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In vitro evaluation of the antimicrobial activity of VemoZyme® Alternase^{+} - A specific muramidase-based product

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Abstract: This study evaluated the *in vitro* antibacterial activity of VemoZyme® Alternase^{+}, a muramidase-containing enzyme preparation, against Gram-negative bacteria relevant to animal health. The specific enzyme was tested using agar diffusion, viability reduction assays, and suspension assays against *Escherichia coli* ATCC 8739 and *Salmonella enterica* ATCC 1304. Agar diffusion assays showed no inhibition, likely due to limited enzyme diffusion. In suspension assays at 0.05 g/mL enzyme concentration and pH 6.5, bacterial viability decreased to 25% for *E. coli* and 60% for *S. enterica* after 90 minutes. These results indicate a measurable reduction in Gramnegative bacterial viability under in vitro conditions, suggesting VemoZyme® Alternase^{+} may have potential for further investigation to modulate gut microbial populations.

Keywords: VemoZyme® Alternase^{+}; muramidase; antimicrobial activity; pH; *Escherichia coli*; *Salmonella enterica*

INTRODUCTION

The growing antibiotic resistance of pathogenic bacteria is a major global concern. A critical task for scientists worldwide is the identification of alternative antimicrobial agents, which are not affected by bacterial resistance mechanisms. Such alternatives must be non-toxic to animals, humans, and the environment, yet effective in suppressing the proliferation of pathogenic microorganisms, and suitable for the prevention and treatment of various infections. Lysozyme is a natural enzyme with antimicrobial activity that has the potential to be used in various industries. It is considered a natural antimicrobial agent with potential applications in the food, pharmaceutical and other industries (Khorshidian et al., 2022; Pilevar et al., 2022).

Among the various factors contributing to the proper functioning and homeostasis of the gastrointestinal tract, the composition and metabolic activity of the gut microbiota play a fundamental role in the overall health of the host. This is due to their influence on digestion, nutrient absorption, intestinal physiology, immune system modulation, and resistance to pathogenic colonization (Sais et al., 2020). An optimally functioning gastrointestinal tract is characterized by a state of stability and mutual interaction between the microbiota and host cells, supporting physiological processes, maintaining homeostasis, and contributing to improved health and productivity (Celi et al., 2017).

One potential approach is the inclusion of exogenous enzymes in feed. VemoZyme® Alternase^{+} is a product that contains a specific 1.4-β-N-acetylmuramidase (muramidase or lysozyme). This enzyme hydrolyzes the β-(1→4) glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in bacterial peptidoglycan, an essential component of the cell wall (Schleifer and Kandler, 1972; Vollmer et al., 2008).

Muramidase plays a key role in the degradation and remodeling of peptidoglycan, influenc-

ing bacterial growth and cell division (Typas et al., 2011). Its catalytic mechanism involves acid-base catalysis, where glutamate and aspartate residues act as proton donors and acceptors to facilitate hydrolysis. This process involves cleavage of the glycosidic bond and formation of a covalent enzyme-substrate intermediate, as supported by recent crystallographic and molecular dynamic studies (Davies and Henrissat, 1995; Vocadlo et al., 2001; Zhang et al., 2024).

Fragments such as muramyl dipeptides, released during enzymatic degradation, serve as activators of host immune responses by binding to nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptors, thereby modulating the immune system (Girardin et al., 2003; Wang et al., 2021).

Studies show that muramidase supplementation in poultry, pigs, and rabbits improves nutrient digestibility and gut health, reduces colonization by pathogens such as *Escherichia coli* and *Clostridium perfringens*, and promotes the growth of beneficial gut microbiota (Lichtenberg et al., 2017; Sais et al., 2020; Goes et al., 2022; Omar et al., 2023). In addition, muramidase contributes to mucosal protection and modulation of the intestinal immune environment (Brugaletta et al., 2022; Amer et al., 2023).

Muramidase produced from microbial strains, such as *Trichoderma reesei*, has been found to be safe and effective in animal feed, and with no reported toxicity even at high doses (Lichtenberg et al., 2017). In broilers, VemoZyme® Alternase$^+$ supplementation has been associated with improved growth, feed efficiency, and enhanced overall gut function.

The aim of the present study was to evaluate the *in vitro* antibacterial activity of the enzymatic product VemoZyme® Alternase$^+$, containing muramidase, against *E. coli* and *S enterica*.

MATERIALS AND METHODS

Material

The antimicrobial effect of VemoZyme® Alternase$^+$, a specific microbial muramidase preparation produced by *Trichoderma reesei* (VEMO Corp., Sofia, Bulgaria), was tested. Stock solution was prepared at 0.5 g/mL in sterile distilled water. Final concentrations in assays ranged from 0.001 to 0.05 g/mL. The antimicrobial activity of the enzyme was tested at three different pH values of the enzyme solutions, 6.5, 6 and 5, respectively.

The product is a natural antibacterial agent tailored to improve gut health, digestion, and nutrient absorption. The recommended practical dosage is 500 g/ton of complete feed. It is manufactured through controlled fermentation using a specially selected strain of *T. reesei*. The guaranteed minimum enzymatic activity is ≥ 300 000 000 U/kg. The physicochemical and microbiological specifications are shown in Table 1.

Microorganisms

Pure cultures of two Gram-negative microbial strains were used: *Escherichia coli* ATCC 8739, *Salmonella enterica* subsp. *enterica* ATCC 1304. The clinical strains originated from the Microbial Strain Collection of the Microbiology Laboratory at the Faculty of Veterinary Medicine, University of Forestry, Sofia, Bulgaria, which is part of the Global Registry of Scientific Collections (https://scientific-collections.gbif.org/collection/4a4a2c4e-e035-4bf1-bc5f-c60974897c18, date of last visit 24.10.2025). Suspensions with a McFarland standard density of 0.5 were prepared in sterile physiological saline.

Nutrient Media

Meat-peptone broth and agar, as well as Mueller-Hinton agar (BUL BIO NCIPD - Sofia), were used.

Table 1. Physicochemical and microbiology specifications of VemoZyme® Alternase$^+$ (VEMO Corp., Sofia, Bulgaria)

Microbiological specifications	Total viable count: ≤ 5×10^4 CFU/g Molds and yeasts: ≤ 1×10^3 CFU/g E. *coli, Salmonella* spp., CFU/g: Must not be detected
Heavy metals	Lead (Pb): ≤ 200.0 mg/kg Cadmium (Cd): ≤ 15.0 mg/kg

Source: Authors' own elaboration

Microscopy

Microscopic examination of the microorganisms was performed under oil immersion at 1000× magnification following classical Gram and Pfeiffer staining methods. A digital microscope with a camera (model B-190TV, Optika, Italy) was used.

Antimicrobial testing

Preliminary assessment of the antibacterial activity was conducted using the classic agar-gel diffusion method of Bauer et al. (1966), suitable for fast-growing aerobic organisms, on Mueller-Hinton agar (BUL BIO NCIPD - Sofia) with pH 7.2 - 7.4 and 4 mm thickness. Microbial suspensions were inoculated at 2×10^6 cells/mL. The product was applied by dispensing 0.1 mL into 9 mm diameter wells. The control antibiotic chloramphenicol (Merck KGaA, Darmstadt, Germany) was tested using discs loaded with 30 µg.

Incubation was carried out at 35 - 37°C for 18 - 24 and 72 hours. Inhibition zone diameters were measured in millimeters, including the diameter of the well, using a transparent ruler placed beneath the petri dishes. Interpretations followed the three-tier system of Bauer et al. (1966): resistant (R): < 12 mm, intermediate (I): 13–16 mm, and sensitive (S): > 17 mm. For chloramphenicol the following three-tier system was used: R: < 12 mm, I: 13–17 mm, S: > 18 mm (NCCLS).

Minimum Inhibitory Concentration (MIC)

MIC was determined using the agar dilution method described by Ericsson and Sherris (1971) and NCCLS M7-A. Two enzyme concentrations were tested.

- **0.5 g/mL stock**: 10 g of enzyme product dissolved in 20 mL sterile distilled water.
- **Final agar concentrations**:
  - 0.1 g/mL: 4 mL of the stock added to 20 mL of molten agar (55 - 58°C)
  - 0.01 g/mL: 0.4 mL of the stock added to 20 mL of molten agar Control plates without enzyme were also prepared.

Suspension method

In 50 mL of sterile liquid medium, 25 g of enzyme were dissolved (0.5 g/mL). The solution was aliquoted into tubes (5 mL/tube). Each bacterial strain was tested in duplicate. Tubes were incubated at 37°C, and samples were plated onto agar at 90 minutes, 4 hours, and 24 hours. Colony counts were compared with untreated controls to assess antimicrobial activity.

RESULTS AND DISCUSSION

Using the agar diffusion method, no inhibition zones were observed at the tested concentrations (0.01 g/well and 0.001 g/well). Chloramphenicol, used as a positive control, showed effective inhibition. The likely cause of the enzyme's inactivity in this method is poor diffusion through the agar, as the white-colored enzyme solution remained localized in the wells without dispersing.

MIC testing showed notable effects on Gram-negative bacteria: *E. coli* and *S. enterica*, colony counts were approximately 70% lower on agar with 0.001 g/mL enzyme, and 50% lower on agar with 0.01 g/mL enzyme.

The data presented in Table 2 indicate that *E. coli* exhibited the highest sensitivity to the enzymatic treatment and *S. enterica* followed. These observations are consistent with the results obtained through minimum inhibitory concentration (MIC) determination.

The findings also suggest that the antibacterial activity of the tested muramidase-containing enzyme preparation is at its peak at pH 6.5, which closely corresponds to the physiological pH of the small intestine. A measurable inhibitory effect was also observed at pH 5, however, the activity was lowest at pH 6. Extending the exposure du-

Table 2. Bacterial viability (%) after 90-minute exposure to 0.05 g/mL enzyme in media of different pH

Bacterial strain	pH		
	6.5	6	5
Escherichia coli	25%	50%	40%
Salmonella enterica	60%	70%	60%

Source: Authors' own elaboration

ration of the enzyme did not result in additional reduction in bacterial counts, indicating a plateau in the enzyme's antibacterial action under the experimental conditions.

The specific muramidase enzyme exhibited *in vitro* antibacterial activity even at high bacterial concentrations (10^6 CFU/mL), suggesting a robust mode of action. It is plausible that *in vivo* efficacy may be enhanced due to the dynamic mixing processes facilitated by gastrointestinal peristalsis, which promote more effective interaction between the enzyme and microbial cells. In contrast, during *in vitro* assays, the enzyme tends to precipitate due to its higher molecular weight, thereby limiting its physical interaction with the suspended bacterial cells.

These results align with the observations of Wang et al. (2021), who reported that dietary supplementation with muramidase exerted a moderate modulatory effect on the microbiota of the duodenum, ileum, and cecum in broilers. The enzymatic hydrolysis of bacterial peptidoglycan generates muramyl dipeptides, which act as ligands for nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptors in eukaryotic cells, thereby contributing to enhanced intestinal immune status and overall gut health.

Several external factors are known to modulate muramidase activity. Ellison and Giehl (1991) demonstrated that lactoferrin alters the outer membrane of Gram-negative bacteria, which normally serves as a barrier to muramidase activity. They proposed that the membrane-permeabilizing effect of lactoferrin enhances muramidase's access to the peptidoglycan layer, leading to synergistic bactericidal activity against species such as *E. coli*, *Salmonella Typhimurium*, and *Vibrio cholerae*.

Further support for muramidase efficacy is provided by Myint et al. (2012), who confirmed the enzyme's antibacterial activity against live *S. aureus* and *E. coli* cells, as well as its peptidoglycan-cleaving activity against isolated cell walls from *Micrococcus lysodeikticus.*

Additionally, chemical modifications such as acidification of the enzyme preparation have been reported to attenuate muramidase activity against Gram-positive bacteria while enhancing its action against Gram-negative strains. However, the overall antibacterial efficacy of acid-modified muramidase remains inferior to that of the native enzyme when targeting Gram-positive pathogens (Wang and Wang, 2022). Recently, Abdou and Awad (2025) had developed a preparation of lysozyme peptides, thereby expanding the spectrum of activity of lysozyme. This preparation exhibits strong antibacterial activity against *E. coli, Salmonella Enteritidis* and other Gram-negative bacteria.

Zhou et al. (2017) further demonstrated that muramidase exhibits inhibitory activity against both Gram-negative (e.g., *Vibrio* spp., *E. coli*) to varying degrees. Their findings indicate that muramidase can bind to lipopolysaccharides (LPS) in Gram-negative bacteria. In addition to hydrolytic activity, muramidase may contribute to bacterial inhibition via agglutination mechanisms, enhancing host immune defenses by promoting bacterial clearance.

Moreover, muramidase activity may reduce peptidoglycan burden in the intestinal lumen, potentially improving nutrient digestibility and absorption efficiency, as described by Goes et al. (2022).

The present study demonstrates inhibitory activity of the tested specific muramidase-containing enzyme preparation against key enteric pathogens, including *E. coli* and *S. enterica*, under *in vitro* conditions. The great efficacy against Gram-negative bacteria is of practical significance, particularly given the prominent role of these pathogens in infectious diseases affecting commercially reared swine and poultry. The underlying mechanisms may include enzymatic degradation of peptidoglycan, disruption of membrane integrity, and agglutination-mediated bacterial immobilization, consistent with the findings of Goes et al. (2022) and others.

CONCLUSIONS

The tested VemoZyme® Alternase$^+$ product demonstrated notable antimicrobial activity against the examined Gram-negative bacteria

Escherichia coli and *Salmonella enterica*. At an initial bacterial concentration of 2×10^6 CFU/mL, a significant reduction in viable cell counts was observed within 90 minutes of enzymatic exposure.

Under conditions of 0.05 g/mL muramidase concentration and pH 6.5, the following relative viability levels were recorded: *E. coli* - 25% viable cells and *S. enterica* - 60% viable cells.

These findings confirm a significant bactericidal effect under the tested *in vitro* conditions. The results suggest potential for VemoZyme® Alternase⁺application in targeting common intestinal pathogens, particularly in animal husbandry settings.

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