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Synergistic Antimicrobial Effects of Zeolite and Eggshell Against *Salmonella* Typhimurium and *Listeria monocytogenes*

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ABSTRACT

Turkey is one of the world's leading egg producers, yet eggshells are usually thrown away as waste and not recycled. However, recycling these materials could benefit both the environment and the economy significantly. In poultry farming, bedding material is of critical importance for animal health, with natural, hygienic, inexpensive and environmentally friendly materials being preferred. In this study, the antimicrobial effects of bedding materials obtained by mixing eggshells with zeolite at specific ratios were investigated on *Salmonella* Typhimurium and *Listeria monocytogenes*. Mixtures prepared at ratios of 100% eggshell, 100% zeolite, 75% eggshell + 25% zeolite, and 50% eggshell + 50% zeolite were inoculated with these pathogens and analysed at 0, 6, 12, 24, and 48 hours. The most effective reduction was observed in the 50% eggshell + 50% zeolite mixture for *S*. Typhimurium at 12 hours [1.4 $\log_{10}$ ($p<0.05$)] and in the 50% eggshell + 50% zeolite mixture for *L. monocytogenes* at 6 and 12 hours [1 $\log_{10}$ ($p<0.05$)]. The moisture-absorbing and toxin-binding properties of zeolite, in synergy with eggshell, were effective in reducing the microbial load. These findings suggest that eggshell can be transformed into an effective bedding material when used in combination with zeolite. This study contributes to the development of alternative bedding materials in the poultry industry while providing a meaningful contribution to the literature in terms of re-evaluating waste-based biomaterials.

Keywords: Eggshell, Zeolite, Bedding material, *S*. Typhimurium, *L. monocytogenes*

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INTRODUCTION

In 2023, approximately 97 million tonnes of eggs were produced worldwide [1] and approximately 1.5 million tonnes in Turkey [2]. Turkey ranks 10th in the world and 2nd in Europe in egg production. Given the high production volume of this food product, it is important to recycle its waste materials and contribute them to the economy [3]. Eggshells account for 9-12% of an egg. Eggshells contain 94% calcium carbonate, 1% magnesium carbonate, 1% calcium phosphate, and 4% organic components consisting of proteins (glycoproteins, proteoglycans, etc.) [4]. In free-range poultry farming, a flexible, water-absorbent material called bedding is spread on the floor to prevent heat loss through conduction and to allow chickens to engage in their natural behaviour of scratching. The main reason for using bedding in poultry houses is to provide thermal insulation on the floor. Good bedding should be dry, clean, and odourless so as not to disturb the animals. It should be soft and compressible, that is, flexible. It is important that bedding is not contaminated with chemicals or medications and does not contain harmful organisms. Additionally, bedding should be a good insulator; that is, it should have low thermal conductivity and high water absorption capacity. These properties should be combined with the ability to dry quickly. Otherwise, the bedding may lose its insulating ability in a short time. When selecting bedding material, it should also be considered whether it can be used as fertiliser when removed from the coop at the end of the rearing period. Among these materials, those that are easy to obtain and low in cost should be preferred [5]. Zeolites have attracted attention in various fields, including antimicrobial applications, in recent years due to their unique structural and chemical properties. The physicochemical properties of zeolite minerals (ion exchange, adsorption, dehydration, and rehydration) enable their use in many different fields, particularly in agriculture and animal husbandry, including fertiliser and soil improvement, agricultural pest control, soil contamination control, animal husbandry, animal manure improvement, and aquaculture [6, 7]. Turkey ranks among the top countries in the world with 45.8 billion tonnes of zeolite reserves [8, 9]. Natural zeolites play an important role in the purification of methane gas; odour removal and moisture control are of great importance in providing healthier conditions in animal shelters and in the utilisation of liquid manure [10]. Zeolite has a specific affinity for ammonium ions and ammonia [9]. Zeolite tuffs, due to their high capacity to absorb ammonium and water, help prevent unwanted odours and excessive moisture when used as bedding in poultry houses. Additionally, when administered to chickens and other poultry in rock form, zeolite absorbs urea and odours in their digestive systems, reducing the moisture content of manure and minimising its negative environmental impact. The resulting manure can be used as high-quality fertiliser due to its rich nutrient content [11]. Studies have shown that adding zeolite to bedding has a positive effect on growth rate, but reusing zeolite-containing bedding does not sustain this positive effect, though it does not cause any negative effects either. In terms of bedding quality, it can be said that there may be a slight advantage in terms of moisture and pH levels [12]. Considering the proven antimicrobial efficacy of natural zeolite in poultry meat decontamination [13], its application as a bedding material in poultry farming may offer additional benefits in reducing pathogenic load in the rearing environment.

S. Typhimurium is one of the major causes of non-typhoidal salmonellosis cases commonly seen in humans. It can cause serious gastrointestinal symptoms, especially in children, the elderly, and immunocompromised individuals. In poultry farming, this pathogen colonises the intestines and spreads into the environment via faeces, and can remain viable for long periods of time, especially in litter materials. Bedding materials such as fresh wood shavings create a favourable growth environment for *S.* Typhimurium, increasing both intra-flock transmission and the risk of spread throughout the processing chain. Factors such as moisture, temperature, and organic matter content in the bedding contribute to the bacterium's ability to form biofilms and develop resistance. Maintaining the hygiene of bedding materials is of great importance in preventing the spread of *S.* Typhimurium in poultry systems. Therefore, selecting appropriate materials for bedding, regularly disinfecting them, and supporting them with effective biosecurity measures are critical requirements for both protecting animal health and reducing the risk of zoonotic transmission [14].

L. monocytogenes is a foodborne pathogen that occurs naturally in the environment and can multiply even in low temperatures, high salt and acidic environments, posing a high risk to human health. This bacterium, which can cause septicaemia, meningitis and foetal complications, particularly in the elderly, immunocompromised individuals and pregnant women, is frequently detected in dairy products, meat, seafood and ready-to-eat foods, with mortality rates reaching 20-30% [15]. Although zero-tolerance policies are applied to food, the live animal production processes that are the source of contamination, particularly poultry farming, have not yet been sufficiently researched. Indeed, *L.* monocytogenes has been isolated from various sources in poultry environments, including litter, dust, feed, faeces, and caecum; litter material has played a significant role in the spread of the bacterium. Litter contaminated with faeces and dust facilitates the spread of the bacterium within the poultry house, while improper storage and inadequate cleaning practices further increase this risk. Therefore, the selection of bedding material, its regular replacement, and the meticulous maintenance of hygienic practices are critical in preventing poultry from becoming a reservoir for *Listeria*. Measures taken at every stage of production, following a 'farm-to-fork' approach, are considered a fundamental strategy for ensuring food safety by reducing the risk of transmission to humans [16].

The combination of eggshell and zeolite can combine the benefits of both materials and offer significant advantages as a bedding material. The low water absorption capacity and high calcium content of eggshell, when combined with zeolite, can result in a more effective bedding material by combining zeolite's moisture absorption and odour removal properties. This combination can create a suitable environment for animal health and offer an environmentally sustainable solution. Notably, no studies have been found on the effects of eggshell and zeolite mixtures as bedding material on the viability of pathogenic bacteria. Additionally, no studies have been found on the use of eggshell and zeolite mixtures as bedding material. This study is expected to contribute to the literature and to producers engaged in industrial chicken production in these aspects.

METHODS

Study Material and Preparation

The eggshells used in the study (sourced from a local food business) and the zeolite (containing a minimum of 95% clinoptilolite) were obtained through commercial purchase. Prior to use, the eggshells were wiped with 70% ethanol and then washed with water at 55 °C to reduce microbial load and remove surface contaminants. A 70% ethanol solution is known to be effective for surface disinfection, as it disrupts the cell membranes of microorganisms and causes protein denaturation. Washing with warm water also contributes to the physical removal of pathogens from biological surfaces and aids in lowering microbial load. The zeolite was used without any chemical treatment and ground directly into a fine powder. Both materials were processed using a grinding machine (GG-30B-C1-GELGOOG, *China*) and prepared as mixtures in the proportions specified in Table 1. These preliminary treatments aimed to reduce the microbial risk associated with natural materials and to ensure a more controlled environment for experimental applications.

Table 1. Prepared eggshell and zeolite mixtures

Group	Mixture Type	Mixing Ratio
1	ES	%100
2	Z	%100
3	ES+ Z	%50 ES + %50 Z
4	ES + Z	%75 ES+ %25 Z

ES: *Eggshell*, **Z:** *Zeolite*

Pathogenic Bacteria Inoculum Preparatıon

Eggshell and zeolite groups were inoculated with *S.* Typhimurium (NCTC 12416, 74 and ATCC 14028) and *L. monocytogenes* (N 7144, RSKK 474 and 476) (Refik Saydam National Public Health Institute-Turkey) reference strains were used. Each pathogen was incubated separately in Tryptic Soy Broth (BIOKAR Diagnostics) at 37 °C for 18–24 hours. After incubation, bacterial counts were adjusted to 10^8 CFU ml^{-1} according to the McFarland standard. 1 ml of the adjusted bacterial suspension was added to 9 ml of sterile 0.1% peptone water (BIOSOLUTE, TH. Geyer), and this process was repeated twice to prepare a new suspension with a concentration of 10^6 CFU ml^{-1}.

Pathogenic Bacteria Inoculum for Groups

0.5 ml of a diluted bacterial cocktail at a level of 10^6 CFU ml^{-1} was inoculated into the formed groups. The bacteria were left to adapt to the environment and establish themselves at room temperature (25±1 °C) for 10 minutes. They were then kept at room temperature for 48 hours.

Microbiological Analysis

Microbiological analyses were performed at 0 (immediately after initial contamination), 6, 12, 24, and 48 hours. At each analysis time point, 10 g of samples were taken and placed in a stomacher bag under sterile conditions. 90 ml of 0.1% peptone water was added to the samples. The samples were homogenised in a stomacher (Mayo Homogeniser Hg400v) for 3 minutes to prepare a 10^{-1} dilution. From this dilution, other dilutions up to 10^{-7} were prepared using the serial dilution method with sterile peptone water. A spread plate method was used for inoculation to determine the number of pathogenic bacteria (per gram) in the samples. [17].

***L. monocytogenes* count:** Oxford agar (BIOKAR Diagnostics) was used as the culture medium for *L. monocytogenes* counting. Plates were incubated at 35 °C for 24–48 hours, and specific colonies (blackish-green, brown, black-zoned, depressed-centred colonies) were counted.

***S.* Tyhimurium count:** Xylose Lysine Deoxycholate (XLD) agar (BIOKAR Diagnostics) was used for *S.* Tyhimurium counting. Plates were incubated at 35 °C for 24–48 hours, and specific colonies (black-coloured colonies) were counted.

Statistical Analyses

Three independent replicates of the study were performed. Microbiological data were converted to log10 CFU g^{-1} and statistical analyses were performed using SPSS 24.0 for Windows software [18]. One-way analysis of variance (One-Way ANOVA) was used to determine whether the differences between the groups were statistically significant at a 95% confidence level. Duncan's multiple comparison test was used to determine the difference between the means of the groups. The values of the research results were given as mean ± standard error. Statistical significance level was evaluated as $p<0.05$.

RESULTS

In this study, the effects of zeolite and eggshell mixtures at different ratios on *S.* Typhimurium and *L. monocytogenes* were investigated. Microbiological analyses revealed significant decreases in bacterial load, particularly at 6 and 12 hours ($p<0.05$).

The most pronounced decrease for *S.* Typhimurium was observed in the 50% eggshell + 50% zeolite and 75% eggshell +25% zeolite mixture groups, with reductions of approximately 1.4 and 1.3 log_{10} units, respectively, at the 12th hour. A decrease of approximately 1.3 log_{10} was observed at the 6th hour in the 100% zeolite group, while no significant decrease in bacterial count was observed in the 100% eggshell group. *S.* Typhimurium counts showed a tendency to increase again at the 24th and 48th hours in all groups. Numerical data for *S.* Typhimurium are presented graphically in **Figure 1.**

For *L. monocytogenes*, the most effective reduction was observed in the 50% eggshell + 50% zeolite group at 6 and 12 hours, with a decrease of approximately 1 log_{10} units. Although

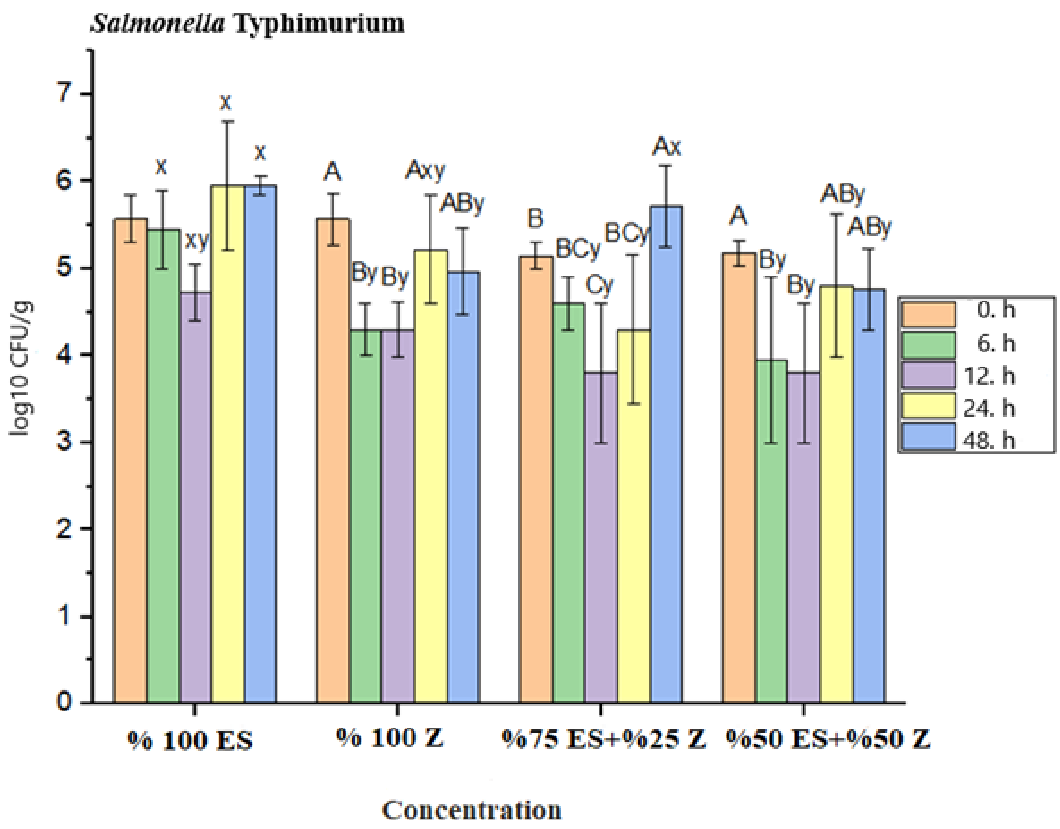

Figure 1. *Salmonella Typhimurium Counts (Mean ± SE)*
ES: *Eggshell,* ***Z:*** *Zeolite,* ^{xy}*: Mean values with different letters between groups are significantly different (p<0.05),* ^{AC}*: Mean values with different letters between sampling periods are significantly different (p<0.05).*

decreases were initially observed in other groups, the bacterial load increased again at 24 and 48 hours. A decrease of approximately 1 log_{10} was determined at 6 hours in the 100% zeolite group; however, no significant effect was observed in the 100% Eggshell group. Although the 25% zeolite + 75% eggshell mixture initially caused a decrease, the microbial load approached the initial levels at 48 hours. Numerical data for *L. monocytogenes* are presented graphically in **Figure 2.**

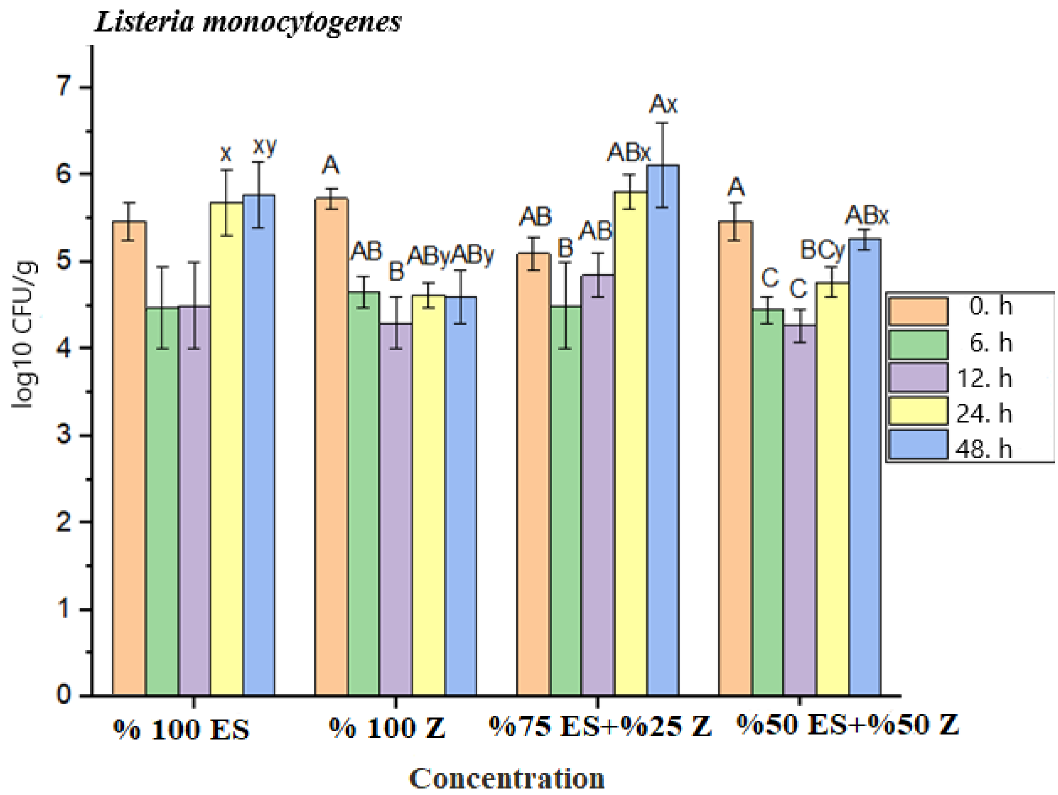

Figure 2. *Listeria monocytogenes Counts (Mean ± SE)*
***ES:** Eggshell, **Z:** Zeolite, ^{xy}: Mean values with different letters between groups are significantly different (p<0.05), ^{AC}: Mean values with different letters between sampling periods are significantly different (p<0.05).*

In general, significant microbial reductions were observed for both pathogens in mixtures with high zeolite content, but this effect was more pronounced for *S.* Typhimurium compared to *L. monocytogenes*.

DISCUSSION

The microbiological results obtained in the study showed significant changes in the number of *S.* Typhimurium and *L. monocytogenes* in different concentration/time groups.

Zeolite can remove moisture and volatile components (e.g., ammonia) from the environment due to its high surface area, ion exchange capacity, and adsorbent properties [19]. This moisture-removing property reduces the water activity necessary for the growth of pathogenic bacteria, thereby inhibiting bacterial growth. Since Gram-negative bacteria, in particular, exhibit the ability to multiply more easily in high-humidity environments, reducing moisture directly affects the life cycle of Gram-negative pathogenic bacteria such as *Salmonella* [20].

Eggshells contain high levels of calcium carbonate (94%), which has the potential to raise the pH of the environment. Increased pH levels can have an inhibitory effect on pathogenic bacteria, which reproduce better in near-neutral environments. Additionally, the antimicrobial proteins and glycoproteins contained in eggshells may contribute to microbial adsorption, making it more difficult for bacteria to adhere to surfaces. The active oxygen species produced by eggshells also have the potential to act as antimicrobial agents. [21]

The most effective results on *S.* Typhimurium were observed in the groups with 50% eggshell + 50% zeolite and 75% eggshell + 25% zeolite at 12 hours. Regardless of the groups, a decrease in *S.* Typhimurium was observed at the 6th and 12th hours, followed by a resurgence at the 24th and 48th hours. In the 100% zeolite group, a decrease of approximately 1.3 $\log_{10}$ units in the *S.* Typhimurium population at the 6th hour is noteworthy. This rapid inhibition can be attributed to zeolite's high ion exchange capacity and adsorption surface properties. Previous studies have indicated that zeolite disrupts the ion balance in the bacterial cell membrane, increasing membrane permeability, and captures metal ions (e.g., Ag^{+}, Zn^{2+}) that disrupt intracellular metabolism, thereby inducing bacterial stress [22]. Additionally, the literature emphasises that zeolite is particularly effective against Gram-negative bacteria, a class that includes *Salmonella* [23].

In the 100% eggshell group, no significant decrease was observed during the 48-hour incubation period. This situation shows that calcium carbonate, the dominant component of eggshells, acts on environmental pH values rather than creating an antimicrobial effect on its own [24]. However, the surface properties of this material may have synergistic potential when combined with other antimicrobial components.

In this context, when the 75% eggshell + 25% zeolite and 50% eggshell + 50% zeolite mixture groups were evaluated, significant decreases in *Salmonella* counts were observed, particularly at 6 and 12 hours. In the 75% eggshell + 25% zeolite group, a decrease of approximately 1.3 $\log_{10}$ units was observed at the 12th hour, while in the 50% eggshell + 50% zeolite group, this decrease was approximately 1.4 $\log_{10}$ ($p<0.05$). These results suggest that the pH change and adsorption contribution provided by the eggshell in the environment support the antimicrobial effect of zeolite.

The findings showed that mixtures at specific ratios significantly reduced the bacterial load, particularly during the first 12-hour period. The underlying mechanisms of these effects are thought to be explained by the synergistic interaction resulting from the unique physicochemical properties of both substances.

In the microbiological analysis data for *L. monocytogenes*, another important pathogen examined in the study, the most effective results among the groups were observed in the group with 50% eggshell + 50% zeolite at 6 and 12 hours. Regardless of the groups, *L. monocytogenes* was observed to rise again at 24 and 48 hours.

The findings revealed significant microbial reductions, particularly in formulations with high zeolite content ($p<0.05$). However, this effect was more limited compared to *S.* Typhimurium; this can be attributed to *L. monocytogenes*' more resistant nature to environmental stress factors.

In the 100% zeolite group, the reduction in microbial load observed at 6 hours was approximately 1 $\log_{10}$ units, which can be explained by zeolite's ion exchange properties, adsorptive capacity, and porous structure that controls moisture [25]. Clinoptilolite, found in the natural structure of zeolite, disrupts permeability by creating an ionic imbalance in the bacterial cell membrane and interferes with intracellular metabolism [26]. This mechanism is more pronounced in Gram-negative bacteria but also disrupts ionic homeostasis in Gram-positive bacteria. However, *L. monocytogenes* is more resistant to such surface-based mechanisms due to its thick peptidoglycan layer [27].

No significant antimicrobial effect was observed in the 100% eggshell group. This natural material, based on calcium carbonate, does not function as an antimicrobial agent on its own, but indirectly contributes by regulating the pH of the environment [24]. pH changes have a limited effect on bacteria such as *Listeria*, which have high tolerance to acidic and basic conditions [28]. Indeed, although a decrease was observed at 6 and 12 hours in other groups containing eggshell, the bacterial load increased again at 24 and 48 hours after the bacteria adapted to the environment.

The 75% eggshell + 25% zeolite group showed a short-term decrease at the beginning, but the microbial load rose again at 48 hours and approached the initial levels. This situation indicates that when the zeolite amount falls below a certain threshold, the sustainability of microbial control decreases. Similarly, the literature reports that the antimicrobial effects of additives are mostly dose-dependent [29].

One of the most striking results was observed in the 50% eggshell + 50% zeolite group, where a decrease of approximately 1 $\log_{10}$ units was observed at 6, 12, and 24 hours ($p<0.05$). This finding suggests that zeolite and eggshell may have a synergistic effect when used in combination. The combined use of zeolite's ion exchange and adsorption capacity, with the effect of eggshell on pH, may have provided advantages in terms of both bacterial stress formation and surface adsorption. [25]. Additionally, this synergy may be related to the creation of a microenvironment within the zeolite's microporous structure that suppresses *Listeria*'s metabolic activity. The findings of this study indicate that *L. monocytogenes* can be inhibited by physicochemical interventions using natural minerals, despite its environmental resistance capacity.

The use of such natural and non-toxic materials is expected to become more widespread, particularly in bioprotective applications and industrial livestock farming.

CONCLUSION

In this study, the antimicrobial effects of eggshell and zeolite mixtures were successfully demonstrated, and it was shown that the use of these combinations as bedding material in animal husbandry could be effective in controlling important pathogens such as *S.* Typhimurium and *L. monocytogenes*. Significant microbial reductions were achieved at specific time intervals, particularly at ratios of 50% eggshell + 50% zeolite and 75% eggshell + 25% zeolite. The findings indicate that the combined use of zeolite and eggshell represents an effective and innovative approach for enhancing antimicrobial properties in livestock bedding materials. Additionally, this combination offers a significant advantage in terms of supporting waste conversion in line with environmental sustainability principles and contributing to the development of new-generation bedding materials that prioritise animal health. Considering the economic and environmental advantages provided by eggshell and zeolite mixtures, this composition has high potential to be evaluated as a sustainable and innovative bedding material in the sector. In future studies, it is anticipated that the duration of the antimicrobial effect can be extended and that the inhibition capacity against different pathogens can be increased by adding additional additives. In this context, the integrated use of waste-based natural materials with a focus on biosecurity is expected to remain an important area of research for providing innovative and sustainable solutions in animal husbandry.

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Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

Conflicts of Interest

There is no conflict of interest for the publication of this article.

Disclosure

The authors have reported no conflicts of interest in preparing and publishing this article.

Ethics committee approval

Ethics committee approval is not required in this study. The study was conducted following the international declaration, guidelines, etc.

Referee Evaluation Process

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