EFFECTS OF A BACTERIOCIN OF *BACILLUS METHYLOTROPHICUS* STRAIN BM47 AND PASTEURIZATION ON THE STORAGE LIFE OF FRESH TOMATO JUICE

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Abstract

Bacteriocins are antimicrobial peptides of bacterial origin, which are widely used in the food industry as biopreservatives. Bacteriocins are safe, do not alter the organoleptic characteristics of food and can be applied directly in purified form or indirectly by in situ production in different food matrices. The aim of the current research was to apply a bacteriocin from *Bacillus methylophilus* strain BM47 in fresh tomato juice, and to evaluate the effects of the bacteriocin singly and in combination with pasteurization on the physicochemical and microbiological characteristics of tomato juice.

To conduct the study, four experimental groups of tomato juice were prepared: non-pasteurized; non-pasteurized with a bacteriocin; pasteurized and pasteurized with a bacteriocin, which were stored under refrigeration conditions (4 °C and 75% relative humidity) for 24 days. During the storage, all experimental groups were observed for decay and fungal growth, and samples for physicochemical and microbiological analyzes were taken at 4 days interval. From the physicochemical parameters the total soluble solids (by refractometric method), titratable acidity (by titration method), pH, organic acids (by high pressure liquid chromatographic analysis), total phenolic content (by spectrophotometric method), total chlorophylls and carotenoids content (by spectrophotometric method) and antioxidant activity (by DPPH free radical-scavenging method) were determined. From the microbiological parameters the total plate count of mesophilic aerobic and facultative anaerobic microorganisms (by colony-count technique on plate count agar at 30 °C) and the number of yeasts and fungi (by colony-count technique on chloramphenicol glucose agar at 25 °C) were determined.

The results showed that pasteurized tomato juice with and without addition of a bacteriocin retained lower titratable acidity and higher levels of total soluble solids, compared to non-pasteurized groups. Throughout the storage period, pasteurized tomato juice with and without addition of a bacteriocin kept lower concentrations of citric and fumaric acids, compared to non-pasteurized samples with and without addition of a bacteriocin. The bacteriocin application and pasteurization did not affect the lowering total phenolic content, total chlorophylls and carotenoids, but helped to maintain higher values of antioxidant activity during the entire storage period. The results from the microbiological analysis demonstrated that bacteriocin treat-
ment, especially in combination with pasteurization reduced the total plate count (bacteria) and yeasts in tomato juice. The same treatment effectively inhibited the fungal growth in both pasteurized groups, and no signs of fungal decay were observed until the end of the storage period.

Therefore, the application of a bacteriocin from Bacillus methylotrophicus strain BM47 in combination with pasteurization could be considered as a promising approach for biopreservation and improvement of the storage life of fresh tomato juice.

**Key words:** Biopreservation, Tomato, Tomato juice, Bacteriocins, Bacillus methylotrophicus.

### 1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the world’s most cultivated and consumed vegetable crops, the fruits of which are widely used in fresh form in salads and in processed forms such as juice, sauces, purees, paste and ketchup. Nutritionally, tomato contains large amounts of water, sugars, organic acids, calcium, potassium, vitamins (A, B3, E) and antioxidants (carotenoids, ascorbic acid and phenolic compounds), which play important roles in metabolic activities and possess many health benefits. For example, one of the essential carotenoids in tomato, which is responsible for the red color of the tomato fruit - lycopene, contributes to the prevention of chronic diseases such as cardiovascular disorders and cancer [1 - 3]. In addition to its anticarcinogenic properties, lycopene has been found to protect the skin from the negative effects of ultraviolet rays. Moreover, tomato consumption helps to maintain normal cholesterol and blood sugar levels due to the fiber content and reduces the risk of neurodegenerative diseases [4].

The cultivation of tomato close to the soil as well as the harvesting, distribution and storage in open crates expose the fruit to microbial contamination that can be easily transmitted to tomato products during processing. The large amounts of water, organic acids and other nutrients in tomato fruit create excellent conditions for microbial growth, which is a prerequisite for spoilage. The microbial spoilage of tomato and tomato products alters their organoleptic properties (odor, flavor, color and texture) and reduces shelf-life and nutritional value. In addition, fungal contamination also contributes to the deterioration of product quality and presents a risk of food poisonings due to the production of mycotoxins by the fungi [5]. Mycotoxins produced by the members of genera Aspergillus, Penicillium, Alternaria, Fusarium and other fungal pathogens affecting the raw plant material are able to sustain the different stages of food processing, thus creating a health risk to consumers throughout the food chain [6].

In recent decades, the rejection of chemical preservatives and the growing consumers’ demands for safe and minimally processed foods have led to the adoption of more advanced approaches to biopreservation by natural antimicrobial compounds such as bacteriocins. Bacteriocins are peptides of bacterial origin that inhibit the growth of unwanted microflora, thus extending the shelf-life of foods and beverages [7]. Such an approach would result in a decrease in microbial and, especially, fungal spoilage, thereby reducing food waste and economic losses for manufacturers and consumers. According to the Food and Agriculture Organization of the United Nations (FAO), approximately 1.3 billion tons of food are lost or wasted each year, 40 - 50% of which come from root crops, fruit and vegetable losses [6]. The preservation of fresh and processed fruit and vegetable products by natural antimicrobials would enhance the utilization and recycling of their wastes into other nutritional resources, bioactive compounds or fertilizers, thus minimizing environmental pollution [8].

The application of bacteriocins for the biopreservation of fruit and vegetable products is not a new concept. Bacteriocins are safe, do not alter the organoleptic characteristics of food and can be applied directly in purified form or indirectly by *in situ* production. In recent years, research efforts have been focused primarily on the application of lactic acid bacteria (LAB) and their metabolites to different vegetable products. Numerous studies have revealed the outstanding antimicrobial potential of *Leuconostoc* spp. [1], *Lactobacillus fermentum* YML014 [9], *Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7 and *Pediococcus pentosaceus* KTU05-8 [10] in the biopreservation of various tomato products by direct application of their metabolites or as *in situ* producers of bacteriocins. Despite the broad antimicrobial spectrum of LAB [11, 12], the disadvantages of some LAB bacteriocins and the promising antimicrobial activity of some non-LAB bacteriocinogenic strains [13, 14] have led to the need of more investigations on the application of *Bacillus* strains and their bacteriocins as biopreservatives in fruit and vegetable food matrices.

Therefore, the aim of the current research was to apply a bacteriocin from *Bacillus methylotrophicus* strain BM47 as a biopreservative in fresh tomato juice and to evaluate the effects of the bacteriocin treatment singly and in combination with pasteurization on its physicochemical and microbiological characteristics in order to improve the product storage life.
2. Materials and Methods

2.1 Fruit

Fresh tomatoes (Solanum lycopersicum L.) at a commercial stage of ripeness were purchased from the local fruit market in Plovdiv, Bulgaria. The fruit was selected based on size, shape, color and the absence of physical damage. The tomatoes were placed in brown paper bags and then immediately transferred to the laboratory for the experiment.

2.2 Bacteriocin

A bacteriocin synthesized by the strain Bacillus methylothrophicus BM47 (previously isolated from a natural thermal spring in the Haskovo region of Bulgaria) was used in this study. The bacteriocin, purified by fast protein liquid chromatography (FPLC), contained an antimicrobial peptide with a molecular weight of 19578 Da as characterized in our earlier research [14].

2.3 Culture media

2.3.1 Plate count agar (PCA)

This medium was used for determination of the total plate count of mesophilic aerobic and facultative anaerobic microorganisms. PCA was prepared by the manufacturer’s (Scharlab S.L., Spain) prescription: 23.5 g of the medium (containing 5 g casein peptone, 2.5 g yeast extract, 1 g dextrose and 15 g agar) was dissolved in 1 L of deionized water. The final pH was adjusted to 7.0 ± 0.2, and the medium was autoclaved at 121 °C for 15 min.

2.3.2 Chloramphenicol glucose agar (CGA)

CGA is a selective medium for the enumeration of yeasts and fungi, prepared according to the manufacturer’s (Scharlab S.L. Spain) prescription: 40 g of the medium (containing 20 g dextrose, 5 g yeast extract, 0.1 g chloramphenicol and 15 g agar) was dissolved in 1 L of deionized water. The final pH was corrected to 6.6 ± 0.2, and the medium was sterilized by autoclaving at 121 °C for 15 min.

2.4 Preparation of tomato juice

For this purpose, 2 kg of fresh tomatoes were washed, blanched and peeled, then cut and homogenized with a Polytron blender (Kinematica AG, Switzerland). The obtained tomato juice was divided into two equal portions (1 L each), and the second one was pasteurized at 75 °C for 10 min. The two types of tomato juice (non-pasteurized and pasteurized) were additionally divided into two equal portions. After cooling the pasteurized juice to room temperature, 100 AU/mL (0.15 mg/mL) of the purified bacteriocin of B. methylothrophicus BM47 was added to the second of the two portions and then stirred in a magnetic stirrer IKA® RCT classic (IKA®-Werke GmbH & Co. KG, Germany) without heating at 400 rpm for 10 min. Thus, four types of samples were obtained: non-pasteurized juice (NP); non-pasteurized juice + bacteriocin (NP + B); pasteurized juice (P) and pasteurized juice + bacteriocin (P + B). The samples were dispensed in amounts of 40 mL each into sterile plastic containers (Isolab, Germany) and stored at 4 °C for 24 days. For each sampling day, one container from each experiment group was provided. The samples were tested physicochemically and microbiologically at the beginning of the experiment (day 0). During the storage period, all experiment groups were observed for signs of decay and fungal growth, and samples for physicochemical and microbiological analyzes were taken on the 4th, 8th, 12th, 16th, 20th and 24th day.

2.5 Physicochemical analyses

2.5.1 Total soluble solids, titratable acidity and pH

The total soluble solids (TSS) content was determined by a portable Abbe refractometer (Officine Galileo, Italy). A few drops of tomato juice were placed on the prism glass, and the TSS value was immediately read and recorded. The titratable acidity (TA) was measured by titration of 2 mL of tomato juice with 0.1 N NaOH (Sigma-Aldrich, Merck, Germany) using phenolphthalein (Sigma-Alldrich, Merck) as an indicator until the appearance of a pale pink color persisting for over 1 min. The results were calculated as the mean value of three consecutive experiments and expressed as the percent of fumaric acid. The pH values for each experiment group were measured by a pH-meter WTW pH 7110 (WTW, Germany) at room temperature [15].

2.5.2 Total phenolic content

For evaluation of the total phenolic content and antioxidant activity, water extracts were used. The homogenized tomato samples were weighed and extracted in an ultrasound bath SIEL UST 5.7-150 (Siel, Bulgaria) at 50 °C for 10 min. The extraction was performed in duplicate. The total phenolic content was measured using a Folin-Ciocalteu reagent (Sigma-Aldrich, Merck) by the method of Stintzing et al., [16] as follows: 1 mL of Folin-Ciocalteu reagent (diluted five times) was mixed with 0.2 mL tomato water extract and 0.8 mL 7.5% Na₂CO₃ (Sigma-Aldrich, Merck). The reaction was performed in darkness at room temperature for 20 min. The absorbance was measured by UV/Vis spectrophotometer CamSpec M107 (Spectronic-Camspec Ltd., UK) at 765 nm against a blank, and the results were expressed as mg equivalent of gallic acid (GAE)/100 g of fresh weight (fw), according to a calibration curve [17].
2.5.3 Total chlorophylls and total carotenoids
The concentrations of total chlorophylls and total carotenoids were determined according to the Lichtenthaler and Wellburn method [18]. Samples of tomato juice were mixed with acetone (Sigma-Aldrich, Merck) (1:5 w/v) and extracted in an ultrasound bath SIEM UST 5.7-150 (Siel) at 40 °C for 15 min. The extraction was performed in triplicate. The collected acetone extracts were measured by UV/Vis spectrophotometer Camspec M107 (Spectronic-Camspec Ltd.) at three wavelengths 662, 645 and 470 nm against a blank (acetone). The results were presented as μg/g of fresh weight (fw).

2.5.4 DPPH radical-scavenging ability
Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging method. To perform this analysis, 0.15 mL of tomato water extract was mixed with 2.85 mL of a freshly prepared 0.1 mM methanol solution of DPPH (Sigma-Aldrich, Merck). The samples were incubated in darkness at 37 °C for 15 min. The reduction of absorbance was measured by UV/Vis spectrophotometer Camspec M107 at 517 nm against a blank - methanol (Sigma-Aldrich, Merck), and the percentage of inhibition was calculated. The antioxidant activity was expressed as mmol Trolox equivalents (TE) per 100 g of fresh weight (fw) [17].

2.5.5 High pressure liquid chromatographic (HPLC) analysis of organic acids
The content of organic acids was determined by an Elite LaChrom HPLC-DAD system (VWR™ Hitachi, Japan) according to the method of Ivanov et al., [19]. The separation was conducted on a Discovery® HS C18 column (5 μm, 25 cm × 4.6 mm) (Sigma-Aldrich, Merck) at 30 °C. The isocratic elution was conducted with 25 mM KH₂PO₄ (pH 2.4 with 85% H₃PO₄) as the mobile phase at a flow rate of 0.5 mL/min. The detection of organic acids was monitored as follows: at 244 nm for L-(+)-ascorbic acid, and at 210 nm for citric and fumaric acids. The obtained results were expressed as mg/100 g of fresh weight (fw).

2.6 Microbiological analyses
On each sampling day, 10 mL of tomato juice from each experiment group was taken, the pH of the samples was adjusted to 6.5 - 7.0 using 20% NaOH (Sigma-Aldrich, Merck) and divided evenly (5 mL each) into two tubes (Isolab). A sample from the first half was poured plated onto PCA agar in order to determine the total number of microorganisms (mesophilic aerobic and facultative anaerobic microorganisms) and onto CGA agar to determine the number of yeasts and fungi. The Petri plates (Gosselin™, France) were incubated at 30 °C, and the results were recorded at 24 and 48 h (for fungi at 25 °C for 72 h).

To determine the presence and survival of spore forms, the second half of the sample was heated in a water bath at 80 °C for 20 min, and after cooling was poured plated onto PCA agar and CGA agar media. The Petri plates (Gosselin™) were incubated under identical conditions and the results were recorded at 24, 48 and 72 h.

2.6.1 Enumeration of colony-forming units of mesophilic aerobic and facultative anaerobic microorganisms
The total plate count (TPC) of mesophilic aerobic and facultative anaerobic microorganisms was determined by colony-count technique on PCA at 30 °C according to the Bulgarian State Standard BSS EN ISO 4833-1:2013 [20].

2.6.2 Enumeration of colony-forming units of yeasts and/or fungi
The total number of yeasts and/or fungi was determined by colony-count technique on CGA at 25 °C according to the Bulgarian State Standard BSS ISO 21527-1:2011 [21].

2.6.3 Determination of spore forms
The presence of spore forms of saprophytic mesophilic aerobic microorganisms was determined by colony-count technique on PCA at 30 °C according to the Bulgarian State Standard BSS 6916:1987 [22].

2.7 Statistical analysis
Each analysis in the experiment was independently replicated three times, the data were presented as mean value, and the standard deviation (±SD) was calculated [15].

3. Results and Discussion

3.1 Effects of the bacteriocin of B. methylotrophicus BM47 and pasteurization on the physicochemical characteristics of fresh tomato juice

3.1.1 Effects on total soluble solids (TSS), titratable acidity (TA) and pH
Effects of bacteriocin of B. methylotrophicus BM47 and pasteurization on the total soluble solids, titratable acidity and pH of fresh tomato juice are presented in Table 1.
The results presented in Table 1 show that the addition of a bacteriocin and pasteurization did not affect the physicochemical parameters of tomato juice during the first four days of refrigerated storage. A slight increase in TSS in pasteurized samples with and without a bacteriocin was observed. An increase in the TA and a decrease in pH values was also recorded, which was most pronounced in the non-pasteurized samples without a bacteriocin. On the 8th day of storage, a slight increase in TSS in the pasteurized samples with and without addition of a bacteriocin was recorded. Samples of non-pasteurized tomato juice without a bacteriocin, retained higher TA and lower pH levels, compared with the pasteurized tomato juice and those containing a bacteriocin. On the 12th day of storage, the trend for a gradual increase in TSS in all samples continued. Non-pasteurized tomato juice without addition of a bacteriocin retained highest TA level and lowest pH, compared to the other three samples. On the 16th day of storage, TSS levels remained unchanged, and the trend for an increase in the TA and a decline in pH values of non-pasteurized tomato juice without a bacteriocin continued. The pH values in the other experiment groups remained almost unchanged, compared to the previous measurement. On day 20, a slight increase in TSS values in all samples was observed. The increase in TA levels of non-pasteurized tomato juice without addition of a bacteriocin continued, as well as the decrease in pH values in the same experiment group. On the last day of storage (day 24), TSS increased, especially in the pasteurized samples with and without a bacteriocin. An increase in the TA and lowering in the pH in non-pasteurized tomato juice without a bacteriocin was observed. The same parameters in bacteriocin- and heat-treated samples remained almost unchanged in comparison with the previous sampling day. Based on the obtained results, we can conclude that the application of a bacteriocin of *B. methylotrophicus* BM47, especially in combination with pasteurization, reduced the TA levels and inhibited the decrease in pH values in treated samples, thus contributed to enhance the storage life of fresh tomato juice.

### 3.1.2 Effects on organic acid content

The organic acid content of tomato juice samples was determined at the beginning (day 0), in the middle (day 12) and at the end (day 24) of the storage period (Figure 1).

The results in Figure 1 show the presence of two types of organic acids - citric and fumaric. Ascorbic acid was not detected. At the beginning of the storage period (day 0), higher levels of citric and fumaric acids in non-pasteurized samples of tomato juice with and without a bacteriocin were detected. In the middle of the storage period (day 12), an increase in the concentrations of both types of acids was observed, and the organic acid content was higher in non-pasteurized tomato juice samples. At the end of the storage period, the organic acid content was almost unchanged compared to the previous measurement.
The results in are showing the high levels of antioxidants in all samples of tomato juice at the beginning of the storage period (day 0) as their content was higher in pasteurized samples with and without a bacteriocin. On the 4th day, the antioxidant activity decreased almost twofold, both in non-pasteurized and pasteurized samples. During the storage period, the declining trend in antioxidant levels continued, despite the addition of a bacteriocin. The lowest concentrations of antioxidants were recorded at the end of the storage period. The obtained results indicate that the pasteurization alone and in combination with a bacteriocin had a protective effect on the antioxidant activity of fresh tomato juice during refrigerated storage.

The results obtained by Gahler et al., [23], demonstrated that the thermal processing steps (sieving, homogenization, sterilization and bottling) had different effects on the antioxidant activity of tomato juice, baked tomatoes, tomato sauce, and tomato soup. The authors stated that the homogenization and thermal treatment increased the hydrophilic antioxidant capacity of the studied tomato products. The antioxidant activity increased after the sieving, marked its highest levels after the second step (homogenization), and then decreased during the third and fourth processing steps.
3.1.5. Effects on total chlorophylls and total carotenoids content

The total chlorophylls and carotenoids content in fresh tomato juice samples was determined at the begin-

![Graph showing effects of B. methylotrophicus BM47 and pasteurization on the total chlorophylls and total carotenoids of fresh tomato juice](image)

- **TChl** - total chlorophylls; **TCar** - total carotenoids; **NP** - non-pasteurized; **P** - pasteurized; **B** - bacteriocin; **fw** - fresh weight

![Figure 4. Effects of B. methylotrophicus BM47 and pasteurization on the total chlorophylls and total carotenoids of fresh tomato juice](image)

The results in Figure 4 shows that during the first half of the storage under refrigeration conditions, the amount of total chlorophylls and total carotenoids in all samples decreased slightly. During the second half of the storage period, the decreasing trend in the concentrations of total chlorophylls and carotenoids persisted, both in non-pasteurized and pasteurized tomato juice, regardless of the bacteriocin and heat treatments. The obtained results could be explained by the degradation of carotenoids, in particular lycopene, known as a thermo-, light-, and oxygen sensitive compound, which undergoes significant degradation during processing such as heat treatment, evaporation, cooking, drying, and storage [24, 25].

3.2 Effects of the bacteriocin of *B. methylotrophicus* BM47 and pasteurization on the microbiological parameters of fresh tomato juice

These effects are displayed at Table 2.

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<th>Yeasts, cfu/mL</th>
<th>Fungi, cfu/mL</th>
<th>SF, cfu/mL</th>
<th>Yeasts, cfu/mL</th>
<th>Fungi, cfu/mL</th>
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<td>4 x 10^3</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>NP + B</td>
<td>4 x 10^4</td>
<td>1.3 x 10^4</td>
<td>4 x 10^3</td>
<td>3.3 x 10^3</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>3 x 10^4</td>
<td>2.4 x 10^3</td>
<td>1.1 x 10^3</td>
<td>2.2 x 10^3</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>P + B</td>
<td>3 x 10^4</td>
<td>1.9 x 10^3</td>
<td>&lt; 10</td>
<td>1.3 x 10^3</td>
<td>&lt; 10</td>
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</tr>
</tbody>
</table>

Legend: NP - non-pasteurized; P - pasteurized; B - bacteriocin; TPC - total plate count; SF - spore forms.
3.2.1 Effects on total plate count - TPC (mesophilic aerobic and facultative anaerobic microorganisms)

The results in Table 2 shows that the pasteurization of tomato juice leads to a decrease in the total number of mesophilic aerobic and facultative anaerobic microorganisms compared to the raw material - non-pasteurized tomato juice (day 0). On the 4th day of storage, an increase in TPC in non-pasteurized tomato juice without the addition of a bacteriocin was observed, while in the treated samples with bacteriocin or pasteurization, this parameter remained at lower limits. The lowest TPC value was found in pasteurized tomato juice with and without the addition of a bacteriocin. On the 8th day, the increase in TPC in non-pasteurized tomato juice without a bacteriocin continued, while the values in the treated samples remained lower. The lowest value of TPC was found in pasteurized tomato juice with an added bacteriocin. On the 12th day of the storage period, the same trend was observed in non-pasteurized tomato juice without a bacteriocin. In the samples with a bacteriocin treatment as well as in pasteurized samples, this parameter remained lower. The lowest TPC value was again found in the pasteurized tomato juice with the addition of a bacteriocin. On the 16th day, the TPC in non-pasteurized tomato juice without a bacteriocin continued to increase. The bacteriocin-treated and pasteurized samples maintained lower values of this parameter. The lowest TPC value was found in pasteurized tomato juice with the addition of a bacteriocin. By the end of the storage period (days 20 and 24), the TPC of non-pasteurized tomato juice without a bacteriocin continued to increase, while in the treated samples this indicator remained lower. The lowest TPC value was found in pasteurized tomato juice treated with a bacteriocin from *B. methylotrophicus* BM47, which demonstrates that the combination of the bacteriocin treatment with pasteurization is an effective approach for a reduction of microbial growth and improvement of the storage life of tomato juice.

3.2.2 Effects on yeasts and fungi

At the beginning of the experiment, the presence fungi and yeasts was detected in the raw material (non-pasteurized tomato juice), and their number decreased after the pasteurization process (Table 2). On the 4th day of storage, an increase in the number of fungi in non-pasteurized tomato juice without a bacteriocin was observed, while in the non-pasteurized samples containing a bacteriocin and pasteurized samples, their number was lower. The lowest number of yeasts was noted in pasteurized tomato juice treated with a bacteriocin, while fungi were not found. On the 8th day, an increase in the number of yeasts and fungi in non-pasteurized tomato juice with and without a bacteriocin as well as in pasteurized tomato juice was recorded, while the number of yeasts in the pasteurized samples treated with bacteriocin also increased but remained lower, and fungi were not detected. On the 12th day of the storage, the number of yeasts and fungi in non-pasteurized tomato juice continued to increase, while the number of fungi in non-pasteurized samples with a bacteriocin did not change. In contrast, the number of yeasts and fungi in the pasteurized tomato juice began to decrease slightly in comparison with the previous measurement (day 8). The lowest number of yeasts was detected in bacteriocin-treated pasteurized samples, and fungi again were not found. On the 16th day, the number of yeasts in non-pasteurized tomato juice continued to decrease, while in pasteurized samples, yeast’s number did not change, compared to day 12. The same trend was observed in the number of fungi, as the lowest amount was determined in the pasteurized samples. In pasteurized tomato juice treated with a bacteriocin, fungi were not detected. By the end of the storage period (days 20 and 24) the number of yeasts and fungi in all experiment groups continued to decrease compared with the previous monitoring days, as the lowest number was determined in the pasteurized samples. In the pasteurized tomato juice with the addition of a bacteriocin, fungi again were not detected. The obtained results demonstrated that the application of a bacteriocin of *B. methylotrophicus* BM47 combined with pasteurization effectively inhibited the fungal growth in processed tomato juice, and this method could be used as a means for extending the storage life of the product.

3.2.3 Effects on spore forms

The additional heat treatment of the samples at 80 °C for 20 min demonstrated the presence of spore forms of saprophytic mesophilic aerobic microorganisms, both in non-pasteurized and pasteurized tomato juice, with a small difference between them, showing that spore forms survived the process of initial pasteurization (75 °C/10 min). After the 4th day, the spore forms increased slightly in all samples, and during the entire storage period their number remained relatively constant. After the additional heat treatment until the end of storage period, no yeasts and fungi were detected in any of the samples. The lowest number of spore forms was detected in pasteurized samples, especially in the pasteurized samples with the addition of a bacteriocin. This result could be explained by the antimicrobial activity of the bacteriocin of *B. methylotrophicus* BM47 and its potential application as a biopreservative in fresh tomato juice.

Antimicrobial peptides synthesized by members of the bacterial genus *Bacillus* and other microorganisms are widely used in the food industry. In recent years, the increasing consumer’s preferences for fresh, healthy and minimally processed foods have stimulated research interest in the use of bacteriocins as natural
preservatives. The application of bacteriocins as biopreservatives has been extensively studied in meat and dairy products, but much less research has been done with foods and beverages based on fruit and vegetables. The bacteriocin’s activity in food can be limited by several factors, such as inactivation, binding to nutritional components and uneven distribution in the food matrix [26]. Therefore, in order to determine the activity and stability of bacteriocins used as biopreservatives in fruit and vegetable products, additional studies are warranted before bacteriocins are used in these foods. In this regard, studies on the use of bacteriocins in biopreservation and increasing the shelf-life of fruit and vegetable products remain insufficient [27].

Gupta et al., [28] investigated the effects of a purified bacteriocin from Brevibacillus borstelensis AG1 isolated from Marcha (Indian herbal cake used as a wine starter) on improving the shelf-life of tomato paste. The authors investigated the preserving effect of this bacteriocin for nine days in tomato paste inoculated with the pathogens Listeria monocytogenes MTCC389, Bacillus subtilis CRI and Clostridium perfringens MTCC1739 in amounts of 8.16, 8.13, and 8.18 log cfu/mL, respectively, and compared its antimicrobial activity to that of the commercial bacteriocin nisin and the chemical preservative sodium benzoate. The results showed that the bacteriocin had promising biopreservation potential by reducing the viable cells of L. monocytogenes MTCC389, B. subtilis CRI and C. perfringens MTCC1739 by 2.02, 2.05 and 2.02 log cycles (cfu/mL), respectively, in the treated tomato paste, compared with the control (without a bacteriocin). The purified bacteriocin from B. borstelensis AG1 has also been found to be active over a wide pH range (3 to 11) and able to withstand temperatures up to 100 °C, which demonstrates the efficacy of this bacteriocin as a biopreservative for enhancing the safety and prolonging the shelf-life of acidic foods such as tomato products.

Grande et al., [26], investigated the stability and interaction of the bacteriocin enterocin AS-48 derived from Enterococcus faecalis S-48 with nutrient components in various plant-based foods. The authors found that enterocin AS-48 had different interactions with fruit and vegetable juices, with complete, partial or minimal loss of its activity. In some juices, the loss of activity was improved by increasing the bacteriocin concentration, diluting the juice or applying heat pretreatment. In cabbage, cauliflower, lettuce, green beans, celery and avocado juices, enterocin AS-48 retained its stability for the first 24 - 48 h under refrigeration conditions, after which its activity decreased. In orange, apple, grapefruit, pear, pineapple and kiwi juices, enterocin AS-48 was stable for at least 15 days at 4 °C. More rapid loss of activity of the bacteriocin was observed in juices stored at higher temperatures (15 - 28 °C). In some commercial fruit juices (orange, apple, peach and pineapple) stored at 4 °C, the activity of the bacteriocin was stable and remained unchanged for 120 days, and over 60% of the initial activity was still present in juices stored at 15 °C for the same period. Commercial fruit juices stored at 28 °C for 120 days retained between 31.5% (apple) and 67.71% (peach) of their initial bacteriocin activity. The authors also stated that enterocin AS-48 added to lettuce juice and incubated at 15 °C led to a decrease in the viable cells of the food-borne pathogens Listeria monocytogenes CECT 4032, Bacillus cereus LWL1 and Staphylococcus aureus CECT 976. In another study, the same authors proved that enterocin AS-48 in concentrations of 3 and 6 μg/mL possessed strong inhibitory activity against Bacillus coagulans in three vegetable foods: tomato paste, syrup from canned peaches, and juice from canned pineapple. The authors stated that viable cell count of B. coagulans CECT 12 in tomato paste were reduced significantly by 2.3 (3 μg/mL) and 3 (6 μg/mL) log units during the first 24 h of the storage at 37 °C. Significant reductions were also observed in samples stored at 22 °C and 4 °C as well as on the 3-rd day of storage. After 15 days of storage, no viable cells of B. coagulans were detected [29].

Pei et al., [30], reported the antimicrobial effect of the bacteriocin bifcin G6165 synthesized by Bifidobacterium animalis subsp. animalis CICC 6165 against Acidobacillus acidoterrestris, known as one of the most common spoilage agents in fruit juices. The authors examined the antimicrobial activity of this bacteriocin against sixteen strains of A. acidoterrestris in diluted apple juice, and stated that the effective inhibitory concentration of bifcin G6165 was 40 μg/mL. The inhibitory effect of this bacteriocin was more pronounced at lower pH values (pH 3.5) and at higher temperatures (45 °C). In addition, the electron microscopy examination of bacteriocin-treated vegetative cells demonstrated significant cell damage and bacterial lysis. The authors also found that the encapsulation of the bacteriocin bifcin G6165 with Ca-alginate gel was an effective means for controlling the spoilage microorganism A. acidoterrestris in the food industry.

4. Conclusions
- The results of the present study showed that the bacteriocin of B. methylotrophicus strain BM47 applied in combination with pasteurization to fresh tomato juice led to a decrease in the levels of titratable acidity and a reduction in the total plate count, yeasts, and spore forms.
- Addition of a bacteriocin, especially in pasteurized tomato juice effectively inhibited the fungal growth in the product.
- The beneficial effects on these important physico-chemical and microbiological characteristics revealed...
the potential of the purified bacteriocin from *B. methylotrophicus* BM47 for application as a biopreservative and as a means for extending the storage life of fresh tomato juice.

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**5. References**


