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FUNCTIONAL POTENTIAL OF ORGANIC HONEY VINEGAR: COMPARATIVE PHYSICOCHEMICAL PROPERTIES, ANTIOXIDANT, ANTIMICROBIAL AND BIOFILM-MODULATING ACTIVITIES AMONG COMMERCIAL ORGANIC VINEGARS

Seda ALTUNTAŞ, Aycan YİĞİT ÇINAR

Bursa Technical University, Faculty of Engineering and Natural Sciences, Department of Food Engineering, Bursa, Türkiye

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ABSTRACT

In this study, apple, grape, and honey vinegars (AV; GV; HV) were comparatively investigated in terms of their physicochemical properties, antioxidant capacity, antibacterial activity, and antibiofilm effects. Differences were observed among the vinegars in pH, soluble solids, and phenolic-associated characteristics, while total acidity levels were similar. These variations were reflected in the functional outcomes. Antioxidant activity varied according to the assay; AV and HV showed higher DPPH (197.98 and 218.28 µmol TE/L) and FRAP (1170.16 and 1178.29 µmol TE/L) values than GV, while AV exhibited the highest ABTS activity (934.33 µmol TE/L). AV and HV exhibited stronger antibacterial effects than GV, with MIC values of 31.25 and 15.625 µL/mL against *Listeria monocytogenes* ATCC 7644 and *Pseudomonas aeruginosa* ATCC 35032, respectively. All vinegars reduced biofilm formation to varying extents, with AV, GV, and HV inhibiting *P. aeruginosa* biofilm formation by 74.81%, 71.69%, and 67.30%, respectively, at their MICs. In contrast disruption of mature biofilms remained limited, maximum reductions reached 35.60% for *L. monocytogenes* and 33.31% for *P. aeruginosa*. The results emphasize the role of matrix composition beyond acetic acid content in determining bioactivity. Notably, HV emerged as a promising yet underexplored product with functional potential.

Key words: Honey vinegar, Apple vinegar, Grape veinegar, Anti-biofilm activity, Mature biofilm distruption

ORGANİK BAL SİRKESİNİN FONKSİYONEL POTANSİYELİ: TİCARİ ORGANİK SİRKELERİN FİZİKOKİMYASAL ÖZELLİKLERİ, ANTİOKSİDAN, ANTİMİKROBİYAL VE BİYOFİLM ÜZERİNE

ÖZ

Bu çalışmada, elma, üzüm ve bal sirkeleri (AV; GV; HV), fizikokimyasal özellikleri, antioksidan kapasiteleri, antibakteriyel aktiviteleri ve antibiyofilm etkileri açısından karşılaştırmalı olarak incelenmiştir. Sirkeler arasında pH, çözünür kuru madde ve fenolik bileşiklerle ilişkili özellikler bakımından farklılıklar belirlenirken, toplam asitlik düzeyleri benzer bulunmuştur. Bu farklılıklar fonksiyonel sonuçlara da yansımıştır. Antioksidan aktivite kullanılan yönteme göre değişiklik göstermiş; AV ve HV, GV'ye kıyasla daha yüksek DPPH (sırasıyla 197,98 ve 218,28 µmol TE/L) ve FRAP (sırasıyla 1170,16 ve 1178,29 µmol TE/L) değerleri gösterirken, en yüksek ABTS aktivitesi AV'de (934,33 µmol TE/L) belirlenmiştir. AV ve HV, GV'ye kıyasla daha güçlü antibakteriyel etki göstermiş; *Listeria monocytogene*s ATCC 7644 ve Pseudomonas aeruginosa ATCC 35032'ye karşı sırasıyla 31,25 ve 15,625 µL/mL MİK değerleri belirlenmiştir.. Tüm sirkeler biyofilm oluşumunu farklı düzeylerde azaltmış; MİK değerlerinde AV, GV ve HV, *P. aeruginosa* biyofilm oluşumunu sırasıyla %74,81, %71,69 ve %67,30 oranında inhibe etmiştir. Buna karşılık, olgun biyofilmlerin parçalanması üzerindeki etki sınırlı kalmış ve maksimum azalma *L. monocytogenes* için %35,60, *P. aeruginosa* için ise %33,31 olarak belirlenmiştir. Sonuçlar, biyoaktivitenin belirlenmesinde yalnızca asetik asidin değil, matris bileşiminin de önemli rol oynadığını ortaya koymaktadır. Özellikle HV, fonksiyonel potansiyele sahip ancak henüz yeterince araştırılmamış umut verici bir ürün olarak öne çıkmıştır.

Anahtar kelimeler: Bal sirkesi, Elma sirkesi, Üzüm sirkesi, Antibiyofilm aktivite, Olgun biyofilm parçalanması

Yazışmalardan sorumlu yazar / Corresponding author: Seda ALTUNTAŞ
ORCID No: 0000-0003-1126-6405 **E-mail:** seda.altuntas@btu.edu.tr

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INTRODUCTION

Microbial biofilms are recognized as structured, surface-associated microbial communities embedded within a self-produced extracellular polymeric substance (EPS) matrix. Biofilm formation begins with reversible attachment, during which microbial cells interact with biotic or abiotic surfaces through weak physicochemical forces, including electrostatic interactions, van der Waals forces, and hydrodynamic effects. As microorganisms approach the surface more closely, these interactions transition into irreversible attachment, driven by the activation of adhesins and early matrix components. Subsequent maturation involves extensive EPS secretion, microcolony formation, and the establishment of chemical gradients that support biofilm stratification. Finally, regulated dispersal enables biofilm propagation and colonization of new niches, thereby contributing to ecological persistence (Sauer et al., 2022; Sharma et al., 2023).

Bacteria residing within biofilms display markedly enhanced tolerance to antibiotics, disinfectants, and environmental stresses compared to their planktonic counterparts. This elevated resilience underlies the role of biofilms in a broad range of persistent infections and industrial challenges (Maillard & Centeleghe, 2023). *Pseudomonas aeruginosa*, a prominent opportunistic pathogen, remains a key model organism in biofilm research and is implicated in chronic respiratory infections, wound infections, and complications associated with cystic fibrosis. Its rising antimicrobial resistance has placed it among the World Health Organization's top-priority pathogens for novel antimicrobial development (Thomassen et al., 2023). Similarly, *Listeria monocytogenes*, although less prevalent than other foodborne pathogens, poses a major public health concern due to its high mortality rate and ability to form biofilms on food-processing surfaces (Paiva et al., 2025).

Vinegar, produced via a two-stage fermentation process involving alcoholic fermentation by yeasts followed by acetic acid fermentation by acetic acid bacteria, has long been used as a natural antimicrobial agent. Its antimicrobial efficacy is primarily associated with its organic acid content-especially acetic acid-whose activity depends on pH, acid concentration, ionic strength, and microbial physiology. Vinegar-based solutions have therefore gained increased attention as consumers seek alternatives to chemical preservatives in food processing (Bai et al., 2024; Kiliçgün & Yangilar, 2024). Among various vinegar types, honey vinegar (HV) has attracted renewed scientific interest due to its unique biochemical composition arising from honey fermentation. Honey vinegar is produced when honey undergoes sequential lactic/alcoholic fermentation and acetic acid fermentation by yeasts and acetic acid bacteria. Previous studies have shown that honey-derived fermented products possess complex microbial and biochemical dynamics, including the activity of lactic acid bacteria, yeasts, and acetic acid bacteria, which contribute to their sensory characteristics and functional properties. In particular, honey vinegar contains distinct volatile and phenolic compounds derived from honey, and recent reviews highlight its potential health-promoting, antimicrobial, and functional properties (Margaoan et al., 2020). Despite several studies demonstrating antimicrobial and antibiofilm effects of apple and grape vinegars (Kahraman et al., 2021; Ozturk et al., 2015), honey vinegar remains insufficiently investigated. Moreover, no comprehensive studies have evaluated its total phenolic content, antioxidant capacity, antimicrobial activity, or antibiofilm properties collectively. This lack of empirical evidence highlights a significant research gap regarding the functional potential of honey-derived vinegars.

The present study aims to evaluate the total phenolic content, antioxidant, antimicrobial and antibiofilm activities of different commercial organic vinegars-apple vinegar (AV), grape vinegar (GV), and honey vinegar (HV)-against major foodborne and opportunistic pathogens. In addition to these properties, comprehensive physicochemical analyses, including Brix value, titratable acidity, pH, and color parameters, were performed to characterize the vinegars and to explore potential correlations between their compositional attributes and antimicrobial efficacy. To the best of our knowledge, there is a lack of detailed comparative studies on the antimicrobial and biofilm inhibitory capacities of honey vinegar, highlighting the novelty and significance of this investigation.

MATERIAL AND METHODS

Materials

Three different types of organic vinegars, including apple vinegar (AV), grape vinegar (GV) and honey vinegar (HV) were purchased from a local supermarket in Bursa, Türkiye. The products were selected based on their certified organic

status to minimize variability. According to the product labels, all vinegars were certified organic products and contained no preservatives or other additives. The products were labeled as being produced in accordance with Turkish organic farming legislation (Organic Agriculture Law No. 5262, dated December 1, 2004, and the Regulation on the Principles and Implementation of Organic Agriculture, No. 27676, dated August 18, 2010). All commercial vinegars were stored under refrigerated conditions (6±1°C) prior to analysis.

Physicochemical properties

pH values of the vinegars were measured using a pH meter (Mettler-Toledo S210 Uni) calibrated with buffer solutions before analysis. Five milliliters of vinegar was mixed with 20 mL of distilled water and the mixture was titrated using 0.1 N NaOH. Equivalence points were later verified using phenolphthalein indicator. Total acidity was expressed as acetic acid equivalent (Bakir et al., 2016). Brix values of the vinegars were measured using a refractometer (RA-600, KEM Kyoto Electronics) calibrated with distilled water. Color parameters were measured directly on the surface of vinegars after the vinegar samples were poured into plastic Petri dishes by using a colorimeter (PCE Instruments, CSM3, Turkey). The color attributes included lightness (L^*), redness-greenness (a^*), yellowness-blueness (b^*), chroma (C^*) and hue angle (h^o) were extracted with the instrument. Five measurements were performed for each vinegar sample.

Total phenolic content and antioxidant activity

Vinegar samples were filtered through a 0.22 μm membrane filter and subsequently diluted with methanol to appropriate concentrations for analysis. Total phenolic content (TPC) of the vinegars was analyzed using the Folin-Ciocalteu colorimetric method (Altuntas & Korukluoglu, 2024). Absorbance was measured at 740 nm, and quantification was performed using a gallic acid calibration curve (10-100 mg/L). TPC values were expressed as mg gallic acid equivalents (GAE)/L vinegar. Antioxidant activity of vinegars was determined with 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays according to Altuntas & Korukluoglu (2024). For the DPPH assay, absorbance was measured at 515 nm. For the ABTS assay, the ABTS radical solution was prepared and incubated for 12-16 h in the dark at room temperature. Following reaction with the samples, the mixture was incubated for 6 min in the dark and absorbance was measured at 734 nm. For the FRAP assay, the working reagent consisted of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ prepared in 40 mM HCl, and 20 mM $FeCl_3$ at a ratio of 10:1:1 (v/v/v). The freshly prepared reagent was equilibrated at 37°C for 10 min, and the reaction mixture was subsequently incubated at 37°C for 30 min before absorbance measurement at 593 nm. For all antioxidant assays, antioxidant capacity was calculated using a Trolox calibration curve (10-250 μmol/L) and expressed as μmol Trolox equivalents (TE)/L vinegar.

Preparation for bacterial suspension

In this study, *Listeria monocytogenes* ATCC 7644 and *Pseudomonas aeruginosa* ATCC 35032 were used as test organisms. These microorganisms were inoculated into 10 mL of sterile tryptic soy broth supplemented with 6 g L-1 yeast extract (TSBYE) and tryptic soy broth (TSB), respectively, and incubated at 37°C for 18-24 h (Ferreira and Domingues, 2016; Mashhady et al., 2016). After incubation, the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard (1.5 x 10^8 CFU/mL).

Antibacterial activity

Antimicrobial activity of the vinegars was determined using the microdilution method and disk diffusion assay (Cruz-Galvez et al., 2013). Vinegar samples were filter sterilized with a 0.45 μm membrane filter and were serially two-fold diluted in TSBYE (for *L. monocytogenes* ATCC 7644) and TSB (for *P. aeruginosa* ATCC 35032). The prepared 0.5 McFarland units bacterial suspension was further diluted. The final concentration of microorganisms was adjusted to 5.0 x 10^5 CFU/mL in each well. To assess the minimum inhibitory concentration (MIC) for the vinegars, all the wells of microtiter plates were filled with 100 μL bacterial suspension and 100 μL of different concentrations of vinegar. Plates were incubated at 37°C for 24 h and the MICs were determined as the lowest concentration of additive that demonstrated no visible growth. For minimum bactericidal concentration (MBC) determination, 10 μL of the broth from wells without visible growth were plated onto tryptic soy agar supplemented with yeast extract (TSAYE) and were incubated for 24 h at 37°C. MBC was defined as the minimum concentration of vinegar capable of killing all bacteria.

Bacterial suspensions (100 μL of about 1.0 x 10^8 CFU/mL)

were spread onto Petri dishes containing agar medium. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of the sterile-filtered vinegar samples and placed on the inoculated plates. Plates were incubated at 37°C for 24 h and the diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate.

Inhibition of the cell attachment assay

The effects of AV, GV and HV in inhibiting *L. monocytogenes* ATCC 7644 and *P. aeruginosa* ATCC 35032 biofilm formation were assessed using a crystal violet assay according to the methodology described by Sandasi et al. (2010). One hundred microliters of the different concentrations of vinegars were added to the 96-well microplates, and equal volumes of water were added as a negative control. The standardized culture (1.0 x 10^6 CFU/mL) (100 µL) was then pipetted into the wells to yield a final volume of 200 µL in each well. The cultures were added into the wells in triplicate, and sterile TSB/TSBYE was added as an additional control to ensure that the media remained sterile during the experiment. The plate was incubated at 37°C for 24 h without shaking to allow cell attachment and biofilm development. Following incubation, the modified crystal violet assay was performed to assess biofilm biomass, and the results were expressed as percentage inhibition (Eq. 1).

$$\text{Percentage Inhibition} = \frac{OD_{negative\ control} - OD_{experimental}}{OD_{negative\ control}}$$

(Eq. 1)

The strains were classified as weak (optical density $(OD)_{negativecontrol} < OD \leq 2\times OD_{negativecontrol}$), moderate ($2\times OD_{negativecontrol} < OD \leq 4\times OD_{negativecontrol}$), or strong ($OD > 4\times OD_{negativecontrol}$) biofilm producers, according to Stepanović et al., (2004).

Mature biofilm disruption assay

Biofilm formation was achieved by aliquoting 100 µL of a standardized (1.0 x 10^6 CFU/mL) *L. monocytogenes* ATCC 7644 or *P. aeruginosa* ATCC 35032 into a 96-well microplate. The plates were incubated at 37°C for 24 h to allow cell attachment. Following the 24 h incubation, 200 µL of each vinegar (not diluted) and serial two-fold dilutions were added to each well for different treatment times (30, 60 and 120 min). Equal volumes of water were added as a negative control. The vinegars were removed by pipetting immediately after each treatment period. Subsequently, the crystal violet assay was performed.

Assessment of biofilm biomass (Crystal violet staining assay)

Biofilm biomass was indirectly assessed using the modified crystal violet (CV) assay (Sandasi et al., 2010). The plates were washed three times with 220 µL of sterile distilled water to remove loosely attached cells. The plates were air-dried for 30 min. Following drying, the microplates were fixed with 200 µL of methanol (99.8%) for 15 min. After pouring out the methanol, the plates were dried in the inverted position for 10 min. Then, 150 µL of crystal violet dye (1.0%) was used to stain biofilms and positive and negative control wells for 15 min and removed by pipetting. The plate was rinsed with sterile distilled water until the washing was dye-free. The bound dye was resolubilized in 200 µL of 95% ethanol for 15 min and 150 µL of the resolubilized solution was transferred into a new 96-well plate. The OD of the dye solution was measured at 595 nm.

Statistical analysis

All statistical analyses were conducted using IBM SPSS (version 22). Data normality was evaluated with the Kolmogorov-Smirnov test. Depending on distribution characteristics, parametric analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, while non-parametric data were analyzed using the Kruskal-Wallis and Mann-Whitney U tests. Statistical significance was considered at a 95% confidence level ($P<0.05$).

RESULTS AND DISCUSSION

Physicochemical properties

Physicochemical properties-especially pH and total acidity-are key determinants of the antimicrobial and antibiofilm activities of vinegars. Lower pH values and higher concentrations of organic acids increase the proportion of undissociated acid molecules, which can penetrate bacterial cells and disrupt essential physiological functions. Therefore, assessing pH and acidity provides a meaningful basis for understanding how different vinegars may vary in their inhibitory effects against microorganisms (Kahraman et al., 2021). Table 1 shows the physicochemical properties of the traditional and industrial vinegar samples. HV exhibited the lowest pH (3.06±0.00), followed by AV (3.39±0.01) and GV (3.45±0.01). In a study

of Kiliçgün & Yangilar (2024), the pH values of apple vinegars were reported to range between 3.15 and 3.46. Another study reported a slightly broader pH interval for apple vinegars, varying from 2.84±0.01 to 3.82±0.04 (Abdali et al., 2023). Such variation is expected, as the degree of ripeness of the apple fruit influences the concentrations of sugars and organic acids available for fermentation, which in turn affects the microbiological stability, acidity, and ultimately the pH of the resulting vinegar (Kara et al., 2021). Similarly, grape vinegars have been reported to exhibit pH values predominantly in the low acidic range, often between approximately 2.78 and 3.48 (Cavdaroglu & Ozen, 2022). A lower pH is generally associated with increased antimicrobial efficacy, as acidic environments potentiate the penetration of undissociated organic acids through bacterial membranes. This trend aligns with previous reports showing that vinegars with pH <3.5 tend to exert stronger inhibitory effects on foodborne pathogens (Rebelo et al., 2023). HV's lower pH, therefore, suggests a potentially higher intrinsic antimicrobial activity. In a thesis study evaluating seven honey vinegars (one originating from Italy and six from Turkey, four of which were produced from honeys collected in the Adana-Kozan region), the pH values of the samples were reported to range between 2.19±0.00 and 3.35±0.01 (Alak, 2015). The pH value of HV in the present study (3.06) falls within this reported range, supporting the variability of honey vinegar pH, which may reflect differences in raw material composition and fermentation conditions.

HV showed a markedly higher Brix value (12.20±0.00), followed by AV (6.70±0.14), while GV had the lowest value (1.70±0.00). The higher Brix value of HV may reflect the contribution of residual sugars and other soluble constituents remaining after fermentation, as well as differences in fermentation progress and production conditions. In honey-derived fermentations, soluble solids may also include phenolic compounds and Maillard reaction products, which have been associated with antioxidant and antimicrobial properties (Margaoan et al., 2020). Therfore, the high Brix value of HV likely reflects the combined influence of its raw material composition and fermentation-related changes, resulting in a distinct soluble-solids profile compared with the fruit vinegars.

Total acidity was comparable among samples (3.56±0.12-3.71±0.28%), indicating similar concentrations of acetic acid equivalents despite differences in Brix and pH values. These findings indicate that the vinegar samples differed in their physicochemical profiles despite having comparable total acidity. Such compositional differences may contribute to variations in their functional properties, as further evaluated in the subsequent sections.

Table 1. Physicochemical properties of vinegars

Samples[1]	pH	Brix	Total acidity (%)[2]	Color L*	a*	b*	C	h
AV	3.39±0.01^{b}	6.70±0.14^{b}	3.71±0.28	40.01±3.74	2.44±0.43^{c}	10.80±0.80^{c}	11.09±0.68^{b}	77.08±3.23^{a}
GV	3.45±0.01^{a}	1.70±0.00^{c}	3.56±0.12	39.86±4.05	6.71±1.20^{a}	12.23±0.85^{b}	13.98±1.21^{a}	61.45±3.35^{c}
HV	3.06±0.00^{c}	12.20±0.00^{a}	3.58±0.22	36.90±4.40	5.31±0.48^{b}	14.03±1.07^{a}	15.02±0.92^{a}	69.14±2.84^{b}

1 AV: Apple vinegar, GV: Grape vinegar, HV: Honey vinegar, 2 Total acidity: % (as acetic acid). Results are expressed as mean±standard deviation, n=3. Different lowercase letters in the same column indicate statistically significant differences (P<0.05). Values without superscript letters indicate no statistically significant differences among samples (P>0.05).

HV exhibited the lowest *L** value, indicating a darker appearance, and the highest chroma (C=15.02±0.92) and *a** values, whereas GV showed higher *a** and *b** values than AV. Overall, the differences in color parameters indicate distinct visual characteristics among the vinegar types and may be associated with variations in raw material composition as well as compositional changes occurring during fermentation and processing. Phenolic compounds, pigments, and browning products such as melanoidins may contribute to these color characteristics and could partly account for the differences observed among the vinegar matrices.

The physicochemical data revealed distinct profiles among the vinegar types. HV was characterized by lower pH, higher Brix values, and more intense color attributes, while AV and GV also differed in soluble solids and color characteristics despite their comparable acidity levels. These variations demonstrate the physicochemical diversity of the vinegar samples and

provide a basis for evaluating their antioxidant, antimicrobial, and antibiofilm properties in the subsequent analyses. By integrating physicochemical parameters with microbial assays, this study provides a more comprehensive understanding of how different vinegars may exert variable antimicrobial and antibiofilm activities.

Total phenolic content and antioxidant activity of vinegars

Figure 1 illustrates the total phenolic content (TPC) and the antioxidant activity using DPPH, ABTS and FRAP assays of vinegars. The TPC of the samples exhibited moderate variation, ranging from 360.87 to 409.48 mg GAE/L, indicating that all vinegars possess a relatively high phenolic load. Among the samples, GV (409.48±10.31 mg GAE/L) and HV (405.31±11.91 mg GAE/L) exhibited statistically similar TPC values (P>0.05), whereas AV (360.87±13.18 mg GAE/L) showed a significantly lower phenolic concentration (P<0.05). A similar trend has been reported in several studies, indicating that grape vinegars consistently exhibit higher total phenolic content and stronger antioxidant capacity compared to apple vinegars under comparable experimental conditions (Kahraman et al., 2022; Kelebek et al., 2017). Kadiroğlu (2018) reported that the total phenolic content of apple vinegars was 57.22±2.22 to 484.05±5.75 mg GAE/L, whereas vinegars produced from grapes exhibited higher variability, ranging from 109.66±0.67 to 919.95±2.31 mg GAE/L. In another study, the TPC values of apple and grape vinegar were found to be 948.595±24.54 mg GAE/L and 1583.66±28.42 mg GAE/L, respectively (Budak et al., 2022). Overall, these findings are consistent with the current study. Across apple and grape vinegars, the literature reports a broad range of TPC and antioxidant activity, with values influenced strongly by the fruit material, fermentation method (spontaneous or controlled starter cultures), processing steps, and the particular assay used to measure antioxidant capacity (Bakir et al., 2016; Kara et al., 2021, 2022; Kiliçgün & Yangilar, 2024).

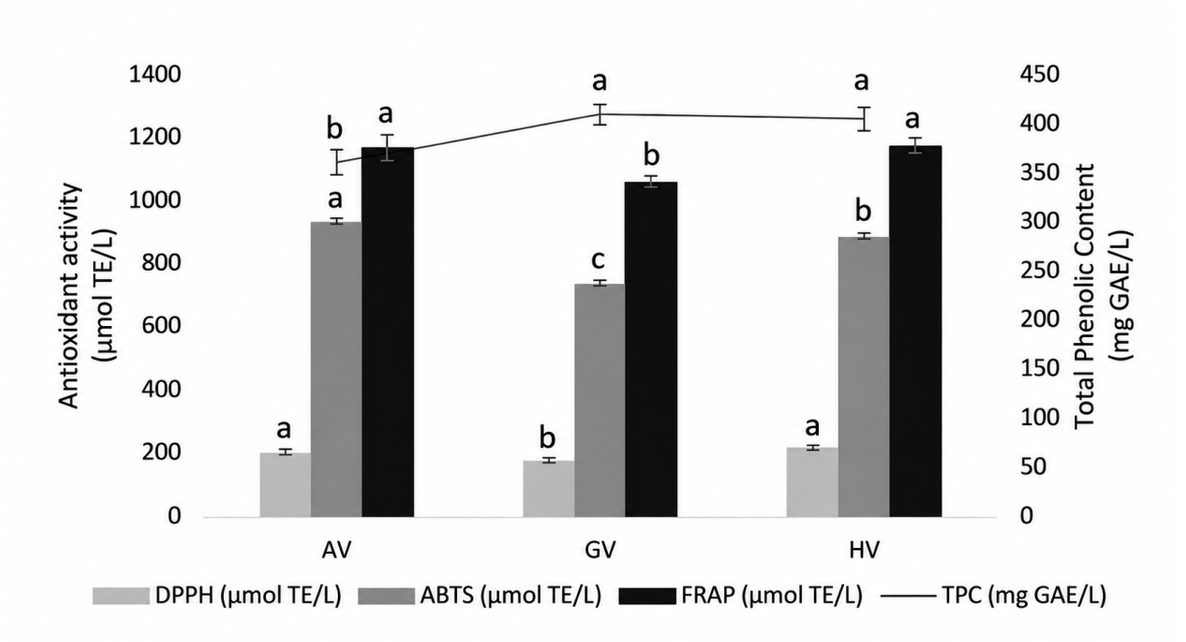


Figure 1. The total phenolic content and antioxidant activity (with DPPH, ABTS and FRAP) of apple vinegar (AV), grape vinegar (GV) and honey vinegar (HV). Different letters indicate statistically significant differences between vinegars (P<0.05)

The antioxidant capacity of the samples varied depending on the assay applied. In the DPPH assay, AV (197.98±11.02 μmol TE/L) and HV (218.28±4.75 μmol TE/L) exhibited comparable radical scavenging activities, both exceeding that of GV (175.81±7.43 μmol TE/L) (P<0.05). A similar pattern was observed for FRAP, where AV (1170.16±43.48 μmol TE/L) and HV (1178.29±23.92 μmol TE/L) showed comparable ferric-reducing capacities and higher values than GV (1060.83±18.83 μmol TE/L) (P<0.05). In contrast, the ABTS assay indicated significant differences among all samples, with AV (934.33±1.21 μmol TE/L) displaying the highest activity, followed by HV (886.71±7.63 μmol TE/L) and GV (738.62±6.42 μmol TE/L). Overall, HV consistently demonstrated strong antioxidant performance across DPPH and FRAP assays, while AV exhibited the highest activity in ABTS, indicating that antioxidant efficacy is not solely determined by total phenolic content but also by the specific composition and reactivity of individual bioactive compounds.

Antimicrobial activity

Table 2 shows the antibacterial activity of vinegar samples against tested microorganisms. The results indicate that AV and HV exhibited stronger inhibitory effects against both *L. monocytogenes* ATCC 7644 and *P. aeruginosa* ATCC 35032 compared to GV. For *L. monocytogenes*, AV and HV shared the lowest MIC value (31.25 μL/mL; 3.12%), whereas GV required a much higher concentration (125 μL/mL; 12.5%), indicating weaker antibacterial activity. The same pattern was observed for bactericidal concentrations, where HV (125 μL/mL; 12.5%) and AV (250 μL/mL; 25%) showed lower MBC values relative to GV (500 μL/mL; 50%). *P. aeruginosa* ATCC 35032 was more sensitive than *L. monocytogenes* ATCC 7644. Consistent with this, AV and HV again reached inhibitory levels at much lower amounts (15.625 μL/mL; 1.56%) for *P. aeruginosa* ATCC 35032, whereas GV required 62.5 μL/mL (6.25%) to achieve growth suppression, reflecting a markedly reduced antibacterial effect. HV also produced the lowest MBC value (62.5 μL/mL; 6.25%), further highlighting its comparatively stronger bactericidal activity. Against *L. monocytogenes* ATCC 7644, HV and AV produced inhibition zones of 11.12±0.01 and 10.95±0.99 mm, respectively, whereas GV showed a smaller zone (6.52±0.19 mm). For *P. aeruginosa* ATCC 35032, AV and HV produced inhibition zones of 8.46±0.79 and 6.69±0.10 mm, respectively, while no detectable inhibition zone was observed for GV. Despite the absence of a measurable zone, GV exhibited MIC and MBC values of 62.5 and 250 μL/mL, respectively, against *P. aeruginosa*. This difference between the assays may be attributed to the diffusion behavior of antimicrobial constituents in the agar matrix, whereas broth microdilution allows more direct and uniform contact between the vinegar components and bacterial cells.

Table 2. Antimicrobial activity of vinegars on tested microorganisms

Sample[1]	*L. monocytogenes* ATCC 7644			*P. aeruginosa* ATCC 35032		
	MIC[2] (μL /mL - % v/v)	MBC[3] (μL /mL - % v/v)	Zone (mm)	MIC (μL /mL - % v/v)	MBC (μL /mL - % v/v)	Zone (mm)
AV	31.25 - 3.12	250 - 25	10.95±0.99	15.62 - 1.56	125 - 12.5	8.46±0.79
GV	125 - 12.5	500 - 50	6.52±0.19	62.5 - 6.25	250 - 25	nd[4]
HV	31.25 - 3.12	125 - 12.5	11.12±0.01	15.62 - 1.56	62.5 - 6.25	6.69±0.10

1AV: Apple vinegar, GV: Grape vinegar, HV: Honey vinegar, 2MIC: Minimum inhibition concentration as μL /mL and %, 3MBC: Minimum bactericidal concentration as μL /mL, 4nd: not determined

The antimicrobial activity of vinegars is primarily driven by their acetic acid content, whose undissociated form can penetrate bacterial membranes and dissociate intracellularly, leading to cytoplasmic acidification, metabolic enzyme inhibition, disruption of ATP synthesis, and membrane potential collapse. Although the vinegars in this study share comparable total acidity values (expressed as acetic acid), their inhibitory performance may also be influenced by matrix-derived minor constituents, including phenolic compounds, organic acids other than acetic acid, Maillard reaction products, and volatile compounds. These components have been reported to enhance or modulate antimicrobial activity through the induction of oxidative stress, membrane disruption, or synergistic effects with acetic acid. Therefore, differences in both pH and

vinegar matrix composition likely account for the variation in antibacterial efficacy observed among the samples.

Previous studies have reported a broad MIC range for different vinegar types (Karatepe et al., 2024; Ousaaid et al., 2021; Sengun et al., 2020). Kara et al. (2021) evaluated apple vinegars produced from different apple varieties and processing methods, assessing their physicochemical characteristics alongside their antibacterial activity against several pathogenic bacteria. Their findings showed that MIC values against P. aeruginosa (clinical isolate) varied widely, ranging from 7.81 to 500 μL/mL, indicating that both production methodology and the botanical origin of apples can markedly influence antimicrobial potency. These values fall within a similar range to those observed in the present study. Sengun et al. (2020) analyzed the physicochemical, bioactive, and antimicrobial qualities of various fruit vinegars, such as those made from blackberry, plum, pomegranate, apple and grape. Most fruit vinegars exhibited strong inhibitory activity against L. monocytogenes (specifically the Scott A strain) at low concentrations. In particular, persimmon, grape, plum, fig, rosehip, pomegranate, and blackberry vinegars showed an MIC of 3.12% (v/v), whereas apple vinegar was the least effective, requiring 12.5% (v/v) to inhibit growth. Moreover, while apricot and apple vinegars failed to produce a bactericidal effect even at the highest concentration tested (>50% v/v), grape vinegar demonstrated an MBC of 50%. In contrast to these findings, the apple vinegar used in the present study displayed comparatively stronger antimicrobial activity than grape vinegar, suggesting that differences in raw material composition and production conditions may substantially alter the inhibitory potential of apple-based vinegars.

To date, the antimicrobial activity of honey vinegar has not been directly investigated in the literature, and available evidence is limited to studies on honey-vinegar mixtures. Oxymel, a traditional preparation combining honey and vinegar, differs from pure honey vinegar in that it includes both components in a defined ratio, often with the addition of water or herbal extracts (Harrison et al., 2023). While honey vinegar represents a single fermented product with its own intrinsic phenolic and antimicrobial profile, oxymel acts as a blended system where the bioactive properties of honey and vinegar may interact synergistically. This distinction is important, as the observed antimicrobial or antioxidant effects of oxymel cannot be directly attributed to honey vinegar alone, thereby emphasizing the need for studies specifically targeting honey vinegar as an individual functional ingredient.

Anti-biofilm potential of vinegars

Biofilm formation represents a critical survival strategy that enhances bacterial persistence under adverse environmental conditions and contributes significantly to contamination risks in food processing environments. The *L. monocytogenes* ATCC 7644 and *P. aeruginosa* ATCC 35032 strains used in this study were classified as a weak and a strong biofilm producers, respectively. Biofilm production is recognized as an important virulence factor for bacteria of the genera *Listeria* and *Pseudomonas. Hence,* formation of biofilms by these pathogens is of major concern for the food industry.

Table 3. Biofilm forming ability of test microorganisms

Strain	OD	Category
L. monocytogenes ATCC 7644	0.22±0.03	Weak
Negative control (TSBYE)	0.20±0.03	-
P. aeruginosa ATCC 35032	1.14±0.20	Strong
Negative control (TSB)	0.16±0.01	-

1 TSBYE: Tryptic soy broth with 6 g L-1 yeast extract, TSB: Tryptic soy broth, OD: Optical density, weak (ODnegativecontrol< OD ≤2×ODnegativecontrol), strong (OD >4×ODnegativecontrol)

The antibiofilm activities of vinegars at MIC and sub-MIC levels (1/2 MIC, 1/4 MIC, 1/8 MIC, and 1/16 MIC) were evaluated using the crystal violet staining assay to determine their effectiveness in inhibiting biofilm formation and bacterial colonization of L. monocytogenes ATCC 7644 and *P. aeruginosa* ATCC 35032. The application of vinegars was effective in reducing the attachment of both strains to the surface (Figure 1); however, none of the samples achieved complete

inhibition of cell attachment at either MIC or sub-MIC levels. *L. monocytogenes* ATCC 7644 exhibited greater resistance compared to *P. aeruginosa* ATCC 35032, as indicated by its lower inhibition percentages. This observation is further supported by the MIC and MBC values obtained. All vinegar samples inhibited the attachment of *P. aeruginosa* ATCC 35032 by at least 62.96±0.70%, whereas the maximum inhibition observed for *L. monocytogenes* ATCC 7644 was limited to 31.53±2.1%, even at the highest acetic acid concentration in vinegars. These findings suggest that effective biofilm inhibition is strongly dependent on concentration and microbial species. Moreover, even within the same species, the efficacy of these compounds can vary significantly across different serotypes and strains, highlighting the need for either broad-spectrum or strain-specific approaches in biofilm control (Gemmell et al., 2022).

In this study, no biofilm inhibition was observed at the lower sub-MIC levels: for *L. monocytogenes* ATCC 7644, inhibition was absent at 1/8 and 1/16 MIC, while for *P. aeruginosa* ATCC 35032, inhibition was not detected at 1/4, 1/8, and 1/16 MIC. Duzguner & Erbil (2025) reported that pomegranate vinegar exhibited significant antibiofilm activity against major pathogenic strains, including *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and Listeria monocytogenes ATCC 19115, achieving approximately 60-90% biofilm inhibition at concentrations ranging from 25 to 12.5 µL/mL. Supporting these findings, another study demonstrated that apple cider vinegar exhibited notable antibiofilm activity against *Bacillus cereus* biofilms, achieving over 50% inhibition of biofilm development, with a commonly observed minimum biofilm inhibitory concentration ($MBIC_{50}$) of 4 µg/mL, further confirming the strong antibiofilm potential of vinegar-based systems across different bacterial species (Mahmoud et al., 2024). In the present study, AV and HV exhibited 74.81±1.30% and 67.30±1.10% biofilm inhibition, respectively, against *Pseudomonas aeruginosa* ATCC 35032 at a concentration of 15.62 µL/mL. These results suggest that different types of vinegar can exert comparable antibiofilm effects even at similar or lower concentrations, likely through the combined action of acetic acid, which disrupts bacterial cell adhesion and extracellular polymeric substance production, and other bioactive compounds such as phenolics that interfere with quorum sensing and biofilm maturation (Mishra et al., 2020).

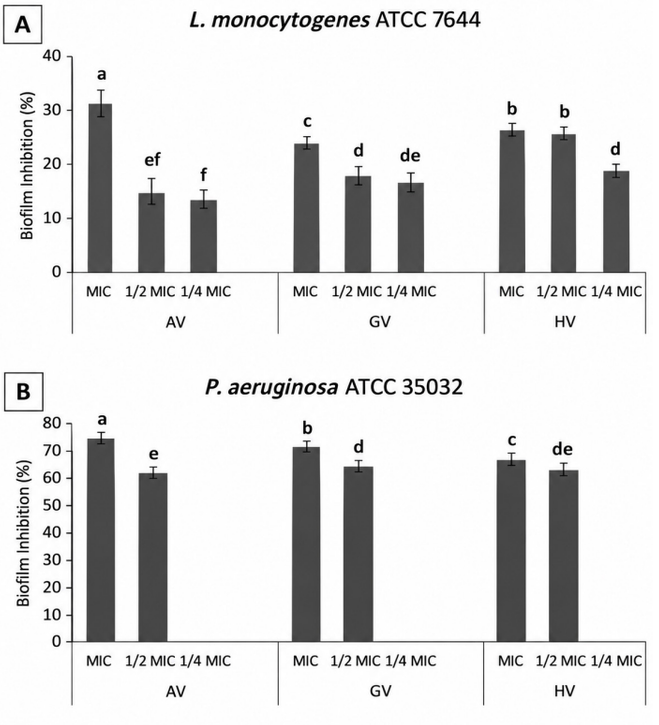


Figure 2. Biofilm inhibition (%) of *L. monocytogenes* ATCC 7644 (A) and *P. aeruginosa* ATCC 35032 (B) treated with different vinegars at MIC and sub-MIC levels. AV: apple vinegar; GV: grape vinegar; HV: honey vinegar; MIC: minimum inhibition concentration. Different letters indicate statistically significant differences ($P<0.05$). The 1/8 and 1/16 MIC levels were not included in the figure, as no biofilm inhibition was observed at these concentrations for either microorganism

Time-dependent disruption of mature biofilms by vinegar treatments

Mature biofilms represent a highly structured and resistant form of microbial growth, characterized by a well-developed extracellular polymeric substance (EPS) matrix that protects embedded cells against environmental stresses and antimicrobial agents. At this stage, bacterial cells exhibit enhanced tolerance and reduced susceptibility to antimicrobial treatments compared to their planktonic counterparts. Therefore, disrupting preformed (mature) biofilms is considerably more challenging than preventing initial attachment (Sharma et al., 2023).

In the present study, the mature biofilm disruption assay revealed a clear time- and vinegar-type-dependent effect. The vinegars (100% and two-fold serial dilutions) applied to preformed (24-h-old) biofilms of *L. monocytogenes* ATCC 7644 and *P. aeruginosa* ATCC 35032 for 30, 60, and 120 minutes resulted in partial biofilm disruption with maximal values reaching 35.60±2.63% and 33.31±1.76%, respectively. These findings contrast with several reports in the literature that demonstrate a more potent efficacy of vinegar and its primary component, acetic acid, against established biofilms. Bjarnsholt et al. (2015) reported that acetic acid at concentrations as low as 0.5% to 1.0% could completely eradicate 3-day-old (72-h) mature biofilms of *P. aeruginosa*. Similarly, in studies focusing on oral health, Ja and Hannig (2020) observed that even a brief 5-second exposure to commercial vinegar was sufficient to physically disrupt 24-h mature biofilms and significantly reduce bacterial viability. However, the literature also presents a different perspective, highlighting the significant resilience of mature biofilms against organic acids and natural extracts, which aligns more closely with our observations of partial disruption. In a study investigating the effects of the flavonoid morin on *Listeria* biofilms, Sivaranjani et al. (2016) reported that while the compound highly inhibited biofilm formation, it remained limited in disrupting preformed (mature) biofilms at sub-MIC and MIC levels. Similarly, Sakarikou et al. (2020) reported that, although plant-derived extracts exhibited strong antimicrobial activity against planktonic cells, they were considerably less effective in disrupting the structural integrity of 24-48 h mature biofilms. The authors emphasized that the mature biofilm matrix (EPS) acts as a protective barrier against chemical agents, resulting in only a limited reduction in biofilm biomass, typically in the range of 20-40%.

As previously noted, vinegar inhibited biofilm formation in a dose-dependent manner. For *L. monocytogenes*, MIC concentrations of AV, GV, and HV reduced biofilm formation by 31.5%, 23.7%, and 26.9%, respectively, with partial inhibition observed at 1/2 and 1/4 MIC. Against *P. aeruginosa*, MIC concentrations of AV, GV, and HV achieved 74.81±1.30%, 71.69±0.60%, and 67.30±1.10% inhibition, whereas 1/4 MIC was ineffective. These results contrast with the mature biofilm disruption data. The observed differences between mature biofilm disruption and formation inhibition are expected, as mature biofilms possess a dense EPS matrix and coordinated quorum-sensing networks that confer high tolerance to antimicrobial agents. Specifically, while AV exhibited the highest efficacy in inhibiting initial biofilm formation, mature biofilm assays revealed a shift in relative effectiveness, where HV demonstrated greater disruption against *L. monocytogenes* biofilms and GV showed comparatively higher activity against *P. aeruginosa* biofilms than AV. Notably, HV showed only limited disruption of mature P. aeruginosa biofilms despite its pronounced inhibitory effect on biofilm formation. This difference may reflect the distinct mechanisms involved in preventing biofilm development and disrupting an established biofilm. While HV may effectively interfere with bacterial growth or the early stages of biofilm formation, its active constituents may have limited ability to penetrate or destabilize the structured EPS matrix of mature *P. aeruginosa* biofilms. Thus, the effectiveness of a vinegar in preventing biofilm formation may not necessarily correspond to its ability to disrupt established biofilms.

Overall, these findings demonstrate that vinegar exhibits significant antibiofilm potential, both by preventing biofilm formation at MIC and sub-MIC levels and by partially disrupting mature biofilms under extended exposure, with efficacy influenced by bacterial species, vinegar type, concentration, and contact time.

Table 4. Mature biofilm disruption (%) of *L. monocytogenes* ATCC 7644 and *P. aeruginosa* ATCC 35032 after treatment with different types and concentrations of vinegar for 30, 60, and 120 minutes

Vinegar	Concentration	Mature Biofilm Disruption (%)					
		L. monocytogenes ATCC 7644			*P. aeruginosa* ATCC 35032		
		30 min	60 min	120 min	30 min	60 min	120 min
AV	1	1.42±0.08^{c}	7.84±1.03^{b}	12.85±0.99^{et}	nd	9.94±1.28^{a}	30.61±2.79^{b}
	1/2	nd	5.32±0.28^{c}	11.12±1.09^{f}	nd	nd	26.74±1.96^{c}
	1/4	nd	nd	10.70±0.47^{f}	nd	nd	19.21±1.09^{d}
	1/8	nd	nd	nd	nd	nd	nd
	1/16	nd	nd	nd	nd	nd	nd
GV	1	1.90±0.10^{b}	8.75±1.22^{b}	28.98±3.47^{b}	nd	6.81±0.85^{b}	33.31±1.76^{a}
	1/2	nd	4.12±0.74^{c}	21.76±2.88^{c}	nd	nd	20.54±1.22^{d}
	1/4	nd	nd	15.90±2.71^{de}	nd	nd	13.41±0.74^{e}
	1/8	nd	nd	4.03±0.07^{g}	nd	nd	5.89±0.52^{f}
	1/16	nd	nd	3.60±0.05^{g}	nd	nd	nd
HV	1	5.40±0.32^{a}	10.98±1.12^{a}	35.60±2.63^{a}	nd	nd	5.04±0.47^{f}
	1/2	2.10±0.07^{b}	7.43±0.67^{b}	22.10±2.97^{c}	nd	nd	1.48±0.08^{g}
	1/4	nd	nd	17.90±1.41^{d}	nd	nd	nd
	1/8	nd	nd	nd	nd	nd	nd
	1/16	nd	nd	nd	nd	nd	nd

AV: Apple vinegar, GV: Grape vinegar, HV: Honey vinegar, Results are expressed as mean±standard deviation, n=3. Different lowercase letters in the same column indicate statistically significant differences (P<0.05).

CONCLUSION

This study provides a comparative evaluation of the physicochemical characteristics and functional properties of organic apple, grape, and honey vinegars, with particular emphasis on their antioxidant, antibacterial, and antibiofilm activities. Despite their comparable total acidity, the vinegars differed considerably in pH, soluble solids, color characteristics, and phenolic content, demonstrating the compositional diversity associated with their different raw materials. GV and HV exhibited similar and higher total phenolic contents than AV; however, this pattern was not consistently reflected in antioxidant activity. AV and HV showed higher DPPH and FRAP activities than GV, whereas AV exhibited the highest ABTS activity, indicating that antioxidant capacity cannot be explained by total phenolic content alone. Differences among the vinegars were also evident in their antibacterial properties, with AV and HV showing lower MIC and MBC values than GV against *Listeria monocytogenes* ATCC 7644 and *Pseudomonas aeruginosa* ATCC 35032. All three vinegars inhibited biofilm formation in a concentration- and species-dependent manner, although their effectiveness varied according to vinegar type and test microorganism. Biofilm inhibition was more pronounced against *P. aeruginosa* ATCC 35032, whereas *L. monocytogenes* ATCC 7644 showed greater resistance. In contrast to the inhibition of biofilm formation, disruption of established biofilms remained limited for all vinegar types, highlighting the greater resistance of mature biofilms to vinegar treatments. Overall, the findings reveal distinct functional properties among the vinegar types, with variations in antioxidant, antibacterial, and antibiofilm activities. The results also provide new information on the biological properties of the relatively underexplored HV alongside conventional apple and grape vinegars. Further studies in real food systems are needed to assess the potential

use of these vinegars as natural antimicrobial and biofilm-control agents in food safety and preservation.

AUTHOR CONTRIBUTIONS

Seda ALTUNTAS: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing - original draft; Aycan YIGIT CINAR: Conceptualization, Methodology, Writing - review & editing. All authors read and approved of the final manuscript.

ETHICAL STATEMENT

This study does not require ethics committee permission or any special permission.

CONFLICTS OF INTEREST

No conflict of interest or common interest has been declared by the authors.

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