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RESEARCH ARTICLE

Effect of Pine Cone Vinegar on The Survival of *Listeria monocytogenes and Salmonella typhimurium* and Some Physico-Chemical Properties in Raw Beef

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ABSTRACT

Pine cones from pine (Pinus) trees contain various bioactive compounds with antibacterial and antioxidant activity. However, the use of pine cone products as functional foods or food additives is limited and there is a paucity of research in the literature. The objective of this study was the evaluation of pine cone vinegar as a marinade against *Listeria monocytogenes* and *Salmonella typhimurium* in raw beef. For this purpose, raw beef was marinated with pine cone vinegar at three different levels (25%, 50% and 75%) and three different marination times (2, 6 and 24 hours) and *L. monocytogenes, S. typhimurium* counts, pH and colour values (L*, a*, b*) were determined. Concentration*time interaction was significant for pH and the lowest pH value was found at 75%*24 hours interaction. Although the marinating process had a significant effect on the colour values of the meat, the concentration*time interaction was not significant for the a* value ($p>0.05$). The number of *S. typhimurium* in pine cone vinegar decreased by 3.7 log10 in 2 hours and by 5.26 log10 in 6 hours ($p<0.05$). The number of *L. monocytogenes* decreased by 4.17 log10 in 2 hours and 5.29 log10 in 6 hours ($p<0.05$). The number of *S. typhimurium* and *L. monocytogenes* decreased significantly in marinated beef samples, with the greatest decrease being observed at 24 hours for all concentrations. After 24 hours of marination, *Salmonella* counts decreased by 1.69, 1.68 and 1.73 log10, and *L. monocytogenes* counts decreased by 3.6, 3.43 and 3.13 log10, respectively. The findings of this study indicate that pine cone vinegar can serve as an effective decontaminant for meat and meat products, making it a valuable component in marinades.

Keywords: Beef, *Listeria monocytogenes*, Marinating, Pine cone, *Salmonella typhimurium*

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Çam Kozalağı Sirkesinin Çiğ Sığır Etinde *Listeria monocytogenes* ve *Salmonella typhimurium*'un Hayatta Kalması ve Bazı Fiziko-Kimyasal Özellikler Üzerine Etkisi

ÖZ

Çam (Pinus) ağacı türlerinden elde edilen çam kozalakları antibakteriyel ve antioksidan aktiviteye sahip çeşitli biyoaktif maddelere sahiptir. Ancak çam kozalağından elde edilen ürünlerin fonksiyonel gıda veya gıda katkı maddesi olarak kullanımı sınırlıdır ve literatürde bu konuda çok fazla araştırma bulunmamaktadır. Bu çalışmanın amacı da çam kozalağı sirkesinin çiğ sığır etlerinde *Listeria monocytogenes* ve *Salmonella typhimurium* karşı bir marinat olarak değerlendirmektir. Bu amaçla çam kozalağı sirkesi ile üç farklı konsantrasyon (%25, %50 ve %75) ve 3 farklı marinasyon süresininde (2, 6 ve 24 saat) çiğ sığır eti marine edilip, *L. monocytogenes*, *S. typhimurium* sayıları, pH ve renk değerleri (L*, a*, b*) belirlendi. pH için konsantrasyon*zaman etkileşimi önemli olduğu, en düşük pH değeri %75*24 saat etkileşiminde sapandı. Marinasyon işleminin etin renk değerleri üzerinde önemli etkileri olmasına rağmen, a* değeri için konsantrasyon*zaman etkileşimi önemli olmadığı belirlendi ($p>0,05$). Çam kozalağı sirkesinde S. typhimurium sayısının 2 saatte 3,7 $\log_{10}$, 6 saatte ise 5,26 $\log_{10}$ azaldığı tespit edildi ($p<0,05$). *L. monocytogenes* sayısının ise 2 saatte 4,17 $\log_{10}$, 6 saatte ise 5,29 $\log_{10}$ azaldığı tespit edildi ($p<0,05$). Marine edilmiş sığır eti örneklerinde *S. typhimurium* ve *L. monocytogenes* sayısının önemli düzeyde azaldığı en çok azalma tüm konsantrasyonlarda 24 saatte tespit edildi. 24 saat marinasyon sonrası *Salmonella* sayısı sırasıyla 1,69, 1,68 ve 1,73 $\log_{10}$ azaldığı, *L. monocytogenes* sayısı ise sırasıyla 3,6, 3,43 ve 3,13 $\log_{10}$ azaldığı saptandı. Bu çalışmanın sonuçları, çam kozalağı sirkesinin et ve et ürünlerinin dekontaminasyonu için kullanılabileceği ve marinatların önemli bir bileşeni olabileceğini ortaya koymuştur.

Anahtar Kelimeler: Çam kozalağı, *Listeria monocytogenes*, Marinasyon, *Salmonella typhimurium*, Sığır eti

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INTRODUCTION

Meat and meat products play a crucial role due to their nutrient content, high pH and water activity, which create an environment conducive to microbial growth (Aminzare et al. 2016). According to the Zoonoses Report published by EFSA and ECDC (2022), three of the most common zoonotic agents in humans are *Salmonella* spp, Shiga toxin-producing *Escherichia coli* and *L monocytogenes*. These pathogens are responsible for a significant proportion of foodborne diseases associated with meat consumption. Therefore, substances such as antimicrobials must be used to reduce the risk and protect human health (Kaur et al. 2023).

Marinating is a crucial step before consuming meat, serving both sensory quality and safety purposes. Not only does marination enhance tenderness and flavor, but it also protects against microbial and chemical spoilage, making meat safer for consumption. One popular method is immersion marinating, where pieces of meat are soaked in a liquid containing various ingredients such as acidic marinade plant extracts and spices (Lopes et al. 2022). Recently, there has been a growing demand for natural preservatives in response to consumer preferences for organic additives and green-labeled foods. Vinegar, rich in bioactive compounds, has emerged as an excellent candidate for meat marinades. Studies have reported that vinegar-based acidic marinades improve the flavour and safety of meat products (Park et al. 2014; Karam et al. 2020; Şengün et al. 2021; Fencioğlu et al. 2022). As a fermented product, vinegar has a long history of use as an acidulant, sauce, and flavoring in the food industry (Sengun et al. 2020; Pashazadeh et al. 2021).

Vinegar is produced not only from commonly used fruits like grapes and apples but also from various grains and other fruits. Fruit vinegars in particular are rich in organic acids and bioactive compounds (Luzón-Quintana et al. 2021; Özen et al. 2020). These bioactive compounds in vinegar have high antibacterial and antioxidant properties (Budak et al. 2014).

Pine cones obtained from various pine (Pinus) tree species contain bioactive substances such as flavonoids, polysaccharides, and phenolic compounds. These compounds exhibit anti-tumor, anti-inflammatory, antioxidant activities and antibacterial (Wang and Hong 2016; Yi et al. 2017). In traditional medicine, pine cones are considered non-toxic and have been used for purposes such as moisturizing the lungs, relieving coughs, and reducing fever (Zhang et al. 2010). However, the use of pine cone products as functional foods or food additives remains limited, especially regarding the effectiveness of pine cone vinegar in meat marinades. While various acidic marinades have been studied extensively, the use of pine cone vinegar as a marinade has not been widely explored. Considering that marination significantly affects the microstructure and properties of meat such as microbiological composition and chemical structure, a comprehensive approach is required to overcome the current knowledge gaps in this field.

This study was designed to investigate the efficacy of pine cone vinegar as a marinade against *L. monocytogenes* and *S. typhimurium* in raw beef. In this study, the effect of marinating with pine cone vinegar on the microbiological and physicochemical properties of raw beef was elucidated.

MATERIALS and METHODS

Musculus longissimus dorsi samples were obtained from local butchers in Şanlıurfa on the day of the experiment. The meat samples were immediately brought to the laboratory under cold chain conditions on the day of the experiment and the experiments were carried out as soon as possible. During all these processes, the meat samples were kept at 4±1 °C. Organic pine cone vinegar (Karşı köyden®) was purchased from a local market in Şanlıurfa. This vinegar is made from pine cones grown from the *Pinus brutia* pine tree species, water and salt and fermented for at least one year.

Preparation of inoculum

S. typhimurium (NCTC 74, 12416 and ATCC 14028) and *L. monocytogenes* (N 7144, RSKK 474 and 476) reference strains were used for inoculum preparation. These strains were incubated in tryptic soya broth (TSB) overnight at 37 °C to obtain fresh cultures. After incubation, the liquid cultures were centrifuged at 4200 x g for 10 min and the supernatant discarded. The resulting pellets were washed twice with sterile 0.1% peptone water (PW) (Merck, Darmstadt, Germany) and collected in a tube. The final volume was adjusted to 10 mL with 0.1% sterile PW (Merck, Darmstadt, Germany) and diluted decimally with PW. An inoculum of approximately 6.0 log10/mL was used for survival testing of pathogens in pine cone vinegar and meat samples.

Pathogens survival experiment in Pine cone vinegar

To assess the antibacterial effect of pine cone vinegar, approximately 6.0 log10/mL of *S. typhimurium* and *L. monocytogenes* were added to the vinegar (at 100% concentration). The pathogen counts were determined immediately after inoculation and at 2, 6, and 24 hours of incubation at 4 °C. The experiment was conducted in triplicate.

Preparation of the groups

To prepare the meat samples, they were first cut into small pieces (50±5 g) using a sterile lancet. Next, 500 µL of a diluted bacterial inoculum cocktail was spread

onto the meat samples using a cell spreader. After inoculation, the bacteria were allowed to adhere to the meat for 30 minutes at 4±1 °C. The samples were then randomly divided into ten groups: a control group (no treatment and 0. hours), three different pine cone vinegar concentrations (25%, 50%, and 75%), and three different marinating times (2, 6, and 24 hours). The marination process was carried out using the immersion method. While determining the concentrations of pine cone vinegar, the meat marinated with different concentrations was cooked and sensory analysis (colour, smell, taste, appearance, general appreciation) was performed as a preliminary study before starting the study. In the study, the ratios that did not adversely affect the sensory quality of the meat were prioritised. Pine cone vinegar was diluted with sterile distilled water to achieve the desired concentrations. The ratio of marinade to meat sample was 2:1 (100 mL marinade to 50 g tenderloin), and marination was conducted in sterile glass jars at 4±1 °C.

Microbiological analyses

Each marinated meat sample (25±1 g) was collected under aseptic conditions and transferred to sterile sampling bags. Next, 225 mL of 0.1% peptone water (PW) was added to the sampling bags, and the mixture was homogenized using a stomacher (BagMixer Interscience, France) for 3 minutes. For the detection of *L. monocytogenes*, Oxford agar (Biokar, France) was used, while xylose-lysine-deoxycholate agar (XLD agar) (Biokar, France) was employed for *S. typhimurium*. The XLD and Oxford plates were incubated at 37±1 °C for 24 hours, and the number of colonies with specific morphology was recorded (İncili et al. 2021).

pH analyses

The pH values of the pine cone vinegar and meat samples were measured using a pH meter (HI 11310, Hanna Instruments, USA). The pH of the pine cone vinegar was measured by dipping the probe directly into the vinegar. When measuring the pH of meat samples, 10 g of meat samples were homogenized in 90 mL of distilled water, and pH measurements were made using a digital pH meter (İncili et al. 2021).

Colour analysis

The color characteristics of the meat samples were measured using a Chroma Meter (model CR-5, Konica Minolta, Osaka, Japan) set to a standard observer angle of 10° and illuminator type D-65. The parameters assessed included Hunter L* (whiteness/darkness), a* (redness/greenness), and b* (yellowness/blueness) (Aydemir et al. 2024).

Statistical analyses

The microbial counts, pH, L*, a*, and b* values of the samples underwent statistical analysis. Microbiological data were logarithmically transformed for statistical purposes. We employed a general linear model (GLM) for the analysis. In the GLM procedure, we treated pine cone vinegar concentrations (25%, 50%, and 75%) and marination times (2, 6, and 24 hours) as fixed effects, while replications were assigned as random effects. Multiple comparisons were conducted using the Tukey test ($p<0.05$). Throughout this study, all data were obtained from three independent replicates, and the results are presented as mean ± standard error of the mean. We performed all statistical analyses using IBM SPSS Version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULT

pH value

The pH of the pine cone vinegar used in the study was 3.8±0.06 and the pH of the meat was measured at 5.8±0.08. The average pH values of the meat samples are given in Figure 1. Marinating time and pine cone vinegar concentration did not cause a statistical difference between the groups in terms of pH. The pH value of the tenderloin samples decreased as the marinating concentration and time increased.

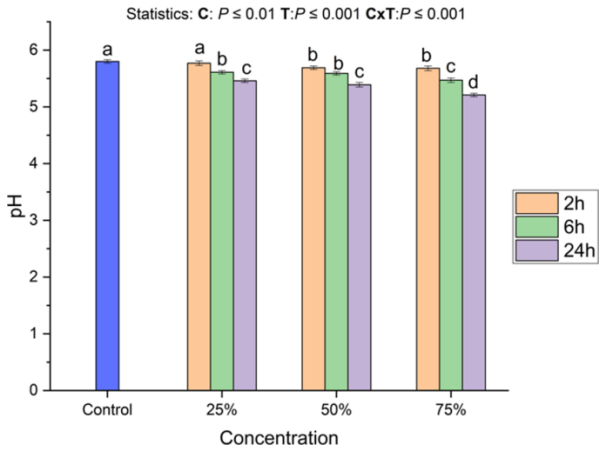

Figure 1: The mean pH values of the raw beef during storage period at 4 °C (Mean value±SE).[a-d] : Different superscripts indicates the statistical significance among the groups ($p<0.05$). *C:* Concentration of pine cone vinegar; *T:* Marination time; *C* × *T:* Interaction between concentration and time.

Colour value

Figure 2 shows that pine cone vinegar significantly changed the colour characteristics (L*, a* and b*) of the meat samples ($p<0.05$). The marinating process significantly decreased the a* value. However, increasing the vinegar concentration and extending the time to 24 hours slightly increased the L* and b* values compared to the control group.

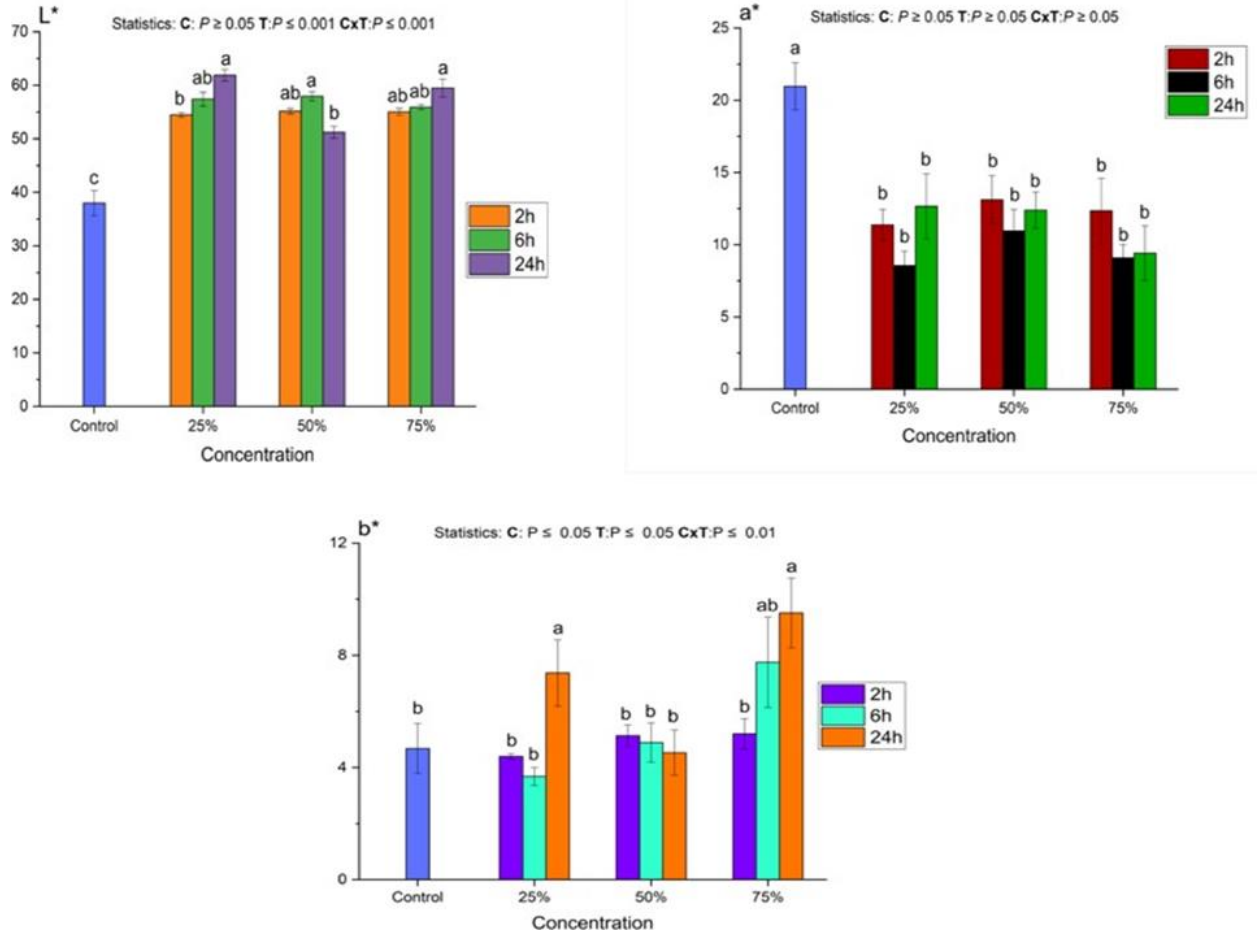

Figure 2: Average L*, a*, and b* values of the non-marinated and marinated beef samples at 4 °C. (Mean value±SE).[a-c] **:** Different superscripts indicates the statistical significance among the groups ($p<0.05$). ***C:*** Concentration of pine cone vinegar; ***T:*** Marination time; ***C × T:*** Interaction between concentration and time.

Salmonella typhimurium

The number of *S. typhimurium* in pine cone vinegar decreased by 3.7 log10 and 5.26 log10 after 2 and 6 hours, respectively ($p<0.05$). Although the number of *S. typhimurium* continued to decrease, the difference between 6 h and 24 h was not significant ($p>0.05$). Rapid reduction of *S. typhimurium* occurred in the pine cone vinegar, while a lower rate of decrease was observed in the marinated beef samples (Table 1). Compared to the control group, the number of *S. typhimurium* was significantly reduced in the marinated beef samples (Table 1). Specifically, *Salmonella* counts were reduced by 1.69, 1.68, and 1.73 log10 after 24 hours of marination at concentrations of 25%, 50%, and 75%, respectively. Although the time interaction was significant for *Salmonella* counts, the concentration*time interaction was not significant.

Listeria monocytogenes

The number of *L. monocytogenes* in pine cone vinegar decreased by 4.17 log10 after 2 hours and by 5.29 log10 after 6 hours ($p<0.05$). Although the number of *L. monocytogenes* continued to decrease, the difference between the 6-hour and 24-hour time points was not significant ($p>0.05$). Rapid reduction of *L. monocytogenes* occurred in the pine cone vinegar, while a lower rate of reduction was observed in the marinated beef samples (Table 1). Compared to the control group, the number of *L. monocytogenes* was significantly reduced in the marinated beef samples (Table 1). The greatest reduction was observed at 24 hours for all concentrations. Specifically, at 25%, 50%, and 75% concentrations, the number of *L. monocytogenes* decreased by 3.6, 3.43, and 3.13 log10, respectively, after 24 hours of marination. Both time

interaction and concentration*time interaction were significant for *L. monocytogenes* counts ($p<0.05$).

Table 1. Average counts for *S. typhimurium* and *L. monocytogenes* in non- marinated and marinated with pine cone vinegar beef at 4 °C (Mean log10 CFU/g±SE).

Concentration	Time	*L. monocytogenes*	*S. typhimurium*
Control	0h	5.66±0.28[a]	5.50±0.39[a]
25%	**2h**	5.14±0.06[a]	4.60±0.04[ab]
	6h	3.51±0.18[b]	4.23±0.15[b]
	24h	2.03±0.03[c]	3.81±0.11[b]
50%	**2h**	5.27±0.04[a]	4.16±0.53[ab]
	6h	3.53±0.06[b]	4.10±0.13[ab]
	24h	2.23±0.23[c]	3.82±0.04[b]
75%	**2h**	4.70±0.02[a]	4.22±0.17[ab]
	6h	3.38±0.08[b]	4.15±0.20[ab]
	24h	2.53±0.23[c]	3.77±0.24[b]
Statistics	**C**	p≥ 0.05	p≥0.05
	T	p≤ 0.01	p≤ 0.05
	CxT	p≤0.05	p≥ 0.05

[a-c]: Different superscripts indicates the statistical significance among the groups ($p<0.05$). ***C:*** Concentration of pine cone vinegar; ***T:*** Marination time; ***C × T:*** Interaction between concentration and time.

DISCUSSION

Effect of marination on physicochemical quality

Effect of marination on pH value

As marination concentration and time increased, the pH of the tenderloin samples decreased, with the lowest pH recorded at the 75% concentration*24 h interaction (Figure 1). The low pH of pine cone vinegar (3.8) is believed to influence the pH decrease of meat samples after marination. In fact, İncili et al (2023) reported a significant decrease in pH values when increasing the concentration of marinade used in meat samples. The decrease in pH due to prolonged marinating can be explained by the entry of more marinade into the meat and consequently the decrease in pH. As a matter of fact, Aydemir et al. (2024a) associated the decrease in the pH of beef tenderloin samples with the absorption of more marinade into the meat due to the prolongation of the marinating process. Consistent with our findings, other researchers also reported changes in pH in meat samples marinated with acidic marinades (İncili et al. 2023; Karatepe et al. 2023; Aydemir et al. 2024a; Aydemir et al. 2024b). A decrease in pH in meat samples is a desirable situation. This is because pH values below the isoelectric point (~5.3) of muscle have a direct effect on the physicochemical and textural properties of meat samples. In particular, a pH below the isoelectric point causes more marinade to enter the myofibrils. This causes the muscle fibres to swell and more water to be retained in the myofibrils. This softens the meat and improves its texture (Siroli et al. 2020; Karatepe et al. 2023). Lowering the pH also improves the microbiological quality of the meat. This is because low pH limits the survival of bacteria.

Effect of marination on color properties

Changes in colour are expected with increasing marination time. The colour of the meat surface is caused by the distribution of light reflected from the meat, as well as the selective absorption of myoglobin caused by major constituents such as muscle fibres and proteins and is also reported to be influenced by the amount of free liquid (Purslow et al. 2020). The absorption of marinade increases as the marinating time increases. In this case, as explained above, the pH of the meat decreases and colour changes occur in the meat samples. Indeed, de Avila Souza et al (2022) reported that myoglobin, which is responsible for the colour appearance of muscle, undergoes denaturation in acidic marinades, with myoglobin being converted to metmyoglobin under acidic conditions. In particular, the decrease in the a* value

of meat is more closely related to this situation. It was found that increasing the vinegar concentration and extending the marinating time to 24 hours resulted in a slight increase in L* values compared to the control group. The results of the study confirm the inverse relationship between pH and L*. Acid marinades reduce the water holding capacity of meat (Mazaheri Kalahrodi et al. 2021). As the water holding capacity of the meat decreases, the meat appears pale, soft and exudative (PSE). A high L* value has previously been reported to characterise pale meat (Barbut et al. 2008). Significant colour changes in meat samples marinated with acidic marinades have also been reported by other researchers (Gargi and Sengun 2021; İncili et al. 2021; Karatepe et al. 2023; Aydemir et al. 2024a; Aydemir et al. 2024b).

Effects of marination against *S. typhimurium, L. monocytogenes*

Vinegar is a special product that shows antimicrobial and antioxidant activity thanks to the bioactive compounds it contains (Bakır et al. 2017). Properties of organic acids in vinegar show antimicrobial effect by disrupting the integrity of the cell wall and membrane of bacteria (Lytou et al. 2019). Therefore, acidic marinades have been used to ensure and protect the microbial safety of meat and meat products. When the results of this study are examined, it is seen that pine cone vinegar caused a significant decrease in the number of pathogenic bacteria (Figure 1, Table 1). This reduction may be attributed to the low pH of pine cones (3.8) and their rich organic acids and bioactive compounds. It has been reported that pine cones are rich in bioactive compounds and have antimicrobial activity (Kim et al. 2019).

In the literature, there are studies investigating the antimicrobial effects of different vinegars against different microorganisms (Kelebek et al. 2017; Öztürk et al. 2015; Sengun et al. 2020; Karatepe et al. 2023). However, despite the widespread use of vinegar in meat marination, very limited research has been conducted on the effect of vinegar on pathogens on meat or meat products. To the best of our knowledge, the effect of pine cones on pathogenic bacteria in meat has not been investigated. Şengün et al (2021) Blackberry, rosehip reported that marinating beef with pomegranate and grape vinegar reduced the number of *S. typhimurium*, *L. monocytogenes* in meat samples by 1.420-1.913 log CFU/g and the highest reduction was found in rosehip vinegar marinade. Karatepe et al. (2023) reported that hawthorn vinegar marinating beef reduced the number of *S. typhimurium* in meat samples from 4.95 log CFU/g to 1.13 log CFU/g (100% concentration*24 *h)*, and *L. monocytogenes* from 4.98 log CFU/g to 1.39 log CFU/g (100% concentration*24 h). In addition, when meat products (chicken, lean boneless beef, etc.) were marinated with different acidic marinades (Rheum ribes L. juice, cucumber juice, V-made acidic pickles), different reduction moments (ranging from 0.54 to 3.3 log 10 CFU/g) in the number of these pathogens were reported (İncili et al. 2020; Şengün et al. 2020; İncili et al. 2021). The differences between the results of this study and the results of other studies can be explained by the type of meat used, the composition of the marinade used, the organic acid content and ratio of the marinade, the concentration of acidifying agent in the marinade, the time of keeping the meat in the marinade and the acid resistance of the pathogenic strains used in the studies.

The numbers of *S. typhimurium* and *L. monocytogenes* in pine cone vinegar decreased significantly after 2 h and 6 h, but not after 24 h (Figure 3). This can be explained by the fact that the bacteria develop resistance to the organic acids in the pine cone vinegar after a certain period of time and adapt to the pH of the pine cone. Indeed, Wang et al. (2019) environmental conditions such as exposure to acids can damage bacteria. However, bacteria that are not fatally injured can enter the stationary phase and/or regain the ability to regrow. It has also been mentioned that bacteria may show high adaptability to acidic environments (Chung et al. 2018). Similar to our findings, İncili et al. (2020) reported that the number of *S. typhimurium* bacteria in the homemade marinade they prepared decreased significantly until the 6th hour, but did not decrease at the 24th hour and was similar to the number of bacteria at the 6th hour. The situation was slightly different for the beef samples marinated in pine cone vinegar. The numbers of *S. typhimurium* and *L. monocytogenes* decreased most after 24 hours (Figure 3). This can be explained by the fact that the longer the time, the more the marinade is absorbed into the meat, the more organic acids enter the meat and the pH of the meat decreases. Thus, pine cone vinegar has a greater antibacterial effect on meat samples after 24 hours. In fact, Karatepe et al. (2023) emphasised that the longer the marinating time, the more the marinade is absorbed and the more organic acids are released into the meat. They also reported that marinating meat for 24 hours was the most effective treatment against bacteria.

The viability of *S. typhimurium* and *L. monocytogenes* in pine cone vinegar was found to be the same and decreased to a similar extent. However, *L. monocytogenes* was found to be more inactivated than *S. typhimurium* when beef was marinated in pine cone vinegar. This may be explained by the fact that bioactive compounds in pine cones are more effective against gram positive bacteria. This may also be explained by the better adhesion of Salmonella bacteria to meat compared to *Listeria b*acteria, thus providing protection from antimicrobial agents. In a study by Ilhak et al. (2018), explained that the reason why *Salmonella* was less inactivated in marinated chicken pieces was due to the protection of the

bacteria from the effects of antimicrobials due to the tight attachment of the bacteria to the meat.

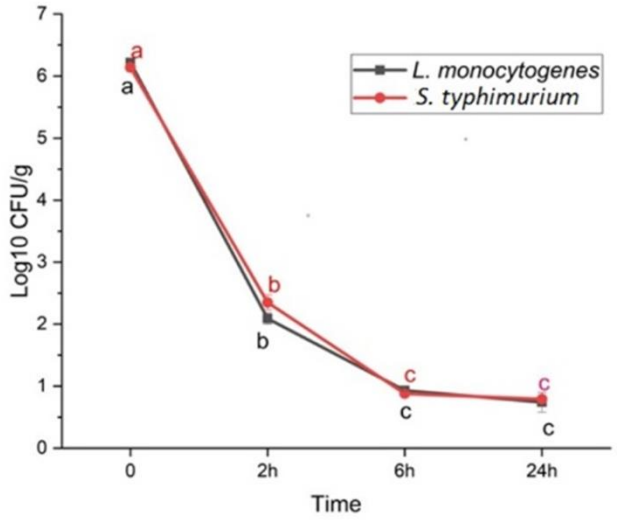

Figure 3. Survival of *S. typhimurium* and *L. monocytogenes* in pine cone vinegar at 4 °C for 24 hours (log10 CFU/g±SE).[a-c] : The mean values with different letters among the sampling hour are significantly different ($p<0.05$).

CONCLUSION

In conclusion, the pine cone vinegar marinade used in this study was found to have antibacterial activity against *S. typimurium* and *L. monocytogenes*. Pine cone vinegar, which has been used in folk medicine for many years against some diseases, may also have the potential to transform foods into functional foods. It can be suggested that pine cone vinegar marinade can be used to reduce microbial risks especially in red meat and its products. The results of this study may provide useful information for further studies investigating the effects of marinating meat and meat products with natural ingredients on the survival of foodborne pathogens and for all food handlers. Further studies are needed to evaluate the potential effects of pine cone vinegar on extending shelf life, improving chemical quality and sensory properties in different food matrices.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: MEA and ES contributed to the project idea, design and execution of the study. MEA, ES and MNG contributed to the acquisition of data. MEA and MNG analysed the data. MEA and ES drafted and wrote the manuscript. MEA, ES and MNG reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

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