

Original Article

Antibacterial Activity of *Apis mellifera* Bees Honey, Garlic Extracts and Their Combinations against Salmonella in Wolayta Sodo, Southern Ethiopia

Rediet Wolde¹, Samatar Abshir Mahamed^{2*}

¹ Jimma University, School of Veterinary Medicine, College of Agriculture and Veterinary Medicine, P.O. Box 307, Jimma, Ethiopia; ² Jigjiga University, College of Veterinary Medicine, P. O. Box 1020, Jigjiga, Ethiopia

ABSTRACT

Background: The global emergence of antibiotic-resistant Salmonella strains poses a significant threat to public health, necessitating the development of alternative antimicrobial agents. Although natural substances, such as honey and garlic, have shown promise in combating various pathogens, their efficacy against Salmonella, particularly in combination, remains understudied. **Objective:** This study aimed to evaluate the antibacterial activity of *Apis mellifera* honey, garlic extracts, and their combination against Salmonella in Wolayta Sodo, southern Ethiopia. **Methods:** Aqueous honey and garlic extracts were prepared at concentrations of 25, 50, and 100 g/100 mL. Antibacterial activity against Salmonella was assessed via the agar well diffusion method to measure zones of inhibition. The efficacies of honey, garlic, and their combinations were compared at different concentrations. All the data were statistically analyzed via one-way ANOVA, with a probability of 0.05 and a 95% significance level, via SPSS Inc. Chicago, IL version 21.0. **Results:** Honey exhibited the strongest antibacterial activity against Salmonella, with inhibition zones ranging from 13.67 mm to 26.33 mm across all concentrations. Garlic extracts showed moderate efficacy, producing inhibition zones between 12.00 mm and 15.67 mm. Unexpectedly, the combination of honey and garlic did not have synergistic effects and consistently underperformed compared with honey alone. The antibacterial activity of all the treatments was concentration dependent, with the highest efficacy observed at 100 g/100 mL ($P < 0.001$). **Conclusion:** This study provides evidence of the potent antibacterial activity of *Apis mellifera* honey against Salmonella, which surpasses that of garlic extracts alone or in combination. These findings highlight the potential of honey as a natural antimicrobial agent, particularly at high concentrations.

KEYWORDS

Apis mellifera, antibacterial agents, honey, *Allium sativum*, drug resistance, one health

ARTICLE INFO

Received Sept 6, 2024

Revised Sept 20, 2024

Accepted Sept 29, 2024

Online Sept 30, 2024

EDITOR

I Made Dwi Mertha
Adnyana, S.Si.,
M.Ked.Trop.

CORRESPONDENCE

Samatar Abshir
Mahamed, Jigjiga
University, College of
Veterinary Medicine;
email: samedeeq@
yahoo.com

CITE THIS ARTICLE

Wolde R., and Mahamed S.A. (2024). Antibacterial Activity of *Apis mellifera* Bees Honey, Garlic Extracts and Their Combinations against Salmonella in Wolayta Sodo, Southern Ethiopia. *Svāsthya: Trends in General Medicine and Public Health*, 1(2): e25. <https://doi.org/10.70347/svsthya.v1i2.25>



Supplemental data for this article can be accessed online at <https://doi.org/10.70347/svsthya.v1i2.25>

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INTRODUCTION

The global emergence and spread of antibiotic-resistant bacterial strains, particularly *Salmonella*, poses a significant threat to both animal and human health [1]. This concern is particularly acute in developing nations, where unsanitary conditions, close animal-human interactions, and cultural practices involving the consumption of undercooked or raw animal products increase the risk of infection [2]. *Salmonella* strains that are resistant to various antimicrobial agents, particularly fluoroquinolones and third-generation cephalosporins, are considered an emerging problem worldwide, resulting in higher morbidity and mortality rates and higher overall treatment costs [2,3]. The increasing prevalence of antibiotic-resistant *Salmonella* strains has created an urgent need for alternative treatment strategies [4–6]. Given this challenge, natural remedies, such as honey and garlic, which have historically demonstrated antimicrobial properties, offer potential effective alternative treatments. These natural substances have been the subject of scientific inquiry owing to their long-standing use in traditional medicine [7–10].

Honey has been shown to combat foodborne pathogens, such as *Salmonella* and *Escherichia coli* [2,6,11], whereas garlic has demonstrated antibacterial properties against a variety of gram-positive and gram-negative bacteria, including drug-resistant strains [12]. Although studies have explored the individual antimicrobial properties of honey and garlic, comprehensive research examining their combined efficacy against *Salmonella* is lacking [13–15]. The most important chemical components of garlic that have been linked to antimicrobial activity are allicin and other organosulfur compounds [16–18]. The physical (acidity and osmolarity) and chemical (hydrogen peroxide, volatiles, beeswax, nectar, pollen, and propolis) components of honey are responsible for its antibacterial properties [19–22]. Previous studies have investigated the effectiveness of honey and garlic extracts, individually and in combination, against *Salmonella* strains isolated from calves in Wolayta Sodo, southern Ethiopia.

Despite the individual antimicrobial properties of honey and garlic, there is a critical knowledge gap regarding their potential synergistic effects against antibiotic-resistant *Salmonella* strains in livestock [17,23,24]. This study aimed to address the urgent need for alternative treatments by assessing the antibacterial effects of *Apis mellifera* bee honey and garlic extracts (*Allium sativum*) and their combinations on *Salmonella* spp. isolated from calves. The urgency of this research is underscored by the rapid increase in antibiotic-resistant *Salmonella* strains and the limited development of new antibiotics, creating a pressing need for alternative treatment strategies [12,18,25,26].

By exploring these traditional remedies, this study contributes to the development of novel strategies for combating antibiotic-resistant *Salmonella* infections in livestock. Furthermore, this study aligns with the broader goal of integrating traditional knowledge with modern scientific approaches to enhance animal healthcare practices, particularly in regions where access to conventional veterinary services may be limited. This research addresses a critical gap in our understanding of natural antimicrobials and has potential implications for sustainable livestock management and public health, particularly in resource-limited settings.

MATERIALS AND METHODS

Study design and setting

The experimental design focused on evaluating the in vitro antibacterial activity of *Apis mellifera* honey, garlic (*Allium sativum*), and their combination against *Salmonella* isolates. All tests were conducted in triplicate to ensure reliability and reproducibility, with three independent replication sets. This robust experimental approach allowed for statistical validation of the observed antibacterial activity.

Ethical considerations

This study was approved by the Research Ethics Review Committee of the College of Agriculture and Veterinary Medicine, School of Veterinary Medicine, Jimma University. The study was conducted in compliance with the ARRIVE 2.0 guidelines. All methods were performed in

accordance with relevant guidelines and regulations. Before the study was conducted, the objectives, expected results, and benefits of the study were explained to the farm owners, and permission was obtained from all farm and cattle owners for the use of their animals in the study.

Study area

This study was conducted from November 2017 to April 2018 in Wolaita Sodo, which is situated in SNNPR, southern Ethiopia, approximately 330 km from Addis Ababa. The research site is characterized by an elevation ranging between 1,600 and 2,100 m above sea level, with geographic coordinates of 6°54'N latitude and 37°45'E longitude. The region experiences a stable climate, with temperatures fluctuating between 24°C–30°C during the day and 16°C–20°C at night throughout the year. This consistent climate provided an ideal setting for maintaining stable experimental conditions.

Collection and preparation of honey and garlic samples

This study utilized processed *Apis mellifera* honey (100% pure Ethiopian honey) and garlic bulbs, both of which were sourced from the local market, to ensure authenticity and freshness. To verify its purity, honey was cultured on blood agar to detect bacterial contamination. Garlic preparation involves meticulous cleaning, peeling, and chopping using sterilized equipment to maintain aseptic conditions [27,28]. Honey and garlic samples (200 g each) were prepared at three concentrations: 25 g/100 mL, 50 g/100 mL, and 100 g/100 mL, with sterile distilled water used as a diluent. The samples were filtered through sterile gauze in a sterile conical flask, followed by appropriate storage and preservation for subsequent analysis. Notably, the incubation process was conducted in the dark to protect the light-sensitive components of honey, including hydrogen peroxide and glucose oxidase [20].

Bacterial isolate bioassay, isolation and identification of Salmonella

This study focused on Salmonella as the test microorganism isolated from fecal samples of calves suspected of having Wolaita Sodo Agricultural College Farm. Morphological and biochemical characteristics of Salmonella isolates presented in Figure 1. The isolation and identification process was conducted at the Wolaita Sodo Regional Laboratory, adhering to aseptic techniques. The protocol involved a series of culture steps and biochemical tests to ensure accurate identification of Salmonella at the genus level. Initially, the fecal samples were cultured in selenite broth, an enrichment medium specifically designed for Salmonella recovery from fecal matter and intestinal tract samples. Incubation was performed at 37°C for 24 h. Positive growth was indicated by a cloudy appearance, foul odor, and gas production upon opening the test tube [29,30].

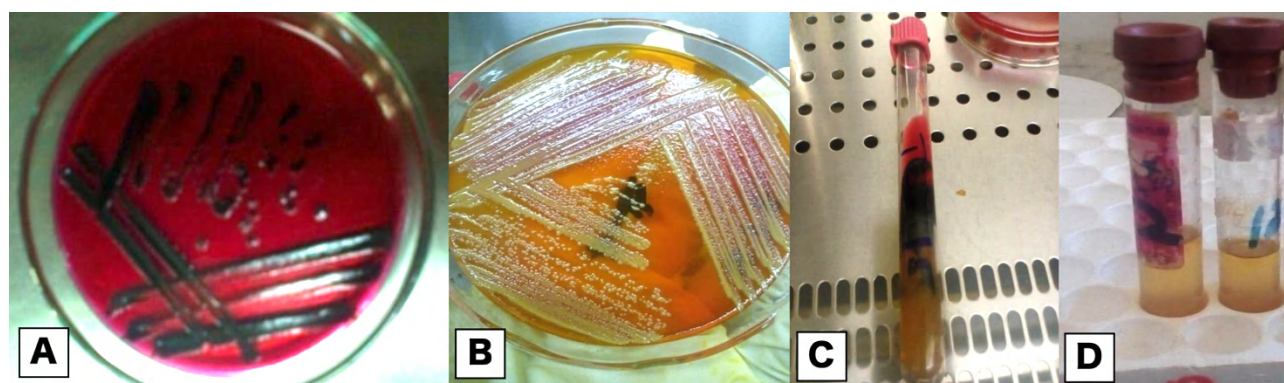


Figure 1. Morphological and biochemical characteristics of Salmonella isolates. (a) Distinctive red colonies with black centers on xylose lysine deoxycholate (XLD) agar, indicative of Salmonella growth. (b) Nonlactose fermenting, pale colonies on MacConkey agar, characteristic of Salmonella spp.; (c) Triple Sugar Iron (TSI) agar slant showing typical Salmonella reactions: alkaline (red) slant, acidic (yellow) but with visible gas production, and hydrogen sulfide (black) formation. (d) A negative indole test result, demonstrated by the absence of red ring formation, is consistent with the biochemical properties of Salmonella. Collectively, these results support the identification of Salmonella isolates at the genus level.

The subsequent culture steps included (a) primary characterization on blood agar; (b) subculturing on nutrient agar; (c) culturing on MacConkey agar (yielding yellowish, nonlactose fermenting colonies); (d) culturing on Brilliant Green agar (producing red to pink colonies); and (e) culturing on xylose lysine deoxycholate (XLD) agar, a moderately selective and differential medium for isolating and differentiating gram-negative enteric pathogens [31]. On xylose lysine deoxycholate agar (XLD agar), *Salmonella* colonies appeared red with black centers, which is a characteristic feature of this genus. Further confirmation involved culturing on triple sugar iron (TSI) agar, where *Salmonella* typically ferments glucose, but not sucrose or lactose, produces gas, and generates H₂S. Additional biochemical tests, including catalase, KOH, indole, and motility tests, along with Gram staining, were performed to identify the isolates comprehensively [22,29,32].

Preparation of inocula and susceptibility tests

Antimicrobial susceptibility testing was performed via the filter paper disc diffusion method, which is a widely accepted technique for evaluating antibacterial activity. The process began with the bacterial inoculum being prepared by transferring a loopful of isolated bacteria into a 10 ml sterile tube of nutrient broth, followed by incubation at 37°C for 24 h. Whatman No. 1 filter papers were utilized to create standardized 6 mm discs, which were subsequently sterilized in a hot air oven to ensure aseptic conditions. Mueller-Hinton agar plates, which are recognized as standard media for antimicrobial susceptibility testing, were prepared and inoculated with the bacterial culture via sterile cotton swabs.

The prepared discs were then immersed in 1 ml of honey, garlic, and combination solutions at concentrations of 100, 50, and 25 grams/100 ml for one minute. Using sterile long forceps, the discs were strategically placed on inoculated Mueller-Hinton agar plates, ensuring appropriate spacing from the edge of the Petri dish and other discs to prevent overlapping of inhibition zones. The experimental setup involved placing five discs on each of the four Mueller-Hinton agar plates, and the remaining seven discs were distributed across two additional plates. Following a 15-minute drying period, the plates were inverted and incubated at 37°C for 24 h to allow optimal bacterial growth and zone formation inhibition. After incubation, the diameters of the inhibition zones were meticulously measured via a ruler and recorded in millimeters, providing quantitative data for subsequent analysis.

Statistical analysis

This study employed a comprehensive statistical approach to analyze the antibacterial effects of honey, garlic extracts, and their combinations. The results are expressed as the means $\pm$ standard deviations, providing a measure of central tendency and variability. Descriptive statistics were used to compare the inhibition zones produced by different treatments. Statistical Package for Social Sciences (SPSS) Inc. Chicago, IL version 21.0 software was used for all the statistical analyses, ensuring accuracy and reliability in data processing. One-way analysis of variance (ANOVA) was used to compare the overall mean inhibition across the different treatment groups, with a probability of $P < 0.05$ and a 95% significance level [33].

RESULTS

Antibacterial activity

Aqueous extracts of honey (*Apis mellifera* bee honey), garlic (*Allium sativum*), and their combination inhibited the growth of *Salmonella* bacteria (Table 1). At the highest concentration tested (100 g/100 mL), the aqueous extracts demonstrated varying degrees of inhibition of *Salmonella* growth. Honey exhibited the most potent antibacterial activity, producing the largest zone of inhibition (26.33 ± 0.577 mm). This effect was significantly greater than that observed with garlic (*Allium sativum*) extracts or the combination of honey and garlic ($P < 0.001$). Interestingly, garlic extract alone had the least inhibitory effect (13.67 ± 2.309 mm) at this concentration, suggesting that honey may possess superior antimicrobial properties against *Salmonella* at relatively high concentrations.

At an intermediate concentration (50 g/100 mL), the inhibitory patterns showed some variation. Honey continued to demonstrate the strongest antibacterial activity (19.67 ± 5.132 mm), although the difference was not statistically significant compared with the other treatments ($p = 0.55$). Notably, the combination of garlic and honey resulted in the smallest inhibition zone (11.33 ± 1.155 mm), which was unexpected given the individual performance of honey. This suggests a potential antagonistic effect when garlic and honey are combined at this concentration. At the lowest concentration tested (25 g/100 mL), honey maintained its superior inhibitory effect against *Salmonella* (13.67 ± 3.215 mm), although the differences between treatments were not statistically significant ($p = 0.149$). The combination of honey and garlic again resulted in the least inhibition (9.67 ± 1.528 mm), which was consistent with the trend observed at higher concentrations. This finding reinforces the hypothesis that combining these two natural products may not enhance their antibacterial properties against *Salmonella*.

Table 1. Inhibitory effects of honey, garlic, and their combination on *Salmonella* growth

Extracts and concentration	Mean $\pm$ SD	F	p value
Extracts (100 g/100 ml)			
Garlic	13.67 $\pm$ 2.309	42.310	<0.001*
Honey	26.33 $\pm$ 0.577		
Garlic and Honey (Combination)	18.67 $\pm$ 2.00		
Extracts (50 g/100 ml)			
Garlic	15.67 $\pm$ 2.082	4.885	0.55
Honey	19.67 $\pm$ 5.132		
Garlic and Honey (Combination)	11.33 $\pm$ 1.155		
Extracts (25 g/100 ml)			
Garlic	12.00 $\pm$ 1.000	2.659	0.149
Honey	13.67 $\pm$ 3.215		
Garlic and Honey (Combination)	9.67 $\pm$ 1.528		

Remarks: * indicates a significant difference ($p < 0.05$) with a level of evidence of 95%.

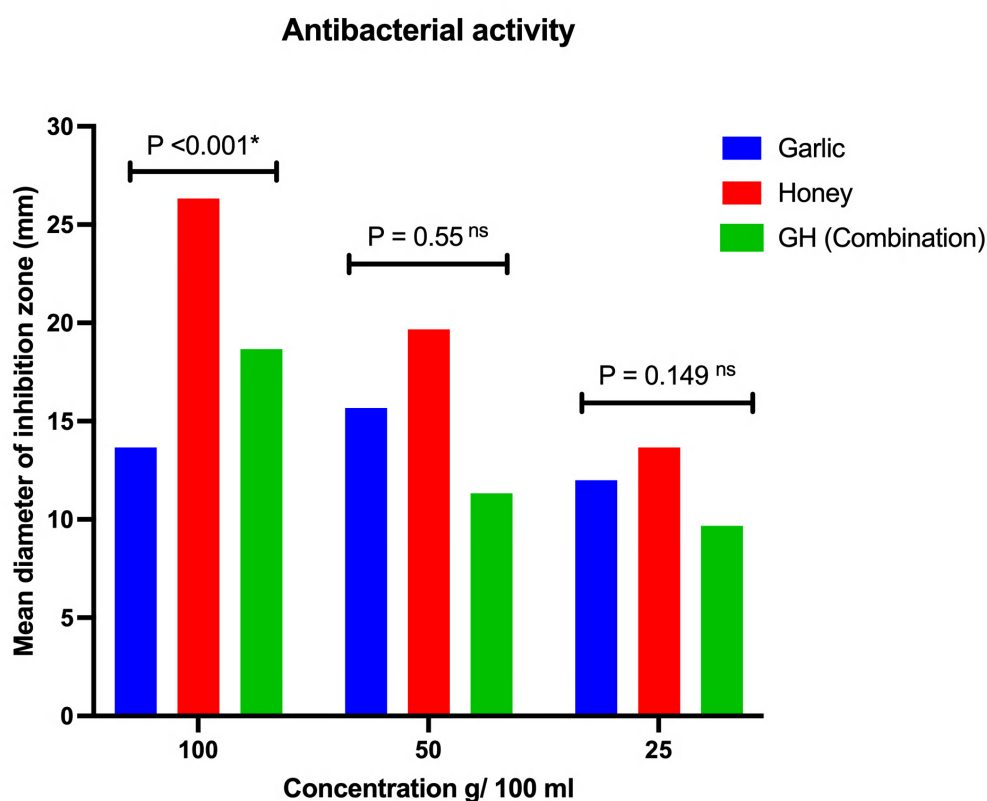


Figure 2. Antibacterial activity of garlic, honey, and their combination at 100, 50, and 25 g/100 ml in distilled water. Remarks: The p value shown is the result of the one-way ANOVA test, with a probability of 0.05 and a significance level of 95%; and ns indicates not significant ($p > 0.05$).

Concentration-dependent activity

Analysis of the concentration-dependent effects revealed that honey consistently outperformed both garlic and the honey-garlic combination across all the tested concentrations. The antibacterial activity of honey was particularly pronounced at the highest concentration (100 g/100 mL), where it demonstrated significantly greater inhibition than the other treatments did. These findings suggest that antimicrobial compounds in honey may have concentration-dependent effects on *Salmonella* growth inhibition.

Comparative analysis of treatments

At 100 g/100 mL, the superior antibacterial activity of honey was clearly evident, with nonoverlapping error bars indicating significant differences from those of the other treatments (see Figure 2). While this clear distinction was less pronounced at lower concentrations (50 g/100 mL and 25 g/100 mL), honey consistently tended toward greater inhibition. The combination of honey and garlic consistently underperformed compared with honey alone, suggesting that the anticipated synergistic effect was not realized under the experimental conditions employed. Collectively, these findings indicate that honey possesses potent antibacterial properties against *Salmonella*, particularly at relatively high concentrations. The unexpected performance of the honey-garlic combination warrants further investigation into the potential interactions between these natural products when they are used in combination for antimicrobial purposes.

DISCUSSIONS

The antimicrobial properties of garlic and honey have been extensively studied, and recent research has elucidated their mechanisms of action against various pathogens, including *Salmonella* spp [1,2,15]. Recent studies have expanded our understanding of the antimicrobial efficacy of garlic, attributing it to a complex composition that includes not only phenolic compounds, organic sulfur compounds, and allicin but also newly identified bioactive compounds, such as ajoene and diallyl trisulfide [26,34–36]. These compounds contribute to the broad-spectrum antimicrobial activities of garlic [37–40]. Similarly, while the antimicrobial properties of honey have long been attributed to its unique physicochemical characteristics, such as high sugar content, low pH, and the presence of hydrogen peroxide [41], recent research has highlighted the significant role of bee-derived defensin-1 and other antimicrobial peptides in the antibacterial activity of honey [22,28,32,42].

The results of the present study demonstrated the promising antibacterial effect of garlic against *Salmonella*, with inhibition zones ranging from 12.00 mm to 15.67 mm across different concentrations. These findings align with those of previous studies, such as Andualem [28], who reported inhibition zones of 14–22 mm against *Salmonella* NCTC 8385. However, our results revealed slightly lower inhibition zones, which may be attributed to differences in the garlic preparation methods or *Salmonella* strains used [7]. Guedes [5] research also corroborated the efficacy of garlic against various *Salmonella* species, with inhibition zones of up to 38 and 15 mm, respectively.

Our investigation of the antibacterial activity of *Apis mellifera* honey revealed substantial inhibition of *Salmonella* growth, with zones ranging from 13.67 mm to 26.33 mm. These results are consistent with the findings of Mohapatra et al. [29], who reported a 37.94 mm inhibition zone against *S. typhi*. The observed variations in inhibition zones across studies can be attributed to differences in honey composition, which has been shown to be significantly influenced by geographical and botanical factors as well as environmental conditions during nectar collection and honey production [6]. A novel aspect of our study was the evaluation of honey and garlic combinations, which demonstrated inhibition zones between 9.67 mm and 16.00 mm. This synergistic approach represents an emerging area of research on natural antimicrobial agents [43,44]. Although direct comparisons with previous studies are limited due to the scarcity of research on this specific combination against *Salmonella*, our findings suggest the potential for further exploration of synergistic natural antimicrobial treatments [17,23,25].

Recent metabolomic analyses have revealed significant variations in the bioactive compound profiles of honey and garlic from different geographical regions, providing a more nuanced understanding of the factors that influence their antimicrobial efficacy [4]. Additionally, methodological differences in extract preparation, concentration, and bacterial strain selection contributed to the variability in the results. Importantly, our study focused on *Salmonella*, which may have limited direct comparisons with species-specific investigations. However, this broader approach provides valuable insights into the general efficacy of natural antimicrobials against *Salmonella*.

This study confirmed the antibacterial effects of honey, garlic, and their combination against *Salmonella*, with honey demonstrating superior inhibitory activity across all concentrations tested [12,45,46]. These findings not only corroborate the literature but also extend our understanding of natural antimicrobial agents. Future research should include in-depth investigations into the molecular mechanisms underlying the observed antibacterial effects, with a particular focus on the potential synergistic or antagonistic interactions between honey and garlic compounds [47–50]. Additionally, exploring potential clinical applications for addressing antibiotic-resistant *Salmonella* strains remains a critical area for further study, especially given the growing global concern regarding antimicrobial resistance [1,6,51].

Clinical implications

From a clinical perspective, the potent antibacterial activity of honey against *Salmonella* offers a promising avenue for therapeutic application. Considering the increasing global concern over antibiotic resistance, honey presents a potentially valuable alternative or adjunct treatment for *Salmonella* infections, especially in cases where conventional antibiotics are ineffective [1,15]. The findings of this study may guide the development of topical treatments for wound infections caused by *Salmonella*, leveraging the antimicrobial properties of honey. Moreover, recent clinical trials have demonstrated the efficacy of medical-grade honey in treating antibiotic-resistant wound infections, suggesting broader application potential for honey-based treatments [52–57]. Additionally, the varying efficacies of different concentrations emphasize the importance of standardization in clinical applications. Although garlic has moderate antibacterial activity, its potential role in combination therapies warrants further investigation, particularly given the unexpected lack of synergy with honey observed in this study. This finding underscores the complexity of interactions between natural products and the need for careful evaluation of combination therapies [58,59].

Practical implications

The findings of this study have several practical implications for the use of natural antimicrobials against *Salmonella*. The superior antibacterial activity of honey, particularly at relatively high concentrations, suggests its potential as a natural preservative in food products susceptible to *Salmonella* contamination. This could be especially valuable in regions with limited access to modern food preservation techniques. Furthermore, the concentration-dependent efficacy of honey highlights the importance of proper dosing. The unexpected performance of the honey-garlic combination underscores the need for careful consideration when combining natural antimicrobials because synergistic effects cannot be assumed. These results may inform the development of novel food preservation strategies and natural medicinal products that target *Salmonella* infections.

Limitations

This study has several limitations. First, the broad focus on the *Salmonella* genus, rather than on specific species or strains, may limit the direct applicability of these findings to clinically relevant pathogenic strains. Future studies should consider testing against specific *Salmonella* serovars known to cause human infections, such as *S. Typhimurium* or *S. Enteritidis*. The in vitro nature of the study, while providing valuable insights, does not account for the complex interactions that may occur in vivo, such as the influence of the host immune system or gastrointestinal environment on antimicrobial efficacy. Although the use of zones of inhibition

measurements is a standard approach, it may not fully capture the dynamics of bacterial growth inhibition over time. Future studies could benefit from incorporating time-kill assays or minimum inhibitory concentration (MIC) determinations to provide a more comprehensive understanding of antibacterial effects.

Additionally, the limited range of tested concentrations may not reveal the full spectrum of concentration-dependent effects. The absence of investigations into the specific bioactive compounds responsible for the observed antibacterial activity limits our understanding of their underlying mechanisms. Employing advanced analytical techniques such as high-performance liquid chromatography (HPLC) or mass spectrometry could help identify and quantify key active compounds in future studies [53]. Finally, the potential variability in honey and garlic composition based on geographical and botanical origin may affect the generalizability of these results to samples from different sources. To address this limitation, future research could involve a comparative analysis of honey and garlic samples from diverse geographical locations to provide a more comprehensive understanding of the variability in antimicrobial efficacy [10,60].

CONCLUSIONS

This study provides compelling evidence for the antibacterial efficacy of *Apis mellifera* honey, garlic extracts, and their combination against *Salmonella* in Wolayta Sodo, southern Ethiopia. The results demonstrated that honey consistently exhibited superior inhibitory activity against *Salmonella* across all the tested concentrations, with inhibition zones ranging from 13.67 mm to 26.33 mm. Garlic extracts also showed promising antibacterial effects, albeit to a lesser extent than honey, with inhibition zones between 12.00 mm and 15.67 mm. Interestingly, the combination of honey and garlic did not produce the anticipated synergistic effect and consistently underperformed compared with honey alone. These findings highlight the potential of honey as a natural antimicrobial agent against *Salmonella*, particularly at high concentrations. These results underscore the need for further investigation into the molecular mechanisms underlying the antibacterial activity of honey and its potential applications in clinical settings, especially in the context of combating antibiotic-resistant *Salmonella* strains. Future research should address the limitations of this study and explore the practical implications of these findings for food preservation and therapeutic applications.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: RW, SAM; Methodology: SAM; Software: RW, SAM; Validation: RW, SAM; Formal analysis: RW, SAM; Investigation: RW, SAM; Resources: RW, SAM; Data Curation: RW; Writing - Original Draft: RW, SAM; Writing - Review & Editing: RW, SAM; Visualization: RW, SAM; Supervision: SAM.

FUNDING

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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