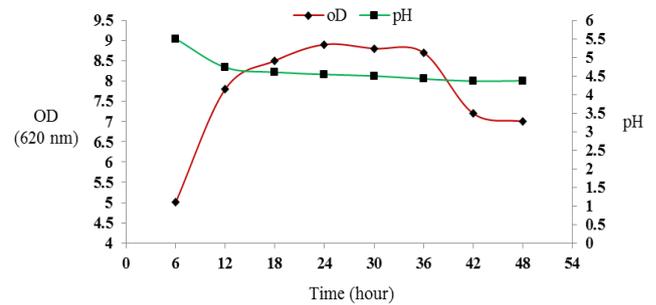


Figure 4. Acid production (detected by pH decline) according to growth stages of *Lactobacillus plantarum*.

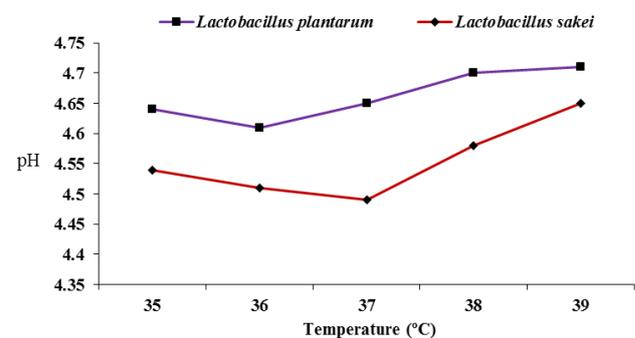


Figure 5. The best temperature in which bacteria showed maximum acid production; detected by the most declines in pH of meat.

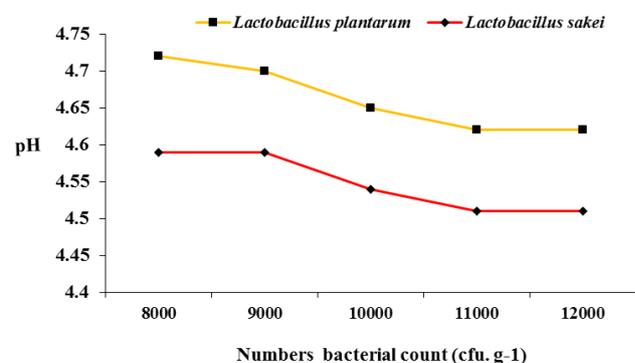


Figure 6. The best inoculated number of bacteria which resulted in maximum acid production; detected by the most declines in pH of meat.

significant effect on pH reduction (60.2%), and acid production (73.7%) by *L. sakei* (Table 2), and *L. plantarum* (Table 3), respectively.

Simultaneous optimization of two bacterial strains

According to the results of optimization experiments, meat samples with 10% glucose supplementation were inoculated with different bacterial counts and subsequently incubated at 36 °C. The results of acid production are shown in Table 4. No significant difference was observed but in comparison to the results

of acid production optimization by each bacterium (Table 1) 6.9% improvement in pH decline was observed in simultaneous application of both strains.

Microbial analysis of the fermented sausage

At the end of heating stage, the total bacterial count was 190 CFU. g⁻¹. Furthermore, lactic acid bacteria count was 180 CFU. g⁻¹, mold/yeast count was 7 CFU. g⁻¹, and *S. aureus* count was 3 CFU. g⁻¹. The amount of LABs was increased four times after fermentation,

Table 1. The results of 16 trials which were designed for experiments by *Taguchi* method (*Qualitek 4* software) for *Lactobacillus sakei* and *Lactobacillus plantarum*.

| Trial number | Temperature (°C) | Glucose (%) | Inoculation (CFU. g ⁻¹) | pH value(inoculation by <i>Lactobacillus sakei</i>) | pH value (inoculation by <i>Lactobacillus plantarum</i>) |
|--------------|------------------|-------------|-------------------------------------|--|---|
| 1 | 36 | 10 | 8000 | 4.37 | 4.57 |
| 2 | 36 | 20 | 9000 | 4.47 | 4.60 |
| 3 | 36 | 30 | 10000 | 4.61 | 4.66 |
| 4 | 36 | 40 | 11000 | 4.61 | 4.73 |
| 5 | 37 | 10 | 9000 | 4.42 | 4.36 |
| 6 | 37 | 20 | 8000 | 4.47 | 4.60 |
| 7 | 37 | 30 | 11000 | 4.47 | 4.62 |
| 8 | 37 | 40 | 10000 | 4.61 | 4.82 |
| 9 | 38 | 10 | 10000 | 4.45 | 4.56 |
| 10 | 38 | 20 | 11000 | 4.40 | 4.57 |
| 11 | 38 | 30 | 8000 | 4.62 | 4.76 |
| 12 | 38 | 40 | 9000 | 4.84 | 4.92 |
| 13 | 39 | 10 | 11000 | 4.57 | 4.59 |
| 14 | 39 | 20 | 10000 | 4.51 | 4.58 |
| 15 | 39 | 30 | 9000 | 4.65 | 4.70 |
| 16 | 39 | 40 | 8000 | 4.88 | 4.97 |

Table 2. The results of variance analysis for detection of the effects of different factors on acid production by *Lactobacillus sakei*.

| Factor | Degrees of freedom | Sum of squares | Variance | Variance Ratio | Total net | Percent |
|----------------------------|--------------------|----------------|----------|----------------|-----------|---------------|
| Temperature | 3 | 0.189 | 0.063 | 16.431 | 0.177 | 17.8 |
| Carbon source | 3 | 0.613 | 0.204 | 53.238 | 0.602 | 60.2 |
| Inoculated bacterial count | 3 | 0.051 | 0.17 | 4.454 | 0.039 | 4.0 |
| Other/error | 38 | 0.145 | 0.003 | | | 18.0 |
| Total | 47 | 1 | | | | 100.00 |

Table 3. The results of variance analysis for detection of the effects of different factors on acid production by *Lactobacillus plantarum*.

| Factor | Degrees of freedom | Sum of squares | Variance | Variance Ratio | Total net | Percent |
|------------------------|--------------------|----------------|----------|----------------|-----------|---------------|
| Temperature | 3 | 0.43 | 0.014 | 5.472 | 0.35 | 4.0 |
| Carbon source | 3 | 0.654 | 0.218 | 81.876 | 0.646 | 73.7 |
| The number of bacteria | 3 | 0.077 | 0.025 | 9.663 | 0.069 | 7.9 |
| Other/error | 38 | 0.1 | 0.002 | | | 14.4 |
| Total | 47 | 0.876 | | | | 100.00 |

Table 4. pH reduction in meat samples inoculated by different bacterial counts in the best fermentation conditions.

| Experiment number | <i>Lactobacillus sakei</i> (CFU. g ⁻¹) | <i>Lactobacillus plantarum</i> (CFU. g ⁻¹) | pH after 30h |
|-------------------|--|--|--------------|
| 1 | 5000 | 5000 | 4.06 |
| 2 | 7000 | 3000 | 4.04 |
| 3 | 3000 | 7000 | 4.04 |

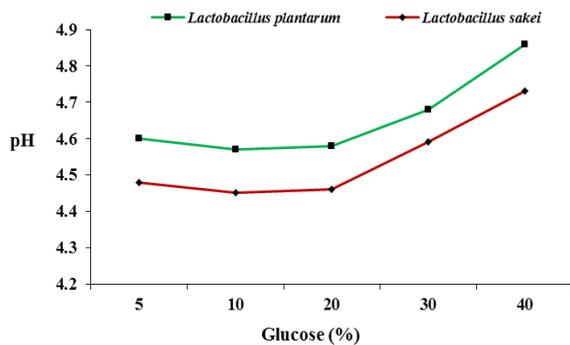


Figure 7. The best supplemented amount of glucose which resulted in maximum acid production; detected by the most declines in pH of meat.

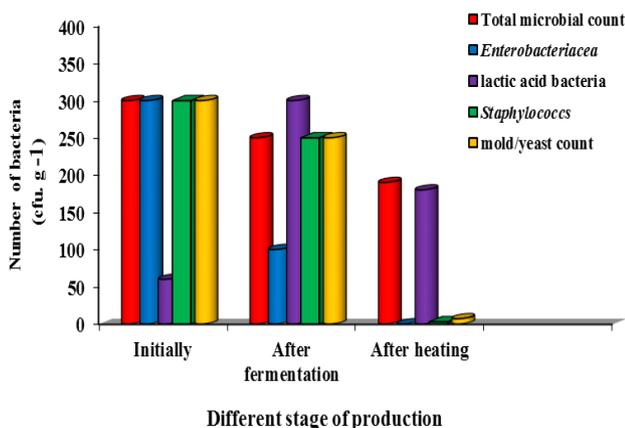


Figure 8. The results of microbial analysis of sausage samples in different stages of production.

and 60% of their viability maintained after heating stage. Fermentation and heating stages completely inhibited the growth of *Enterobacteriaceae* members in sausage (Figure 8).

Discussion

Fermented sausages are meat products that are significantly accounted for world nutritional requests (20). In contrast to increasing

tendency for safe meat products in Iranian societies, fermentation is not yet considerably attended in meat processing industries in this country. On the other hand, because of global economic and exclusive features of these products, local investigations on lactic acid bacteria as starter cultures in meat processing industries is necessary for the attainment of the best bacterial activity conditions.

In the present study, two lactobacilli strains from Iranian microbial collections were to enhance cow meat quality by optimization of acid production using an orthogonal optimization system in batch fermentation. Fermentation has been among the oldest technologies of meat preservation in long periods of time. In this process, microbes, meat, and technology are converged and resulted in variation of valuable nutrients and flavor compounds.

These variations depend on the type and amount of raw materials, starter cultures, and the processing conditions. Optimization of organoleptic and safety features requires detailed knowledge of the relative contribution of different factors which affect the growth of starter culture bacteria and some of their characteristics such as exopolysaccharides production (20, 21).

The results of present study showed that *Lactobacillus* spp. has powerful acid production activity during 30-36 hours after

initiation of the fermentation process, especially when used as co-cultures. Fourfold increases in count following fermentation, 60 maintenance of viability after the heating stage, and the growth inhibition of *S. aureus*, *Enterobacteriaceae*, and yeast/molds in the product were detected for LABs. Elimination of pathogenic bacteria is an important index for evaluation of meat products (22, 23).

As shown in Figure 8, the number of *Enterobacteriaceae* decreased down to 100 cfu. g⁻¹ (33.3% of initial count) after fermentation process which is comparable to other studies such as Stimbirys *et al.*, (2015) which reported the reduction of *Escherichia coli* count down to 38.2% after fermentation of minced pork meat (17). Also, there was a decrease down to 83.3% in *S. aureus* count after fermentation of cow meat in the present study which is lower than the reports of other studies. For example, Stimbirys *et al.*, reported a 50% reduction in *S. aureus* count after fermentation of minced pork meat (17).

However, this pathogenic bacterium was eliminated down to 1% of initial count after heating stage in our study. It is shown previously that rapid decrease of pH is an advantage for inhibition of pathogenic microorganisms in fermented meat (24, 25).

Also, most *Lactobacillus* spp. are able to produce bacteriocins which help to eliminate unfavorable microorganisms in fermented products (26-28).

In the present study, carbon source had the most significant effect on acid production by *L. sakei*, with a share of 60.2% and for *L. plantarum*, with a share of 73.7%. Other factors had fewer effects. The remarkable

aspect of the results is that both bacteria represented approximately the same conditions for cow meat fermentation and in terms of cost, such conditions including temperature of 36 °C, and the amount of carbon source (10%) were minimal for optimal growth and acid production. Glucose which was used in this study is known as the best carbon source for acceleration of fermentation because of homolactic fermentation, in contrast to other sugars such as ribose which undergoes heterolactic fermentation (7).

On the other hand, it is shown that glucose, in contrast to fructose and sucrose, has greatest impact on growth and bacteriocin production by *L. sakei* subsp. *sakei* 2a (27). One of the advantages of the present study is designation of experiment by *Taguchi* method using *Qualitek 4* program.

Taguchi is a powerful, simple and effective method for experimental design in order to optimize different biological productions. Key factor in selection of *Taguchi* method is high quality achievement without increasing cost. Another advantage is the ability to perform parallel tests by one basic engineering design. Furthermore, the results obtained from experiments can be statistically analyzed using *ANOVA* test (14).

Conclusion

Increasing attention to fermented products including different types of meats in the world, especially in developing countries, necessitate further study on starter cultures to improve meat quality using more cost-effective conditions which minimize preservative compounds usage. The best advantage of the present study is that *Lactobacillus* strains

showed approximately the same conditions for cow meat fermentation, and synergistic activity for acid production, when used simultaneously in batch fermentation.

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