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Antimicrobial activity assessment of an innovative powdered honey production approach: a comparative study

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Abstract

Working with honey in laboratory or applied settings presents practical challenges that extend beyond its well-known antimicrobial properties. Honey is viscous and sticky, tends to harden into crystals over time, and can make it tedious to apply the same amount repeatedly. These everyday issues have led researchers to seek powdered alternatives, though many published approaches involve heating steps or require so much carrier material that parts of honey's natural properties may be altered unintentionally. In this study, a powdered honey (P.H.) mixture supported with water-insoluble microcrystalline cellulose (MCC) was evaluated alongside liquid honey, ensuring that the actual amount of honey applied in each experiment was equivalent. Two commercially available honeys with different declared botanical origins (chestnut and polyfloral) were included to assess the applicability of the approach across market-available samples. Antimicrobial activity was assessed against Gram-positive *Staphylococcus aureus* and Gram-negative *Salmonella typhimurium* using both disc and well diffusion assays. Before testing, powdered samples were rehydrated so that each application delivered a quantity of honey equivalent to that present in 10 µL of the liquid form. The resulting inhibition zones were measured using calibrated digital image analysis. Under matched-dosing conditions, antimicrobial activity was preserved in both liquid and powdered honey, with inhibition patterns varying depending on the microorganism and experimental conditions. Overall, the findings suggest that this non-thermal, MCC-assisted powdering strategy can preserve antimicrobial performance while improving handling, storage, and dose reproducibility, and that inhibition outcomes may be shaped by physical form–dependent diffusion behavior under equivalent honey content.

Keywords: Powdered Honey, Polyfloral Honey, Monofloral Honey, Antimicrobial Properties, Physical Form

Yenilikçi toz bal üretim yaklaşımının antimikrobiyal etkinliğinin değerlendirilmesi: karşılaştırmalı bir çalışma

Öz

Bal ile laboratuvar veya uygulamaya yönelik çalışmalarda, iyi bilinen antimikrobiyal özelliklerinin ötesinde bazı pratik zorluklarla karşılaşılmaktadır. Balın yüksek viskoziteye ve yapışkan bir yapıya sahip olması, zamanla kristalleşerek sertleşmesi ve her seferinde aynı miktarın tekrarlanabilir şekilde uygulanmasının güç olması, deneysel süreçleri zahmetli hâle getirebilmektedir. Bu günlük pratik sorunlar, araştırmacıları toz formdaki alternatiflere yöneltmiş; ancak literatürde bildirilen birçok yaklaşımın ısıl işlem içermesi ya da yüksek miktarda taşıyıcı madde gerektirmesi nedeniyle, balın doğal özelliklerinin istemeden değişime uğrayabildiği görülmektedir. Bu çalışmada, suda çözünmeyen mikrokristalin selüloz (MCC) ile desteklenmiş bir toz bal (P.H.) karışımı, sıvı bal ile birlikte değerlendirilmiş ve her bir deneyde uygulanan gerçek bal miktarının eşdeğer olması sağlanmıştır. Yöntemin piyasada bulunan farklı örnekler için uygulanabilirliğini değerlendirmek amacıyla, beyan edilen botanik kökenleri farklı olan iki ticari bal (kestane ve polifloral) kullanılmıştır. Antimikrobiyal aktivite, Gram-pozitif Staphylococcus aureus ve Gram-negatif Salmonella typhimurium suşlarına karşı disk ve kuyu difüzyon yöntemleri kullanılarak test edilmiştir. Deneyler öncesinde, toz örnekler yeniden hidratlanmış ve her bir uygulamanın, sıvı formdaki 10 µL bala eşdeğer miktarda bal içermesi sağlanmıştır. Oluşan inhibisyon zonları, kalibre edilmiş dijital görüntü analiz yöntemi ile ölçülmüştür. Eşleştirilmiş dozlama koşulları altında, antimikrobiyal aktivitenin hem sıvı hem de toz bal formlarında korunduğu; inhibisyon paternlerinin ise mikroorganizma türüne ve deneysel koşullara bağlı olarak farklılık gösterdiği belirlenmiştir.

Genel olarak elde edilen bulgular, bu ısıl işlem içermeyen ve MCC destekli tozlaştırma stratejisinin, antimikrobiyal performansı korurken kullanım kolaylığı, depolama ve doz tekrarlanabilirliğini artırabildiğini; ayrıca eşdeğer bal içeriği sağlandığında, inhibisyon sonuçlarının fiziksel forma bağlı difüzyon davranışlarından etkilenebileceğini göstermektedir.

Anahtar Kelimeler: Toz bal, Polifloral Bal, Monofloral Bal, Antimikrobiyal Özellikler, Fiziksel Form

INTRODUCTION

Honey is a natural product with a long history of application in nutrition and traditional medicine, and its antimicrobial properties have been consistently demonstrated in both experimental and clinical studies. Its antibacterial activity does not rely on a single mechanism; rather, it results from the combined effects of multiple physicochemical and biochemical factors. High osmotic pressure and reduced water activity create an unfavorable environment for microorganisms, while the inherently acidic pH further limits bacterial survival. Additionally, hydrogen peroxide generated upon dilution and non-peroxide bioactive constituents, including phenolic compounds and flavonoids, contribute to the overall antimicrobial profile of honey (Almasaudi 2020; Al-Kafaween et al. 2023). Unlike conventional antibiotics, which act on specific cellular targets, honey's multi-target mode of action may reduce the risk of microbial resistance development (Combarros-Fuertes et al. 2020).

The inhibitory effect of honey varies among microbial species. Gram-positive bacteria are generally more susceptible than Gram-negative ones, largely due to differences in cell envelope structure and wall complexity (Anand et al. 2019; Almasaudi 2020). Consequently, antimicrobial studies frequently include representatives of both groups, with *Staphylococcus aureus* (Gram-positive) and *Salmonella typhimurium* (Gram-negative) commonly used as model organisms. Their distinct cell wall architectures allow controlled comparisons of honey's antibacterial effects.

Most laboratory studies to date have employed liquid honey, which presents practical challenges: it is viscous, sticky, prone to crystallization, and difficult to apply in consistent quantities (Samborska 2019). These factors have sparked interest in honey preparations in forms other than liquid, as alternative physical states may ease laboratory handling and product formulation without compromising honey's biological properties. From a technological and practical perspective, converting honey into a powdered form addresses several well-recognized limitations of liquid honey. Honey is prone to crystallization during storage, which reduces flowability and causes handling difficulties. Crystallization may also result in the separation of a free aqueous phase, increasing the risk of fermentation and compromising product quality. In practice, crystallized honey is often reliquefied by heat treatment; however, thermal decrystallization has been shown to promote the formation of hydroxymethylfurfural (HMF) and to cause quality deterioration. Recent work has clearly demonstrated that temperature and duration of heat exposure during decrystallization are key determinants of HMF accumulation (Samples et al. 2025). The debate surrounding HMF formation is current and relevant; however, the importance of the issue can be more clearly demonstrated by stating that HMF is not only an indicator of quality but also a compound with potential toxicological significance. In this context, powdered honey offers advantages in terms of storage stability, dose reproducibility, and industrial applicability, while avoiding the need for repeated thermal processing.

Powdered honey has received increasing attention as a functional alternative. It offers advantages in dosing control, stability, and ease of application (Osés et al. 2022; Cantero et al. 2023). However, several studies have shown that commonly used drying techniques, such as spray drying and freeze drying, may adversely affect heat- and process-sensitive honey constituents, including phenolic compounds, enzymatic activity, and hydrogen peroxide–mediated antimicrobial mechanisms, depending on processing conditions (Samborska 2019; Rivero et al. 2021). At the same time, available evidence indicates that powdering procedures and the carrier systems employed can influence the structural integrity and biological performance of honey. In particular, drying processes that involve thermal exposure have been associated with losses of heat-sensitive bioactive constituents (Samborska 2019; Jiamjariyatam et al. 2024). For this reason, the development of alternative powdering strategies that enable the production of stable honey powders while maintaining inherent biological properties remains an important area of investigation.

Recent studies have explored a variety of carrier materials for honey powder production, such as maltodextrin, gum arabic, protein-based carriers, and cellulose derivatives (Mutlu et al. 2020; Toniazzo et al. 2023). However, honey powder production also poses practical challenges, including hygroscopicity, stickiness under humid conditions, and processing sensitivity, which can negatively affect powder quality and stability (Cantero et al. 2023; Saraugi et al. 2026). Microcrystalline cellulose (MCC) has attracted interest as a carrier material due to its natural origin, chemical inertness, and suitable physical properties for powder formation, enabling the physical conversion of honey with minimal expected chemical interference. In the present study, preservation of honey composition is assumed based on the inert, non-thermal nature of the formulation strategy rather than directly verified through post-processing measurements of phenolics, enzymatic activity, or H_2O_2-

related antimicrobial mechanisms. MCC is not introduced here as a novel carrier, as its use in honey powder formulations has been previously reported. The present study differs from earlier MCC-based approaches by avoiding thermal processing and by evaluating antimicrobial performance under strictly equivalent honey dosing, allowing inhibition outcomes to be interpreted in relation to physical form–dependent diffusion behavior.

Cellulose-based approaches have been suggested to facilitate the conversion of honey into a powdered form while largely preserving its original chemical composition (Samborska et al. 2013; Mutlu et al. 2020). Despite these reported advantages, studies specifically addressing the antimicrobial activity of honey powders produced with cellulose-based carriers remain scarce.

Accurate comparisons between liquid and powdered honey require consistent honey concentrations, as differences in applied amounts directly affect inhibition zone measurements in diffusion assays (Hossain et al. 2022). Accordingly, the use of comparable honey amounts in evaluations of liquid and powdered honey is a fundamental requirement for methodological reliability.

Additionally, the botanical origin of honey substantially influences antimicrobial efficacy, alongside concentration-related factors. Chestnut honey has been frequently described as exhibiting strong antibacterial activity, a characteristic commonly associated with its high phenolic content and distinctive chemical composition. By comparison, polyfloral honeys tend to show a wider but less uniform biological activity profile due to contributions from multiple plant sources (Al-Kafaween et al., 2023; El-Meihy et al., 2025). For this reason, including honeys of different botanical origins within the same experimental design enhances the robustness and broader relevance of antimicrobial assessments.

In this study, MCC-supported powdered honey was evaluated alongside liquid honey, ensuring equivalent honey concentrations. Using chestnut and polyfloral honey, the antimicrobial effects of liquid and powdered honey forms against S. aureus and S. typhimurium were compared using disc diffusion and agar well diffusion methods.

The primary rationale for selecting *Staphylococcus aureus* and *Salmonella typhimurium* was that these bacteria represent Gram-positive and Gram-negative microorganisms with distinct cell wall structures. This selection enabled a comparative assessment of how the antimicrobial efficacy of honey and its various physical forms varies across bacterial cell structures. In addition, both bacterial species are important food-safety and public-health pathogens, enabling the investigation of honey's natural antimicrobial potential in a practically relevant context.

An additional objective of this study is to determine whether antimicrobial activity is driven solely by the chemical composition of honey, or whether the physical form and mode of application also contribute to the observed effect. For this purpose, the same honey dose was applied to both liquid and powdered preparations, allowing direct comparison of the findings based on equivalent content. Subsequently, two diffusion approaches—disc diffusion and agar well diffusion assays—were used in parallel within the same experimental framework to evaluate how the mode of application shapes inhibition zone formation; however, these assays primarily measure the diffusible fraction of antimicrobial agents and may not fully represent intrinsic antimicrobial potency, as differences in inhibition zones can also arise from diffusion kinetics rather than true efficacy. Since the chemical profile was kept constant, the aim of this design is to disentangle and reveal the contribution of physical form (and application approach) to antimicrobial performance. Accordingly, this study differs from previous MCC-based honey powder research by combining a non-thermal production approach with an mass-based equivalent honey-dose experimental design, in which the applied amount was standardized according to the weight fraction of honey in the powdered formulation, to specifically examine the role of physical form and diffusion behavior in antimicrobial inhibition.

MATERIALS AND METHODS

Honey Samples

Two commercially available honey types were examined in this study: chestnut honey and polyfloral Bingöl honey. The declared geographical origin of the samples was based solely on manufacturer information, and no independent analysis or certification was performed to verify their geographical authenticity. The chestnut honey was obtained via an online retail platform and declared to originate from the Hopa district of Artvin province (Türkiye), while the polyfloral honey was supplied by a commercial producer operating in the Bingöl region. Both samples were obtained from single commercial batches; therefore, the results may not fully represent regional variability, and their generalizability should be interpreted with caution. Accordingly, the present findings should be interpreted as case-specific observations derived from single-batch samples rather than as representative of seasonal or regional variability. Sample selection was based on commercial availability and relevance rather than a systematic regional sampling strategy. Previous studies on honeys from the Bingöl region have reported, based on ICP-MS analyses, that toxic metal concentrations generally remain below regulatory threshold values (İzol et al., 2021), while investigations on chestnut honey have shown that elements such as Al, As, Cd, and Pb do not exceed the maximum limits established by the World Health Organization and the Turkish Food Codex (Guldas, 2023); these findings are provided for contextual background only, as no elemental analy-

sis was conducted in the present study. These literature data are provided solely for context and should not be interpreted as an analytical confirmation of the specific samples used in this study. Prior to analysis, all honey samples were stored at room temperature in containers protected from light and moisture to minimize potential physicochemical or biological alterations and to maintain conditions comparable to typical commercial storage. However, storage-related quality parameters such as HMF, pH, and moisture content were not monitored during this period; therefore, storage stability was assumed rather than analytically verified, which represents an additional limitation of the study. Because only a single commercial batch was analyzed for each honey type, potential batch-to-batch and seasonal variability could not be assessed; therefore, the findings should be interpreted as indicative rather than fully representative of honeys from the respective regions.

Table 1. Key physicochemical properties of chestnut honey used in the antimicrobial study

Parameter	Result	Unit	Analytical Method	Relevance to the Study
Proline	708.69	mg/kg	Spectrophotometric (DIN 10754)	Indicator of honey maturity and bioactive potential
Diastase activity	26.98	DN	Spectrophotometric (IHC)	Reflects enzymatic activity and absence of heat damage
Electrical conductivity	1.49	mS/cm	IHC (2009)	Characteristic of chestnut honey; associated with mineral and phenolic content
Fructose/Glucose ratio	1.10	–	HPLC	Related to crystallization tendency and diffusion properties
Total sugars	66.73	%	TS 13357	High osmotic pressure contributes to bacterial inhibition
Moisture content	17.0	%	AOAC 969.38	Indicates good microbiological stability
Protein	Not detected	–	AOAC 992.15	Suggests antimicrobial activity is mainly non-protein-based
HMF	9.83	mg/kg	HPLC	Low HMF confirms minimal thermal degradation and preserved bioactivity

The physicochemical analysis results presented in the table were obtained for the purchased chestnut honey used in the study and were conducted in an authorized laboratory on behalf of the producer company. These analyses demonstrate the honey's quality criteria, maturity level, and unprocessed (non-heat-treated) nature. In particular, the low HMF value, high diastase activity, and the high electrical conductivity characteristic of chestnut honey indicate that the sample is suitable for use in antimicrobial activity studies. Relevance to the study was interpreted based on internationally accepted honey quality and bioactivity indicators (Molan, 1992; Snowdon et al., 1996; Codex Alimentarius Commission, 2001; Bogdanov et al., 2004; Oddo et al., 2004).

Preparation of Microcrystalline Cellulose–Based Powdered Honey

To obtain honey in a powdered form without introducing components or processing steps that could chemically or enzymatically alter its native structure, a microcrystalline cellulose–based, non-thermal approach was employed. In this system, MCC functioned as an inert physical carrier and matrix, providing surface area for adsorption and physical entrapment of honey components rather than forming chemical interactions or modifying bioactive constituents; therefore, the formulation was intended to influence only the physical state and diffusion behavior of honey rather than its intrinsic antimicrobial chemistry. Liquid honey (10 g) was combined with water-insoluble microcrystalline cellulose (4 g), with this ratio selected based on preliminary trials to achieve adequate powder formation, flowability, and handling stability while maintaining the highest feasible honey loading; no optimization based on antimicrobial performance was performed. The components were mechanically mixed until homogeneous. The resulting material was maintained under ambient laboratory conditions (22–25 °C) in open glass containers, in a dry environment protected from direct light. Drying was conducted in a clean, low-traffic laboratory environment using covered but aerated glass containers to minimize airborne contamination and particle deposition. During the drying period, the mixture was mechanically stirred 3–4 times per day to facilitate the gradual uptake of honey by the cellulose matrix. Drying was carried out under ambient laboratory conditions without active control of relative humidity; therefore, the process duration (approximately two weeks) reflected natural variations in ambient humidity rather than a fixed drying parameter. The procedure resulted in a free-flowing powdered honey material without visible agglomeration. At no stage were thermal treatment, forced drying, or auxiliary equipment applied, and all procedures were conducted at room temperature, consistent with the previously described non-thermal approach (Kaya, 2023).This distinguishes the present method from commonly reported spray drying or freeze-drying techniques by enabling honey powder formation without heat exposure. The resulting powdered honey samples were stored at room temperature until subsequent analyses. Physicochemical parameters such as pH, moisture content, and water activity were not measured after powdering; therefore, the

relative contributions of intrinsic compositional factors versus physical form–dependent diffusion effects to the observed antimicrobial activity cannot be fully distinguished, which represents an important limitation when interpreting differences between liquid and powdered honey. Therefore, claims regarding the preservation of natural properties are limited to the formulation strategy's non-thermal, inert nature rather than to direct post-processing physicochemical characterization. This represents a limitation of the present study and should be addressed in future work.

Table 2. Physicochemical characteristics of the purchased polyfloral honey used in this study

Parameter	Result	Unit	Analytical Method	Relevance to the Study
Proline	1034	mg/kg	Spectrophotometric (DIN 10754)	Indicator of honey maturity and bioactive potential
Diastase activity	38	DN	Spectrophotometric (IHC)	Reflects enzymatic activity and absence of heat damage
Electrical conductivity	0.3	mS/cm	IHC (2009)	Indicative of mineral content and floral diversity
Fructose/Glucose ratio	2	–	HPLC	Related to crystallization tendency and diffusion properties
Total sugars	71	%	TS 13357	High osmotic pressure contributes to bacterial inhibition
Moisture content	16.0	%	AOAC 969.38	Indicates good microbiological stability
Protein	Not detected	–	AOAC 992.15	Suggests antimicrobial activity is mainly non-protein-based
HMF	<loq	mg/kg	HPLC	Low HMF confirms minimal thermal degradation and preserved bioactivity

The information provided in this table refers to the polyflo. The Bingöl honey examined in this study is derived from standard analytical evaluations routinely performed by an accredited laboratory on behalf of the producer for product characterization. The HMF and diastase values reported are consistent with those expected for non-heated honey and are commonly used indicators of freshness, while the measured proline level reflects a degree of maturity considered suitable for investigations of biological activity. Data on the sugar profile were obtained from the producer's analytical records and presented to support the sample description, without further experimental assessment within the framework of the present study. Relevance to the study was interpreted based on internationally accepted honey quality and bioactivity indicators (Molan 1992; Snowdon et al. 1996; Codex Alimentarius Commission 2001; Bogdanov et al. 2004; Oddo et al. 2004).

Preparation of Test Solutions and Concentration Equivalence

In antimicrobial experiments, comparable amounts of honey were applied in liquid and powdered forms based on mass-based calculations. In the powdered honey formulation, microcrystalline cellulose was treated as an inert carrier and, in accordance with previous reports, was assumed to have no intrinsic antimicrobial activity (Ye et al. 2018; Dong et al. 2021); however, no separate carrier-only control was included, which should be considered a limitation of the study. Accordingly, the inertness of MCC and the absence of chemical or enzymatic interactions were inferred from the literature rather than directly verified experimentally.

Fig 1. General appearance of the powdered honey sample obtained using a microcrystalline cellulose–based alternative production approach. The powdered honey was produced without thermal treatment, preserving its natural structure.

Based on the formulation ratio, honey constituted 71.4% (w/w) of the powdered product (10 g honey in 14 g total formulation). For agar well diffusion assays, 2.0 g of powdered honey was dispersed in 10 mL of sterile distilled water, yielding a suspension containing approximately 142 mg of honey per mL. Accordingly, application of 100 µL of this suspension corresponded to approximately 14 mg of honey, which is mass-equivalent to the 10 µL of liquid honey used in disc diffusion assays. It should be noted, however, that this equivalence was established solely on a mass basis; differences in application geometry, surface contact area, and initial diffusion kinetics between disc-loaded liquid honey and well-loaded suspension may independently influence inhibition zones, independent of dose. The suspension was mixed by vortexing and incubated at 37 °C for

40 minutes to facilitate rehydration and dissolution of honey components prior to application; these conditions were selected to promote solubilization rather than based on a systematic optimization study.

Furthermore, the potential physical effects of MCC—such as altered diffusion behavior within the agar matrix or partial binding of honey constituents—were not specifically controlled, and therefore, the absence of a carrier-only control limits the ability to attribute all observed antimicrobial effects exclusively to honey. The equivalence between liquid and powdered honey applications was therefore derived from theoretical mass calculations and was not independently verified experimentally. This constraint should be regarded as one of the primary methodological limitations when interpreting differences between physical forms.

Microorganisms and Culture Conditions

Staphylococcus aureus (Gram-positive, G+) and *Salmonella typhimurium* (Gram-negative, G–) reference strains obtained from the culture collection of Atatürk University (Erzurum, Türkiye) were used to evaluate antimicrobial activity. These strains were deliberately selected to represent Gram-positive and Gram-negative bacteria rather than to provide broad-spectrum antimicrobial coverage. Accordingly, the antimicrobial assessment was exploratory, and the limited number of bacterial strains limits the generalizability of the findings. After activation in Mueller–Hinton broth and agar media, bacterial suspensions were incubated at 37 °C for 18–24 h under aerobic conditions prior to testing. The bacterial suspensions were adjusted to the 0.5 McFarland standard using a densitometer. All experiments were performed in triplicate using freshly prepared cultures under identical experimental conditions.

Antimicrobial Activity Assay

In this study, agar disc and agar well diffusion methods were combined to enable direct comparison of diffusion behavior and antimicrobial zone formation for honey samples in different physical forms within the same experimental framework. The combined use of these two diffusion techniques was specifically intended to distinguish antimicrobial effects arising from chemical composition from those influenced by physical form and diffusion characteristics. Antimicrobial activity was assessed after surface inoculation of Müller–Hinton agar plates with bacterial suspensions, and all applications were conducted according to the respective methodological requirements of both diffusion techniques. Sterile distilled water and carrier-only (MCC suspension) applications were included as negative controls to exclude solvent- or carrier-derived inhibition effects.

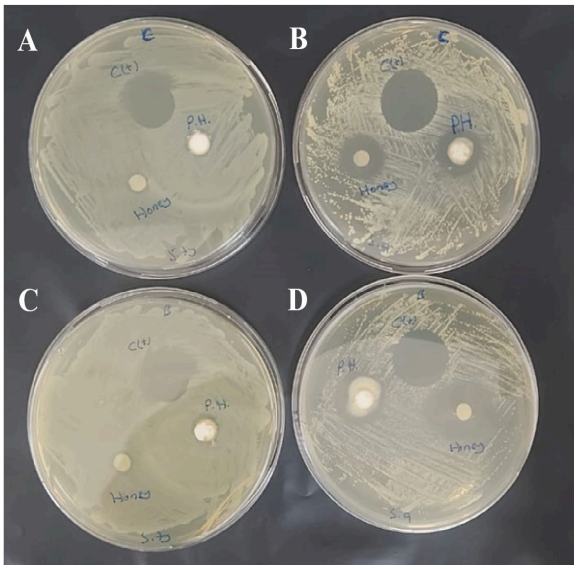

Figure 2. Petri dish images showing the evaluation of the antimicrobial effects of liquid honey and microcrystalline cellulose–based powdered honey against *Staphylococcus aureus* (Gram-positive) and *Salmonella typhimurium* (Gram-negative) using agar well/disc diffusion methods. (A) Antimicrobial effect of chestnut honey against *Staphylococcus aureus*, (B) antimicrobial effect of polyfloral honey against *Staphylococcus aureus*, (C) antimicrobial effect of chestnut honey against *Salmonella typhimurium*, and (D) antimicrobial effect of polyfloral honey against *Salmonella typhimurium*. In all Petri setups, inhibition zones corresponding to liquid honey, powdered honey, and the positive control [C (+), gentamicin] are shown. Equivalent honey concentrations were applied in both liquid and powdered honey samples to enable direct comparison of antimicrobial activity.

The positive control consisted of gentamicin (C+), applied at 5 µL per Petri dish based on commonly used clinical and literature standards and included to provide a consistent reference for comparison rather than to establish susceptibility breakpoints; the antibiotic was applied at a standardized dose of 10 µg per disc to allow direct comparability with conventional disc diffusion assays, and the antibiotic solution was loaded onto sterile paper discs (6 mm diameter) at a constant concentration throughout the experiments. Liquid honey was applied at 10 µL onto sterile discs, while powdered honey was applied by loading 100 µL of the prepared stock solution into agar wells (6 mm diameter, approximately 4 mm depth), a volume selected as the maximum that could be accommodated without overflow or lateral flooding.

It should be noted that disc- and well-based applications differ inherently in contact surface area, agar penetration, and initial diffusion kinetics; therefore, differences in inhibition zone diameters may partly reflect methodological geometry rather than solely antimicrobial efficacy, and this should be considered an important limitation when interpreting comparisons between physical forms. The use of different application formats and volumes reflects methodological constraints imposed by the physical state of the samples rather than an attempt to equalize diffusion geometry; accordingly, the study prioritized mass-

equivalent honey dosing over volumetric equivalence to allow evaluation of physical form–dependent diffusion behavior. Following the application, the plates were incubated at 37 °C for 24 h. All experiments were conducted in three independent replicates to ensure reproducibility (Valgas et al. 2007; Bonev et al. 2008; Balouiri et al. 2016).

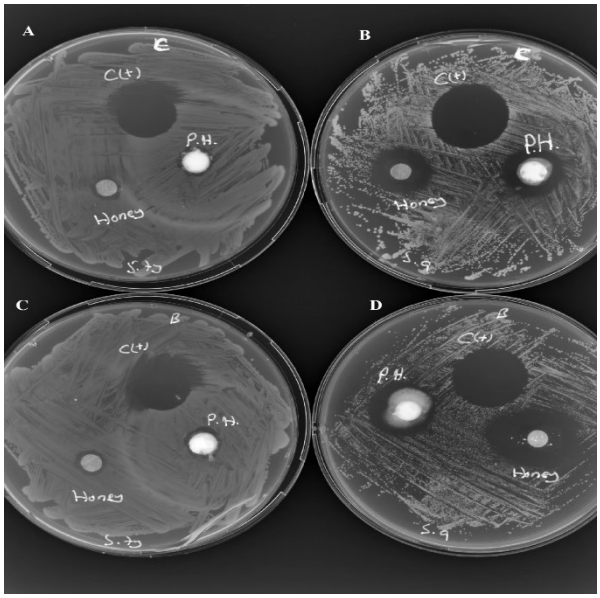

Figure 3. Inhibition zones obtained from disc/well diffusion assays performed on Petri plates were recorded using a Bio-Rad ChemiDoc™ Imaging System in Silver Stain mode with white light (epi-illumination). During image acquisition, automatic exposure (auto optimal exposure) was applied, and imaging was performed directly on a white tray. (A) Petri image showing the antimicrobial effect of chestnut honey against *Staphylococcus aureus* (Gram-positive), (B) Petri image showing the antimicrobial effect of polyfloral honey against *Staphylococcus aureus*, (C) Petri image showing the antimicrobial effect of chestnut honey against *Salmonella typhimurium* (Gram-negative), and (D) Petri image showing the antimicrobial effect of polyfloral honey against *Salmonella typhimurium* are presented. In all Petri setups, inhibition zones corresponding to liquid honey, microcrystalline cellulose–based P.H., and the positive control [C(+), gentamicin] are shown. Powdered and liquid honey samples were prepared to provide equivalent honey concentrations in order to allow a comparable evaluation of antimicrobial activity.

Digital Image Analysis of Inhibition Zones

After incubation, Petri plates were imaged at a fixed distance and under standardized lighting conditions, and inhibition zone measurements were performed using calibrated digital image analysis. Calibration was achieved by referencing the known Petri plate diameters to convert zone diameters to millimeters, and measurements were conducted using the PhotoMeasure online software. To minimize inter-plate variability, the gentamicin inhibition zone on each plate was used as an internal reference (ratio = 1.00). The inhibition zones produced by liquid honey and powdered honey on the same plate were measured proportionally relative to the gentamicin zone, with multiple radial measurements obtained for each inhibition zone and the median value calculated. The resulting proportional values were multiplied by the gentamicin zone diameter (mm) measured on the corresponding plate to obtain inhibition zone diameters (mm). Given the exploratory, method-oriented nature of the study and the limited number of independent replicates (n = 3), formal statistical comparisons and significance testing were not conducted. Accordingly, inhibition zone diameters were reported using descriptive statistics and expressed as median values.

RESULTS

In this study, the antimicrobial activities of liquid honey and microcrystalline cellulose–based powdered honey were evaluated against *Staphylococcus aureus* (Gram-positive) and *Salmonella typhimurium* (Gram-negative) using agar disc and agar well diffusion methods. Inhibition zones were measured by digital image analysis and calculated in millimeters (mm). The reported inhibition zone diameters represent the median values obtained from three independent replicate experiments, rather than measurements from a single Petri dish. Given the exploratory and methodological nature of the study, no formal statistical comparisons between liquid and powdered honey groups were performed, and the results are presented descriptively to illustrate relative trends in antimicrobial performance. Normalization of inhibition zones relative to the gentamicin control was used to reduce interplate variability; however, this approach may limit the direct interpretation of absolute inhibition zone sizes and should be considered when evaluating the results.

Staphylococcus aureus (Gram-positive)

For chestnut honey samples, the inhibition zone measured for the positive control gentamicin was 32.7 mm. In the same Petri dish, the inhibition zone for liquid honey was calculated as 25.8 mm, while that for powdered honey was 25.5 mm. Based on digital image analysis, the calculated ratios were 0.79 for liquid honey and 0.78 for powdered honey. For polyfloral honey samples, the gentamicin inhibition zone was measured as 31.8 mm. The inhibition zone was 30.5 mm for liquid honey and 28.9 mm for powdered honey. The ratios obtained from digital image analysis for this Petri dish were 0.96 for liquid honey and 0.91 for powdered honey.

Salmonella typhimurium (Gram-negative)

For chestnut honey samples, the inhibition zone measured for gentamicin was 33.0 mm. In the same experimental set, the inhibition zone for liquid honey was calculated as 19.5 mm, whereas that for powdered honey was 21.1 mm. The ratios determined by digital image analysis were 0.59 for liquid honey and 0.64 for powdered honey. For polyfloral honey samples, the gentamicin inhibition zone was measured as 32.2 mm. The inhibition zone was calculated

as 20.0 mm for liquid honey and 22.5 mm for powdered honey. The corresponding ratios obtained from digital image analysis were 0.62 for liquid honey and 0.70 for powdered honey. The slightly higher inhibition zones observed for powdered honey compared to liquid honey may be attributed to differences in physical form and diffusion behavior within the agar matrix, rather than differences in honey composition or applied dose. Rehydration of the powdered formulation may facilitate more uniform radial diffusion of antimicrobial components, particularly against Gram-negative bacteria with complex outer membrane structures.

Inhibition zones were measured by digital image analysis using the gentamicin zone in each Petri dish as the reference. Multiple radial measurements were performed for each zone, and median values were used in the calculations. The obtained ratios were multiplied by the gentamicin zone diameters (mm) measured on the corresponding Petri dishes to convert the inhibition zones into millimeters.

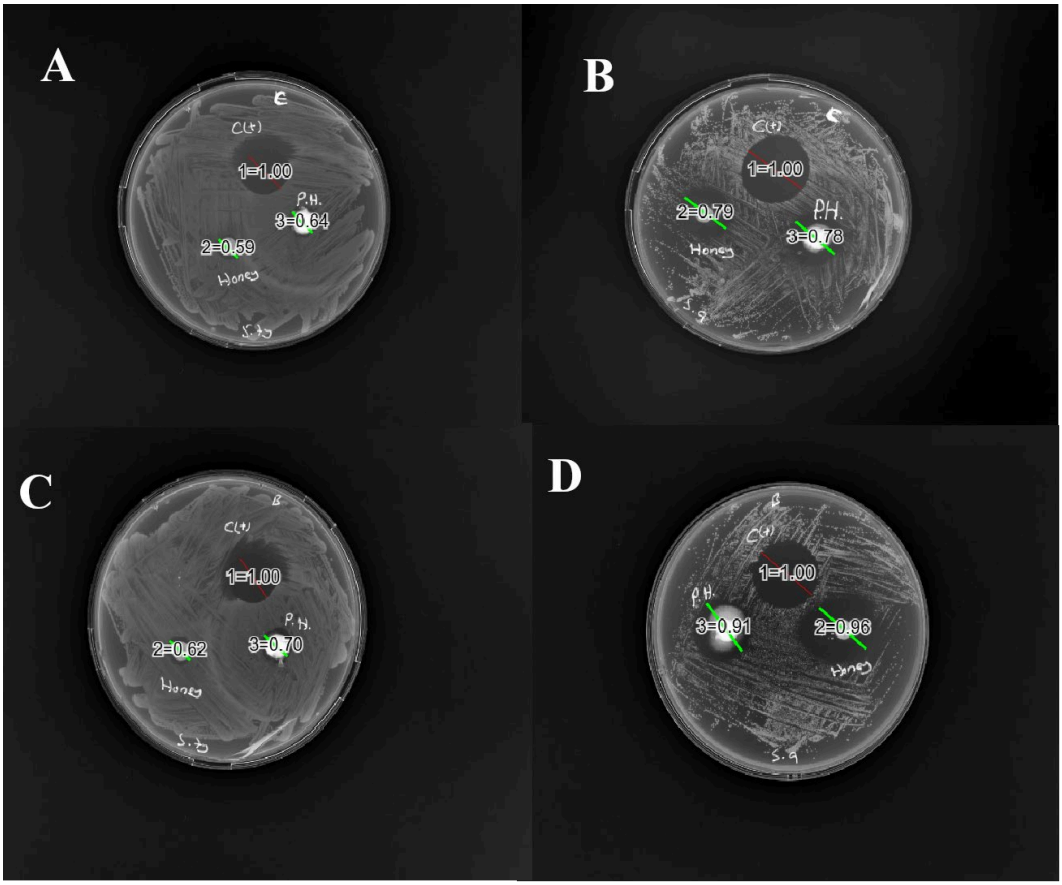

Figure 4. Digital image analysis results of the antimicrobial effects of liquid honey and microcrystalline cellulose–based powdered honey against *Staphylococcus aureus* (Gram-positive) and *Salmonella typhimurium* (Gram-negative) as evaluated by disc/well diffusion methods. (A) The effect of chestnut honey on S. typhimurium, (B) the effect of chestnut honey on S. aureus, (C) the effect of polyfloral honey on S. typhimurium, and (D) the effect of polyfloral honey on *S. aureus* are shown. In the Petri plates, inhibition zones corresponding to the positive control [C (+), gentamicin], liquid honey, and powdered honey are presented in sequence. Inhibition zones were digitally measured using the PhotoMeasure software, and the gentamicin zone diameter in each Petri dish was taken as the reference value (1.00). The green values for liquid and powdered honey represent the ratio of the inhibition zone diameter for each sample to that of gentamicin (relative zone ratio), thereby enabling comparability across different Petri dishes and bacterial strains. In all applications, liquid and powdered honey samples were prepared to provide equivalent honey concentrations in order to assess antimicrobial activity solely as a function of physical form.

Table 3. Antibacterial Activity of Liquid and Powdered Honey (Inhibition Zone Diameters, mm), (Formül: mm = Gentamicin(mm) × ratio)

Bacterium	Gram	Honey type	Sample	Ratio	Inhibition zone (mm)
S. a.	G (+)	C	C (+)	1.00	**32.7**
S. a.	G (+)	C	Liquid honey	0.79	**25.8**
S. a.	G (+)	C	P.H.	0.78	**25.5**
S. a.	G (+)	B	C (+)	1.00	**31.8**
S. a.	G (+)	B	Liquid honey	0.96	**30.5**
S. a.	G (+)	B	P.H.	0.91	**28.9**
S. t.	G (–)	C	C (+)	1.00	**33.0**
S. t.	G (–)	C	Liquid honey	0.59	**19.5**
S. t.	G (–)	C	P.H.	0.64	**21.1**
S. t.	G (–)	B	C (+)	1.00	**32.2**
S. t.	G (–)	B	Liquid honey	0.62	**20.0**
S. t.	G (–)	B	P.H.	0.70	**22.5**

Inhibition zones were measured using the PhotoMeasure software through digital image analysis. For each Petri dish, the gentamicin inhibition zone was accepted as the reference value (ratio = 1.00), and the zones of liquid honey and powdered honey were calculated proportionally relative to this reference. Final zone diameters (mm) were obtained by multiplying the gentamicin zone diameter by the corresponding proportional value. Measurements were based on the median of multiple radial assessments, and all experiments were conducted in triplicate.

Abbreviations: C (+), gentamicin (positive control); P.H., powdered honey; S.a., *Staphylococcus aureus*; S.t., *Salmonella typhimurium*.

DISCUSSION

In this study, an alternative microcrystalline cellulose–based powdered honey system was evaluated as a non-thermal, inert carrier, rather than investigating the antimicrobial properties of honey per se, which are already well established in the literature. The novelty of the present work lies in the conversion of honey into a powdered form using a heatless MCC-based system and in its comparative evaluation with liquid honey at strictly equivalent concentrations. When applied under equivalent dosing conditions, differences in inhibition zone size between liquid and powdered honey were microorganism-dependent: liquid honey produced slightly larger zones against Staphylococcus aureus, whereas powdered honey produced comparable or larger zones against Salmonella typhimurium under certain conditions. The exclusive use of agar diffusion assays was a deliberate methodological choice aimed at evaluating diffusion-related effects associated with physical form, rather than determining MIC or MBC values.

These findings indicate that antimicrobial outcomes associated with honey are influenced not only by chemical composition and applied dose but also by physical form and diffusion behavior within the agar medium. Although reduced inhibition against Gram-negative bacteria was observed, this is consistent with the outer membrane's barrier function and is discussed here in methodological rather than clinical terms. The inclusion of both monofloral and polyfloral honeys demonstrates the applicability of the proposed approach across different botanical origins, rather than providing a quantitative comparison of botanical effects. Previous studies have similarly shown that the physical state of a substance and its diffusion characteristics can influence the accessibility of bioactive components in agar-based systems, thereby affecting measured antimicrobial responses.

As summarized in Tables 1 and 2, the tested honey samples exhibited key physicochemical and functional characteristics relevant to antimicrobial performance, including high sugar content–associated osmotic pressure, low water activity, and acidic pH, all of which are known to contribute to bacterial growth inhibition. In addition, the low HMF levels and preserved diastase activity reported for the honey samples indicate minimal heat exposure and support the use of a non-thermal processing strategy. These parameters provide essential context for interpreting the inhibition zone data, as they reflect both the intrinsic antimicrobial potential of the honeys and their suitability for diffusion-based evaluation.

Accordingly, disc diffusion and agar well diffusion methods were used concurrently in the present work not to establish a direct methodological comparison, but to account for potential differences in diffusion linked to viscosity and physical form under identical experimental conditions. Since inhibition zone size reflects both antimicrobial potency and the capacity of a substance to spread through the agar matrix, the more restricted diffusion of high-viscosity liquid honey compared with the more uniform distribution of adequately rehydrated powdered honey may reasonably contribute to the larger inhibition zones observed for the powdered formulation in certain cases (Rivero et al. 2021; Farooq et al. 2025; Kaya et al. 2025).

The carrier material used in powdered honey production directly affects the final product's physical and functional properties. Many recent studies have

focused on maltodextrin, gum arabic, or protein-based carriers, which have been reported to dilute honey's bioactive components (Ganaie et al. 2021; Chandrakar et al. 2024). In contrast, the use of a natural and inert carrier such as MCC allows the physical transformation of honey without altering its chemical composition. From this perspective, the approach may be considered applicable to different botanical origins.

Experimental studies conducted with various bee products have similarly demonstrated that biological responses depend not only on chemical composition but also on the mode of application. Accordingly, while MCC has previously been used as a carrier material in honey powder formulations, the innovative aspect of the present study lies in the non-thermal formulation strategy and the equivalent honey-dose antimicrobial design, which together enable evaluation of physical form–dependent diffusion behavior. Microcrystalline cellulose is widely reported as an inert and biologically inactive carrier material in food applications.

Literature evidence indicates that MCC alone does not exhibit antibacterial activity (Samborska 2019; Mutlu et al. 2020; Çakar et al. 2025) and the assumption that MCC did not contribute to antimicrobial effects in the present study was evaluated in light of these findings. Most previously reported MCC-based honey powder studies primarily focus on drying performance or physicochemical stability. In contrast, the present study emphasizes antimicrobial performance under strictly standardized honey dosing and interprets inhibition outcomes in relation to physical form–dependent diffusion behavior. Unlike spray-drying–based approaches, the applied non-thermal powdering strategy avoids heat-induced quality deterioration, as evidenced by the preservation of low HMF levels and high diastase activity, indicating minimal thermal damage and maintained enzymatic integrity.

Although the inert nature of microcrystalline cellulose is well documented in the literature, the present study did not include a direct experimental comparison with other commonly used carrier materials such as maltodextrin, gum arabic, or protein-based matrices under identical antimicrobial testing conditions. Similarly, iso-osmotic sugar controls were not employed to experimentally decouple osmotic effects from other honey-derived antimicrobial mechanisms. These aspects therefore represent limitations of the present work. However, the strict standardization of honey mass between liquid and powdered formulations was designed to minimize dilution-related bias and to focus on physical form–dependent diffusion behavior rather than on carrier-specific or purely osmotic effects.

Differences in antimicrobial responses between Gram-positive and Gram-negative bacteria observed in this study are consistent with previously reported findings. The outer membrane of Gram-negative bacteria constitutes a well-known permeability barrier that restricts the intracellular penetration of both hydrophilic and hydrophobic antimicrobial agents, thereby attenuating the antibacterial effectiveness of honey-based treatments (Onyango et al. 2024; Ogwu et al. 2025). From this perspective, the relatively smaller inhibition zones observed for Salmonella typhimurium are expected. Nevertheless, the fact that powdered honey produced wider inhibition zones than liquid honey under certain experimental conditions indicates that physical form and diffusion-related properties may influence antimicrobial performance even in Gram-negative species. Beyond bacterial cell wall architecture, the botanical origin of honey plays a decisive role in shaping antimicrobial activity.

Chestnut honey has been frequently associated with strong antibacterial activity, largely attributed to its high phenolic content and distinctive chemical composition, particularly against *Staphylococcus aureus* and biofilm-forming microorganisms (Koloh et al. 2024; Coppola et al. 2025). Although polyfloral honeys exhibit greater compositional variability, they have been shown to exert broad-spectrum biological effects (Nagy-Radványi et al. 2024; Luca et al. 2025). The maintenance of antimicrobial efficacy in the powdered form for both honey types suggests that the cellulose-based powdering strategy is applicable across different botanical origins. A notable strength of the present study is the strict standardization of honey concentration across liquid and powdered formulations, an aspect that is often insufficiently addressed in comparable studies and can compromise the interpretation of results (Obey et al. 2022; Wadi 2022). Furthermore, the use of calibrated digital image analysis for inhibition zone measurement enhanced data reliability by reducing operator-dependent variability.

Chestnut honey was deliberately selected as a challenging model due to its high biological value, allowing assessment of whether the non-thermal powdering approach preserves antimicrobial activity in a highly bioactive honey. This also demonstrates that the method is applicable to premium honeys, not just those that are technologically problematic. A limitation of the study is that antioxidant activity was not directly evaluated; therefore, the findings should be interpreted as reflecting preservation of antimicrobial functionality, while conclusions regarding antioxidant retention remain beyond the scope of this work.

Although inhibition zones are commonly reported as absolute diameters, this study adopted proportional normalization relative to an internal reference to reduce inter-plate variability. Factors such as agar thickness, inoculum density, and minor incubation differences can influence absolute zone sizes between plates. By expressing inhibition zones of honey samples relative to the gentamicin zone obtained on the same plate, plate-specific variation was minimized, allowing a more reliable comparison of antimicrobial performance across treatments. This

approach was selected to support the study's exploratory and methodological focus rather than to establish standardized susceptibility thresholds.

It should be recognized that agar diffusion assays are inherently limited and do not provide an absolute measure of antimicrobial efficacy; therefore, larger inhibition zones observed with powdered honey should not be interpreted as indicating lower minimum inhibitory concentrations (MICs). In the present study, diffusion-based assays were intentionally employed to evaluate physical form– and diffusion-related effects rather than to determine MIC or bactericidal endpoints. Accordingly, the literature underscores the importance of complementary approaches—such as broth microdilution, time–kill kinetics, and antibiofilm assays—for a more comprehensive assessment of the antimicrobial potential of honey-based formulations (Bezerra et al. 2024; Hosseini-nejad et al. 2025). Future studies should therefore substantiate the antimicrobial performance of cellulose-based powdered honey through MIC and MBC determinations, evaluate its effectiveness in biofilm models, and assess its stability during storage. Within this context, the present study offers a meaningful methodological and practical contribution by demonstrating that an alternative powdered honey system is intended to preserve honey's natural structural characteristics, thereby supporting its potential for broader functional and application-oriented use.

Conclusion

This study demonstrated, comparatively, the effect of a microcrystalline cellulose–based alternative approach to powdered honey production, designed to preserve the natural structure of honey, on antimicrobial activity by ensuring an equivalent honey concentration to liquid honey. The findings showed that the cellulose-based powdered honey form exhibited measurable, reproducible antimicrobial activity against Staphylococcus aureus and Salmonella typhimurium in both chestnut and polyfloral honey. The honey samples used in this study were selected to represent monofloral (chestnut honey) and polyfloral honey structures.

This approach indicates that the observed findings are not limited to a single botanical origin but may also be applicable to honeys with different floral compositions. Accordingly, the applicability of the cellulose-based powdered honey production approach was evaluated independently of botanical diversity. Unlike commonly reported methods in the literature that involve thermal processing or require high proportions of carrier materials, the applied powdered honey production approach offers an alternative, innovative strategy to preserve the chemical composition and natural characteristics of honey. The use of microcrystalline cellulose, an inert and naturally derived carrier, enabled the transformation of honey into a powder while maintaining its antimicrobial activity. In this respect, the study demonstrates that the biological activity of honey is influenced not only by its chemical composition but also by its physical form and mode of application. Furthermore, ensuring equivalent honey amounts in comparisons between liquid and powdered honey strengthened the direct comparability of the results. Inhibition zone measurements performed using a digital image analysis method enhanced methodological transparency and contributed to the reliability of the findings.

In conclusion, this study demonstrates that a cellulose-based powdered honey production approach, validated with both monofloral and polyfloral honey samples and preserving honey's natural structure, can maintain antimicrobial activity while expanding honey's functional use potential. The findings provide a strong methodological foundation for future studies evaluating honey in alternative physical forms and offer a new perspective for the development of honey-based functional products.

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Conflict of Interest: I declare no conflict of interest.

Data Availability Statement: The data supporting the findings of this study are available from me upon reasonable request.

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