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Effects of Nisin and Organic Acid Mixture on *Salmonella enteritidis* Colonization in Experimentally Contaminated Poultry Feed at Different Storage Times

Farklı Depolama Zamanlarında Deneysel Olarak Kontamine Edilmiş Kanatlı Yemlerindeki *Salmonella enteritidis* Kolonizasyonu Üzerine Nisin ve Organik Asit Karışımının Etkileri

Ahmet Önder ÜSTÜNDAĞ 0000-0002-4950-8927

Aydın Adnan Menderes Üniversitesi Ziraat Fakültesi Zootekni Bölümü, Güney Kampüs Koçarlı-Aydın

Corresponding author: austundag@adu.edu.tr

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Keywords:

Nisin, organic acid, salmonella, feed, contamination

ABSTRACT

Objective: This study was conducted to investigate the inhibitory effects of nisin alone or in combination with organic acid on Salmonella enteritidis in poultry feed at different storage times.

Material and Methods: Feeds divided into six experimental groups that consist of control, 150 mg/kg nisin (N150), 300 mg/kg nisin (N300), 3 g/kg organic acid mixture (OA), 150 mg/kg nisin + 3 g/kg organic acid mixture (N150+OA), 300 mg/kg nisin + 3 g/kg organic acid mixture (N300+OA). Then, 10^4 cfu/ml *Salmonella enteritidis* cultures added in the feeds. Feeds were stored in room temperature and *Salmonella* colonies were enumerated at 48^{th} hour (Initial Time), followed on 7^{th}, 15^{th}, 21^{st} and 28^{th} days of the study.

Results: The highest *Salmonella* values were observed in the control group during this study (P<0.05). Nisin that alone and in combination with organic acid had inhibitory effects against *Salmonella enteritidis* at initial time, on 7^{th}, 15^{th}, 21^{st} and 28^{th} days of the study. However, on the 28^{st} day, an increase of *Salmonella* count in the experimental groups was observed.

Conclusion: It is known that the effect of nisin on gram-negative bacteria is low. However, in this study, it was observed that a significant decrease in the number of Salmonella occurred with the addition of nisin to the feeds stored at room temperature for 28 days.

Anahtar Kelimeler:

Nisin, organik asit, salmonella, yem, kontaminasyon

ÖZ

Amaç: Bu çalışma, farklı depolama sürelerinde kanatlı hayvan yemlerinde tek başına veya organik asit ile kombinasyon halinde nisinin *Salmonella enteritidis* üzerine önleyici etkilerini araştırmak için yapılmıştır.

Materyal ve Metot: Yemler; kontrol, 150 mg/kg nisin (N150), 300 mg/kg nisin (N300), 3 g/kg organik asit karışımı (OA), 150 mg/kg nisin + 3 g/kg organik asit karışımı (N150+OA), 300 mg/kg nisin + 3 g/kg organik asit karışımı (N300+OA) dan oluşan 6 deneme grubuna ayrılmıştır. Daha sonra yemlere 10^4 cfu/ml *Salmonella enteritidis* kültürü eklenmiştir. Yemler oda sıcaklığında muhafaza edilmiş ve denemenin 48. saatinde (başlangıç Zamanı), 7, 15, 21 ve 28. günlerinde *Salmonella* sayımı yapılmıştır.

Bulgular: Bu çalışmada en yüksek *Salmonella* değerleri Kontrol grubunda gözlenmiştir (P<0.05). Nisin, tek başına ve organik asit ile kombinasyon halinde, çalışmanın başlangıç zamanında, 7, 15, 21 ve 28. günlerinde *Salmonella enteritidis*'e karşı inhibe edici etkilere sahip olduğu gözlenmiştir. Bununla birlikte, 28. günde, deneme gruplarında Salmonella sayısında bir artış gözlenmiştir.

Sonuç: Nisinin gram negatif bakteriler üzerindeki etkisinin düşük olduğu bilinmektedir. Bununla birlikte, bu çalışmada 28 gün oda sıcaklığında saklanan yemlere nisin ilavesiyle Salmonella sayısında önemli bir azalma olduğu gözlenmiştir.

INTRODUCTION

Animal feeds play a leading role in global food industry, enabling economic production of animal origin products throughout the world (FAO and IFIF, 2010). For this reason, every factor affecting the safety of feeds affects production negatively (Bryden, 2012). Pathogenic bacteria, fungi and mycotoxins in feeds adversely affect the safety of feed and constitute a considerable potential risk to human and animal health. *Salmonella* spp. was identified as the major hazard for microbial contamination of animal feed in The Panel on Biological Hazards (EFSA, 2008). Main sources of *Salmonella* contamination in animal feed are feed ingredients originating from oilseed meals and by-product ingredients originating from animals such as feathers, fish meal and blood meal (Hald et al. 2012; Cegielska-Radziejewska et al. 2013; Andino et al. 2014; Khan and Iqbal, 2016). These feed sources can be contaminated with *Salmonella* either during harvesting, processing or during storage and distribution (Maciorowski et al. 2007; Carrique-Mas et al. 2007; Davies and Wales, 2010; Jones, 2011; Torres et al. 2011; Berge and Wierup, 2012). *Salmonella* persists in a wide range of feedstuffs for a long period (Abd El-Ghany et al. 2015). Therefore, detection and elimination of *Salmonella* in feed are necessary in the processing chain guarantees (Vu et al. 2016). Since all feed ingredients can be potential Salmonella source, decontamination steps are essential to prevent spreading of contaminated feed to production animals (Sauli et al. 2005; Hald et al. 2012; Vukmirović et al. 2017). There are different decontamination procedures in practice to reduce or eliminate *Salmonella* contamination in feed. The most widely used procedures are heat treatments, the use of organic acids and other chemicals preservatives (Vukmirović et al. 2017). Previous studies reported that organic acids have been shown to have a potential to reduce *Salmonella* colonization (Humphrey and Lunning, 1988; Iba and Berchieri, 1995; Al-Natour and Alshawabkeh, 2005; Carrique-Mas et al. 2007; Koyuncu et al. 2013; Abd El-Ghany et al. 2015; Bourassa et al. 2018). Besides organic acids, bacteriocins, which are ribosomally synthesized proteinaceous compound by many bacteria, have become one of the weapons against human and animal pathogens due to having many properties like to be non-toxicity, natural source, heat stable and the availability of both broad and narrow spectrum (Maciorowski et al. 2006; Cotter et al. 2013; Yang et al., 2014; Ahmad et al. 2017). Bacteriocins are widely used as a food preservative agent to control food borne pathogens. Also, recently they are used in agriculture and veterinary medicine as a therapeutic (Ustundag and Ozdogan, 2011; Ahmad et al. 2017; Lagha et al. 2017; Kierończyk et al. 2017). Nisin which produced by lactococcus lactis and approved as an food preservative (E234) by the European Union and the Food and Agriculture Organization/World Health Organization (FAO/WHO), shows a broad antimicrobial spectrum against Gram-positive bacteria such as Listeria monocytogenes (Phongphakdee and Nitisinprasert, 2015; Sangcharoen et al. 2017; Kierończyk et al. 2017; Fernández-Pérez et al. 2018). However, it has activity against Gram-negative bacteria lower degree than Gram positives (Choi et al. 2000; Govaris et al. 2010; Selim et al. 2012; Cotter et al. 2013; Ahmad et al. 2017; Azhar et al. 2017; Kierończyk et al. 2017; Jayaweera et al. 2018). Although Gram-negative bacteria are resistant to nisin, the effectiveness of nisin on Gram-negative bacteria could be enhanced by used at high concentrations and combined with other antimicrobial factors like chelators, polycationic acid, organic acids, other bacteriocins, and essential oils (Todorov and Dicks, 2005; Rattanachaikunsopon and Phumkhachorn, 2010; Govaris, 2010; Galvão et al. 2015; Ay and Tuncer, 2016; Pinilla and Brandelli, 2016; Mills et al. 2017; Sangcharoen et al. 2017; Bingol et al. 2018).

Therefore, the aim of this study was to investigate the effects of nisin and organic acid mixture on reducing or eliminating *Salmonella* in contaminated poultry feeds at different storage time.

MATERIAL and METHOD

Feed Material

Feeds based on corn and soybean meal and balanced to meet the nutrient requirements for quails according to NRC (1994) divided into six experimental groups that consist of control, 150 mg/kg nisin (N150), 300 mg/kg nisin (N300), 3 g/kg organic acid mixture (Selacid® Green Growth MP) (OA), 150 mg/kg nisin + 3 g/kg organic acid mixture (N150+OA), 300 mg/kg nisin + 3 g/kg organic acid mixture (N300+OA). Active ingredients of Selacid ® GreenGrowth MP were sorbic acid, formic acid, acetic acid, lactic acid, propionic acid, ammonium formate, citric acid, 1,2-propanediol, coconut/palm kernel fatty acid distillate, silicondioxide (SiO_2). Nisin was used as bacteriocin in this study.

Method

Preparation of contaminant

An isolate of *S. Enteritidis* was procured from the Refik Saydam National Type Culture Collection Laboratory, Ankara, Turkey. Broth culture of *S. Enteritidis* was prepared in buffered peptone water (BPW) at 37 °C for 18 h. The number of *S. Enteritidis* was confirmed by serial dilution and colony counts on Brilliant Green Agar (Oxoid). The final contamination culture level contained 10^4 cfu/ml *S. Enteritidis*.

Experimental design

150 g of feed samples were weighed into sterile plastic pots and *Salmonella* cultures added by

spraying while vigorously stirring the feed. After application of the *Salmonella* into the feeds, *Salmonella* sp. were isolated and identified using the method described in ISO 6579:2002. 25 g of feed samples were added to 225 ml of buffered peptone water for pre-enrichment at 37 °C for 18 h. Then, samples were serially diluted in sterile saline solution and 10 µl of these solutions were pipetted onto XLD medium in triplicate and plates were incubated at 37°C for 48 h. Salmonella colonies were enumerated and results expressed as cfu/g (Initial Time). This process was repeated on 7th, 15th, 21st and 28th days of the study. Feed samples kept at room temperature during the experiment.

Statistical methods

Because the values did not adhere to normal distribution, data were expressed in $\log_{10}$. Data were analysed by ANOVA using the GLM procedure with SAS 8 software (SAS, 1999). The differences among the means were tested using Duncan's Multiple Range Tests. The differences were considered statistically significant at $P<0.05$.

RESULTS

Efficacy of treatments on *Salmonella* contamination was presented in Table 1.

After the contamination, while the highest *Salmonella* count was found in the Control group at initial time, N150+OA mixture and N300+OA mixture groups were found the most effective against *Salmonella* at the same time. *Salmonella* counts linearly increased in the Control group at day by day and it was observed the highest *Salmonella* values in the Control group during this study ($p<0.05$). Although lower values were observed in the experimental groups than the Control group ($p<0.05$), the differences between experimental groups were not found significant on the 7th, 21st and 28th days. On the 15th day of the study, only N300+OA mixture group was found highest between experimental groups. However, an increase in the number of *Salmonella* was observed in the experimental groups after the 21st day of the study.

Table 1. Effects of feed additives on *Salmonella* colonization in contaminated feed (cfu/g)
Çizelge 1. *Yem katkılarının bulaşık yemlerde salmonella kolonizasyonu üzerine etkileri (cfu/g)*

	Initial Time	7th day	15th day	21st day	28th day
Control	1.01[a]	3.09[a]	3.39[a]	3.58[a]	3.77[a]
N150	0.87[c]	0.72[b]	0.91[bc]	0.54[b]	0.77[b]
N300	0.81[bc]	0.80[b]	0.60[b]	0.45[b]	0.74[b]
OA mixture	0.87[c]	0.90[b]	0.87[bc]	0.57[b]	0.78[b]
N150+OA	0.74[bd]	0.84[b]	0.66[b]	0.60[b]	0.90[b]
N300+OA	0.70[d]	0.77[b]	1.09[c]	0.53[b]	0.72[b]
SEM	0.033	0.259	0.293	0.348	0.337
P value	0.008	0.0005	0.0006	0.0002	0.0003

N150: 150 mg kg^{-1} nisin, N300: 300 mg kg^{-1} nisin, OA: 3 g kg^{-1} organic acid mixture, N150+OA: 150 mg kg^{-1} nisin + 3 g kg^{-1} organic acid mixture, N300+OA: 300 mg kg^{-1} nisin + 3 g kg^{-1} organic acid mixture.
SEM: Standard error of means
a-d Values within the same column with different superscripts differ significantly (P<0.05).

DISCUSSION

Previous studies have shown that organic acid has positive effects in preventing *Salmonella* contamination in poultry feed or feed materials (Rouse et al. 1988; Matlho et al. 1997; Ha et al. 1998; Carrique-Mas et al. 2007; Koyuncu et al. 2013; Axmann et al. 2017; El Baaboua et al. 2018). Similar results were obtained from this study. The number of *Salmonella* in organic acid supplemented feed has significantly decreased compared to the control group. It is thought that the antibacterial effects of organic acids are due to their dissociation into anions and protons after they enter the cell wall. While protons reduce intracellular acidity, anions can affect DNA synthesis. The cell tries to remove excess proton and rebalance the intracellular pH via H + -ATPase. Meanwhile, high level of energy is wasted and the resulting lack of energy causes the death of the cell (Carrique-Mas et al. 2007; Van Immerseel et al. 2008; Doyle and Erickson, 2012; İpçak ve ark., 2017). Nisin has lower antibacterial activity against Gram-negative bacteria, such as *Salmonella*, because they have an LPS (Lipopolysaccharide) layer and this layer causes them to gain resistance against nisin (Ay and Tuncer 2016; Sangcharoen et al. 2017; Bingol et al. 2018). Although *Salmonella* is resistant to nisin, nisin reduced the number of *Salmonella* in this study. This result may depend on whether nisin is applied first and the formation of contamination later (Carrique-Mas et al. 2007). No study investigating the effects of nisin on salmonella contamination in feed has been found. However, similar results were obtained in some studies. Selim et al. (2012) reported that addition of 16 mg ml^{-1} nisin has inhibitory properties on *Salmonella indica*. In other study, it was reported that the addition of 250 IU ml^{-1} nisin in dooghs stored at 4ºC decreased *Salmonella typhimurium* from day 3 (Shahbazi, 2016). Similarly, Jayawera et al. (2018) reported that the addition of 0.2 g kg^{-1} nisin has the inhibitory effect against Salmonella on sausages infected with Salmonella at 10^2 and 10^8.

In the present study, the highest antimicrobial effect was observed in N300+OA group and this result was in agreement with the previous reports declared that antimicrobial activity of nisin against *Salmonella* could be increased by using it in combination with other antimicrobials and preservation strategies such as chelating agent, plant essential oils and organic acids (Ndoti-Nembe et al. 2015; Pinilla, 2016; Silva et al. 2016; Sangcharoen et al. 2017; Nissa et al. 2018; Ashari et al. 2019). In addition, it was reported that various stress factors such as temperature, pH and gamma irradiation increase the effect of nisin on Salmonella (Galvão et al. 2015; Ndoti-Nembe et al. 2015).

In conclusion, the present study demonstrated that bacteriocin (nisin), alone or in combination with organic acid mixture had great efficacy to prevent or inhibition of *Salmonella* contamination in feed at the different storage times.

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