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# *In vitro* antibacterial activity of honey against multidrug-resistant *Shigella sonnei*

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ABSTRACT

*Background and purpose:* The health benefits of honey as an oral therapeutic agent for the treatment of diarrhoea caused by *Shigella sonnei* depend on the ability of honey to withstand human gastrointestinal conditions. This study aimed to investigate whether honey could withstand and inhibit the growth of *Shigella sonnei* under such conditions.

*Materials and methods:* We initially evaluated the survival of *Shigella sonnei* in human simulated gastric conditions (SGC) and simulated intestinal conditions (SIC). This was followed by determination of the susceptibility of *Shigella sonnei* to Manuka and Talah honey under gastrointestinal conditions. The colony forming units (CFU) of *Shigella sonnei* and minimum inhibitory concentrations (MICs) of honey were calculated.

*Results: Shigella sonnei* was unable to survive in the acidic environment of the stomach without food matrix and survived only when inoculated with a food source, resulting in $1.5 \times 10^5 \pm 0.2$ CFU at 60 min and $1.7 \times 10^5 \pm 0.3$ CFU after 120 min of incubation. In SIC, it survived both with and without food matrix at the same CFU ($1.2 \times 10^7 \pm 0.4$) at 60 min and $1.7 \times 10^7 \pm 0.2$ CFU after 120 min of incubation. Growth of *Shigella sonnei* was not observed in SGC in the presence of either honey at different concentrations without a food source. In the presence of a food source, Manuka honey inhibited the growth of *Shigella sonnei* at 10% v/v and Talah honey at 20% v/v dilutions in SGC. In SIC, Manuka honey inhibited the growth of *Shigella sonnei* at 15% and 20% v/v dilutions, whereas Talah honey inhibited *Shigella sonnei* at 20% and 25% v/v dilutions without and with food sources, respectively.

*Conclusion: Shigella sonnei* can survive in the acidic environment of the stomach if inoculated with a food source. Acidic pH and pepsin had no deleterious effects on the antibacterial capability of honey. However, bile reduced the antibacterial activity of honey in the intestinal environment.

## 1. Introduction

Shigellosis is a major global human health problem [1]. It is one of the main causes of diarrhoeal diseases and associated deaths. Shigellosis is caused by bacteria that infect epithelial cells and multiply there, causing ulcers, inflammation, and haemorrhage. These bacteria are transmitted through contaminated food and water from one person to another [2]. *Shigella sonnei* ranks third after Salmonella and Campylobacter among bacteria associated with food-related infections [3]. *Shigella sonnei* is transmitted rapidly through the faecal-oral route in areas of poor hygienic where access to clean water and food is limited. As few as ten bacteria are sufficient to cause shigellosis [2]. Multidrug-resistant (MDR) strains of *Shigella sonnei* are rapidly expanding because of their potential for growth and their ability to transmit exogenous genes linked to mobile genetic components such as transposons, R-plasmids, integrons and genomic islands associated with bacterial chromosomes [4]. Despite improvements in the provision of safe drinking water and food hygiene, shigellosis due to *Shigella sonnei* is

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still endemic in Saudi Arabia [5]. Honey provides various advantages over conventional antibiotics in the treatment of *Shigella sonnei* because antibacterial resistance to honey is unlikely and has not been reported in the literature [6]. This remarkable property of honey could be related to the fact that honey contains multiple antibacterial substances, such as hydrogen peroxide, high osmolarity, acidic pH and plant-derived flavonoids and phenolic substances. These substances have synergistic antibacterial effects on multiple targets in pathogenic bacteria. Honey also contains prebiotics, probiotics and zinc in addition to multiple synergistic antibacterial components [7]. In contrast to antibiotics, when it is consumed orally, honey targets only pathogenic microorganisms without disrupting the development of beneficial gut flora [8]. Moreover, it enhances the growth of normal flora (beneficial bacteria) in the gastrointestinal tract [9]. The antibacterial activity of honey against enteric pathogens, including *Shigella sonnei* has been reported by several researchers [10–12]. Previous studies were performed *in vitro* using agar dilution assays or broth dilution assays and provided good evidence of the effectiveness of honey against gastrointestinal pathogens, including *Shigella sonnei*. However, in these previous studies, the impact of the human gastrointestinal environment on the effect of orally administered honey against *Shigella sonnei* was not addressed, although the human gastrointestinal environment is known to play a substantial role in the ultimate bioavailability of natural products [13]. Therefore, the health benefits of honey as an oral therapeutic agent for the treatment of infectious diarrhoea depend on the ability of honey to withstand human gastrointestinal conditions. The main factors in the human gastrointestinal tract that can affect the antibacterial potential of honey and bacterial viability are the acidic environment of the stomach (pH between 1 and 2), the presence of bile in the duodenum, and the alkaline pH (between 5.1 and 7.5) and enzymes in the intestine [14]. The presence of pepsin and hydrochloric acid in the stomach can cause enzyme- or acid-induced hydrolysis of orally administered honey, resulting in chemical modification and inactivation [15]. Similarly, alkaline pH and bile can alter the chemistry or concentrations of bioactive antibacterial compounds in honey. The main antibacterial characteristics of honey that could be modified or degraded in the gastrointestinal environment are pH, hydrogen peroxide content, osmolarity, flavonoid content and phenolic acid content. Moreover, the presence of food in the gastrointestinal tract could influence the antibacterial activity of honey against the tested pathogens. Therefore, the objective of this study was to examine the effect of honey against *Shigella sonnei* in the context of gastrointestinal factors. In addition, we determined the potential role of food in relation to the antibacterial activity of honey and the survival of *Shigella sonnei*.

## 2. Materials and methods

### 2.1. Sample collection

Seven samples of local honey produced by *Apis mellifera jemenitica* were collected directly from beekeepers from different areas of Saudi Arabia, as shown in Table 1. *A. mellifera jemenitica* is a local bee that can survive in dry and hot conditions [16]. The botanical origin of the honey samples was identified based on geographical areas, blossoming plants, season, and the colour and aroma of each honey sample according to the methods adopted by previous studies [17,18]. Medical-grade Manuka (UMF18+) honey was purchased from a pharmacy in Jeddah and used for comparison.

### 2.2. Sterility of honey

The honey samples were examined for the presence of pathogenic microbial contaminants. One gram of each honey sample was diluted in 10 ml of sterile distilled water. A loopful of diluted honey was inoculated on blood agar and nutrient agar plates, which were incubated for 18 h at 37 °C [19]. The agar plates were examined for any growth.

### 2.3. Simulated honey

To assess the function of sugar with respect to the antibacterial activity of honey, simulated honey was prepared by dissolving 1.5 g of sucrose, 7.5 g of maltose, 40.5 g of fructose and 33.5 g of glucose in 17 ml of distilled water as described by French et al. (2005) [20].

### 2.4. Bacterial strain

Stool culture isolates of *Shigella sonnei* were obtained from King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The pathogen was isolated and identified from a stool specimen of a 26-year-old male patient who presented with a three-day history of diarrhoea and fever. Re-identification of the isolate was performed via colony morphology, culture characteristics and biochemical profiling. Final identification of the organism was performed by 16S rRNA sequencing using GN ID cards and a macrogen system. The isolate was kept at −80 °C in BHI broth (Difco) containing 16% glycerol. A sterile stick was utilized to transfer the culture to a glass tube containing 10 ml of BHI broth one day before the experiment. The culture was incubated for 4 h at 37 °C, transferred to fresh BHI broth and incubated at 37 °C without agitation for approximately 18 h. Ground beef test samples were inoculated with the resulting cultures at the selected dilutions.

### 2.5. Susceptibility testing

In compliance with the protocols of the Clinical and Laboratory Standards Institute, the Kirby-Bauer disc diffusion method was used to determine the antimicrobial profile of *Shigella sonnei* (Oxoid, Basingstoke, UK) [21]. The following antibiotics were used: amikacin (AK) (30 μg), gentamicin (GM) (10 μg), cefepime (CPM) (30 μg), ticarcillin (TC) (75 μg), piperacillin (PRL) (100 μg), imipenem (IMI) (10 μg), norfloxacin (NOR) (10 μg), tobramycin (TM) (10 μg), cephalothin (CEF) (30 μg), cefoxitin (FOX) (30 μg), ciprofloxacin (CIP) (5 μg) and cefotaxime (CTX) (30 μg). For antibacterial susceptibility testing, *E. coli* (ATCC 25922) was used as a control strain.

**Table 1**
Floral source, geographical location and harvesting season of unifloral Saudi honeys.

| S. No | No. of samples | Floral Source | Common name | Botanical name | Geographical location | Harvesting season |
|---|---|---|---|---|---|---|
| 1 | Manuka | Manuka | Manuka | *Leptospermum* | New Zealand | 2017 |
| 2 | SI - 1 | Sidr | Sidr | *Ziziphus spina-christi* | Rotht Krame Riyadh | October 2017 |
| 3 | AC - 1 | Talah | Talah | *Acacia origina* | Hail | April 2017 |
| 4 | SA - 1 | Saffey | Saffey | *Bassia scoparia* | Rotht Krame Riyadh | April 2017 |
| 5 | BA - 1 | Multi-flower | Al- Bahha | *Multi-flowers* | Al- Bahha | April 2017 |
| 6 | SI - 2 | Sidr | Sidr | *Ziziphus spina-christi* | South of Saudi Arabia | October 2017 |
| 7 | ACT - 1 | Sumra | Sumra | *Acacia tortilis* | South of Saudi Arabia | April 2017 |
| 8 | AC - 2 | Talah | Talah | *Acacia origena* | Abha | April 2017 |

SI, Sidr; AC, Acacia; SA, Saffey; BA, Bahha; ACT, *Acacia tortilis*.

### 2.6. Agar well diffusion assay

A primary honey solution was made by adding 2 g of thoroughly mixed honey and 2 ml of sterile water to standard bottles, which were placed at 37 °C for half an hour and stirred intermittently to maintain mixing. A 50% (w/v) solution of each prepared honey sample was diluted to 25% (w/v) by adding 1 ml of each of the primary solutions to either 1 ml of sterile water or 1 ml of catalase solution to obtain secondary solutions. Catalase (C1345-10G 2950 units/mg, Sigma) solution was made by adding 20 mg of catalase to 10 ml of sterile distilled water [18]. The antibacterial activity of the honey samples was determined by agar well diffusion assay as described by Hussain et al. (2015) [8]. For this purpose, seven local honey samples and one medical-grade honey sample, namely, Manuka honey (UMF-18+), were evaluated against *Shigella sonnei*. Briefly, overnight cultures of target bacteria were used to inoculate ($10^7$ CFU) Muller Hinton (MH) agar plates (20 ml/plate). Next, under laminar air flow, the inoculated agar plates were punched with a sterile agar drill to make 9 mm holes. Thereafter, one hundred 40 μL of honey with 50 and 25% dilutions in catalase solution and in sterile water was transferred into each allotted well. Equal volumes of sterilized water and catalase solution were used as negative controls, and 6% phenol was used as a positive control. Finally, the plates were incubated for 17 h at 36 °C ± 1 °C under aerobic conditions. After overnight incubation, the plates were examined for the presence of inhibition zones, and each zone was measured in mm using callipers. Tests were carried out in duplicate on the same day.

### 2.7. Microbroth dilution method

A microbroth dilution method was performed in 96-well microtiter plates (Merck KGaA, Germany) to assess the minimum inhibitory concentrations (MICs) of honey against *Shigella sonnei*. Fifty percent (v/v) honey stock solutions were prepared by adding 13.7 g of honey to MH broth with and without catalase in a volume of 20 ml. It is difficult to pipette honey because of its high viscosity; therefore, the honey was weighed, and its density was considered to be 1.37 g/ml [22]. In the 96-well microtiter plate, twenty-five incremental dilutions (1%–25% v/v) were set up by adding a determined quantity of honey from the 50% (v/v) stock solution and a determined amount of bacterial suspension ($5 \times 10^5$ CFU/mL); each well contained a final volume of 200 μl. In most previous studies, two-fold dilutions of honey were used in a microbroth dilution assay for MIC determination [23,24]. Nonetheless, we used incremental dilutions of 1% to obtain more precise honey inhibitory concentrations [18]. However, for simulated honey, 5% incremental dilutions were prepared, ranging from 5% to 40%. To attain a turbidity corresponding to 0.5 McFarland ($1 \times 10^8$ CFU/ml), five discrete colonies were selected from overnight blood agar plates and inoculated into nutrient broth. Additional dilutions of bacterial suspension with final inoculum concentrations from 5 to $10^5$ CFU/ml were obtained in MH broth. Negative control wells contained MH broth, and positive control wells contained bacterial suspension in MH broth. The microtiter plates were aerobically incubated at 37 °C for 16 h in a stationary incubator and visually analysed for the presence or absence of growth compared to the negative and positive controls. The MICs were determined as the lowest honey concentration that completely prevented visible bacterial growth after overnight incubation. The tests were performed on the same day using three similar wells in triplicate.

### 2.8. Survival of Shigella sonnei in a simulated gastrointestinal environment

The simulated gastrointestinal environment was developed according to the method described by de Melo et al. (2017) [25]. Briefly, simulated gastric fluid (SGF) was prepared in buffered peptone water (BPW). Using one molar hydrochloric acid, the pH was adjusted to 2.5–3.0. Pepsin (Sigma, Madrid, Spain) solution was sterilized by filtering through a 0.22 μm membrane filter and added to BPW to final concentrations of 1000 $U/ml^{-1}$. Simulated intestinal fluid (SIF) was prepared by adding 0.3% (w/v) bile salts, and the pH was readjusted to 7 with sodium hydroxide in BPW.

### 2.9. Bacteria and culture conditions

Bacteria were harvested from a blood agar plate and resuspended in phosphate-buffered saline (PBS) (pH 7.4). The bacterial suspension was added to four different tubes containing 1 ml of SGF or SIF each to achieve a final bacterial concentration of $10^7$ CFU and $10^2$ CFU (representing the infective dose of *Shigella sonnei*) and incubated at 37 °C in a shaking incubator. Samples were taken at 60 min (to represent early gastric and intestinal emptying) and 120 min (to represent late gastric and intestinal emptying). Viable cell counts were calculated by the preparation of serial decimal dilutions in 0.1% (w/v) peptone water (Merck KGaA, Germany); the dilutions were then plated (in duplicate) on MacConkey lactose agar (Oxoid) [26].

### 2.10. Survival and susceptibility of Shigella sonnei to honey under simulated gastrointestinal conditions without a food source

The susceptibility of *Shigella sonnei* to 5% incremental dilutions (ranging from 5% to 25%) of Manuka and Talah honey was evaluated under the same conditions, initially without food matrix. All assays were performed in duplicate on the same day. BPW containing pepsin (1000 U/ml) at pH 7, BPW at pH 7 and bile salts at pH 7 were used as controls. The bacterial concentration in the controls was adjusted to that of the simulation solutions.

### 2.11. Survival and susceptibility of Shigella sonnei to honey under simulated gastrointestinal conditions with a food source

The survival and susceptibility of *Shigella sonnei* to honey under simulated gastrointestinal conditions with a food source were determined according to methods described by de Melo et al. (2017) [25]. Ground beef (5–9% fat) was purchased from a local supermarket. For sterility, the beef was autoclaved, vacuum sealed and stored at −20 °C. Before experimentation, the frozen sterile beef was warmed at 37 °C. The ground beef (0.1 g) was inoculated with 10 μl of overnight *Shigella sonnei* culture, diluted to $10^{-1}$ (approximately $10^6$ CFU) in PBS. The bacteria on the beef surface were allowed to dry at room temperature for 10 min. The inoculated beef was placed in 10 ml of SGF and incubated in a 20 ml plastic tube (Oxoid) at 37 °C with gentle shaking (100 rpm). The SGF was decanted from the fragments of beef after incubation, and the pH was measured. After 60 min and 120 min of incubation, surviving bacteria were retrieved by extracting the ground beef with 10 ml of PBS under intense vortexing. The counts of CFU/ml were determined by preparing serial decimal dilutions in 0.1% (w/v) peptone water that were then plated (in duplicate) on MacConkey agar. The bacteria that survived in SGF were further evaluated in SIF for their survival under simulated intestinal conditions (SIC). The susceptibility of *Shigella sonnei* inoculated on ground beef to Manuka and Talah honey was also determined under the same conditions described above. Five percent incremental dilutions (ranging from 5% to 25%) of Manuka and Talah honey were used, and the test was performed in duplicate on the same day. Under identical conditions, an uninoculated ground beef control was placed in acidified LB and examined for the presence of any contaminating bacteria.

## 3. Statistical analysis

The IBM Statistical Package for Social Sciences software (SPSS 19.0) was used to analyse the data. The mean values of the MICs and the inhibition zone of each honey sample were calculated. The Kruskal-Wallis test was applied to determine differences among the mean MIC values of

**Table 2**
Zone of inhibition (mm) of Saudi honey samples at 50% and 25% (w/v) dilutions in sterile distilled water and 50% and 25% (w/v) dilutions in catalase solution by agar well diffusion assay against *Shigella sonnei*.

| S. No | Honey samples | Zone of inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | 50% in water | 25% in water | 50% in catalase | 25% in catalase |
| 1 | Manuka | 14 ± 0.57[a] | 10 ± 0.28 | 11± 0.57 | 9 ± 0.28 |
| 2 | Sidr (SI-1) | 10 ± 0.57 | NI | NI | NI |
| 3 | Talah (AC-1) | 9.3 ± 0.25 | NI | NI | NI |
| 4 | Saffey (SA-1) | 10 ± 0.3 | NI | NI | NI |
| 5 | Multi-flower (BA-1) | NI | NI | NI | NI |
| 6 | Sidr (SI -2) | NI | NI | NI | NI |
| 7 | Sumra (ACT-1) | NI | NI | NI | NI |
| 8 | Talah (AC -2) | 17.5 ± 0.5 | 12 ± 0.57 | NI | NI |

[a] The values represent the mean ± standard deviation of three replicates; NI, no inhibition.

the tested honey samples. The results were considered significant at $p < 0.05$.

## 4. Results

### 4.1. Resistance profile of Shigella sonnei

The *Shigella sonnei* strain used in this study showed resistance to tobramycin, cephalothin, cefoxitin, and ciprofloxacin. Therefore, it is considered multidrug resistant. However, the strain was sensitive to amikacin, gentamicin, cefepime, ticarcillin, piperacillin, imipenem, norfloxacin and cefotaxime.

### 4.2. Screening antibacterial activity of honey by agar well diffusion assay

Sterility testing of honey resulted in no growth in blood agar medium. The results obtained through the screening assay (agar well diffusion assay) showed much variation in the sizes of the zones of inhibition of the tested honey samples against *Shigella sonnei* (Table 2). The difference in the size of the mean inhibition zone among different honey samples against *Shigella sonnei* was also statistically significant (Kruskal-Wallis test, $p = 0.002$). This means that there was a significant difference in the level of antibacterial activity of indigenous honey against the tested pathogens. The antibacterial activity of the tested honey ranged from 0 to 17.5 ± 0.5 mm (Table 2). Multi-flower honeys collected from the Al-Bahha region (BA-1) and Sidr (SI-2) and Sumra (ACT-1) collected from the southern region of Saudi Arabia did not produce any zone of inhibition at all tested dilutions, indicating that these honey samples did not have antibacterial activity against *Shigella sonnei*.

Talah (AC-2) honey collected in Abha produced the largest zone of inhibition compared to the other local honey samples as well as Manuka honey. This result reveals that Talah (AC-2) honey has even higher total or hydrogen peroxide-related antibacterial activity than Manuka honey. Nonetheless, in catalase solution, none of the indigenous honey samples displayed antibacterial activity. However, in sterile distilled water, at a dilution of 50% (w/v) four indigenous honey samples showed antibacterial activity and one demonstrated antibacterial activity at a dilution of 25% (w/v) (Table 2). Manuka honey exhibited an inhibition zone (antibacterial activity) against *Shigella sonnei* at all tested dilutions in both water and catalase solution.

### 4.3. Minimum inhibitory concentrations of honey

The antibacterial activity of Talah honey (AC-2), which showed a larger zone of inhibition against *Shigella sonnei* than the other tested honeys, including Manuka honey, was further evaluated by a microdilution assay, which is a more sensitive method. The MICs of Talah, Manuka and artificial honey against *Shigella sonnei* were determined by this assay. Manuka honey inhibited the growth of *Shigella sonnei* at a mean of 9 ± 0.57 (v/v %), whereas Talah and artificial honey inhibited the growth of *Shigella sonnei* at 20 ± 0.5 (v/v %) and 30 ± 0.0 (v/v %), respectively, without catalase. These values represent the overall or combined antibacterial activity of the tested honey samples against *Shigella sonnei*, including all factors present in the honey samples. The addition of catalase to the honey samples reduced the antibacterial activity of both Talah honey (22.3 ± 0.5) and Manuka honey (11.6 ± 0.5). The antibacterial activity of artificial honey remained the same after the addition of catalase. There was also a statistically significant difference (Kruskal-Wallis test, $p = 0.023$) among the mean MICs of the tested honeys against *Shigella sonnei*.

### 4.4. Survival of Shigella sonnei under simulated gastrointestinal conditions

The survival of *Shigella sonnei* under simulated gastrointestinal conditions with and without a food source is illustrated in Table 3. This study has shown that when inoculated on ground beef, *Shigella sonnei* was able to survive at pH 2.5–3.0, although these isolates could not survive without food when tested under the same conditions. The pH of the GSF did not change after the inoculation of ground beef. This demonstrates that food sources have some protective role against acidic pH.

The study also demonstrated that bacteria did not survive in the gastric environment at $1 \times 10^2$ CFU, even in the presence of food. This indicates that the survival of *Shigella sonnei* also depends on inoculum size. *Shigella sonnei* survived in ground beef when inoculated at a concentration of $1 \times 10^7$. The bacteria survived in both simulated gastric conditions (SGC) and simulated intestinal conditions (SIC) with high CFU counts when inoculated in food matrix (Table 3). Under identical conditions at pH 2.5, a control ground beef sample that was not inoculated was tested and did not show any contaminating bacteria.

### 4.5. Susceptibility of Shigella sonnei to honey under gastrointestinal conditions

Bacterial growth was not observed in GSF at all tested concentrations of both Manuka and Talah honeys without food sources after 60 and 120 min of incubation (Table 4, Figs. 1 and 2). On the other hand, bacterial growth was observed in SIF in the presence of 5%–20% v/v dilutions of

**Table 3**
Survival of *Shigella sonnei* in simulated gastric conditions (SGC) and simulated intestinal conditions (SIC) with food and without food.

| Availability of food | Without food | | | | With food | | | |
|---|---|---|---|---|---|---|---|---|
| Type of Media | SGC | | SIC | | SGC | | SIC | |
| Colony forming units (CFU) of suspension | $1 \times 10^7$ | $1 \times 10^2$ | $1 \times 10^7$ | $1 \times 10^2$ | $1 \times 10^7$ | $1 \times 10^2$ | $1 \times 10^7$ | $1 \times 10^2$ |
| Colony forming units (CFU) at 60 min | 0 | 0 | $1.2 \times 10^7 \pm 0.4$ | $1.3 \times 10^2 \pm 0.3$ | $1.5 \times 10^5 \pm 0.2$ | 0 | $1.2 \times 10^7 \pm 0.4$ | $1.4 \times 10^2 \pm 0.3$ |
| Colony forming units (CFU) at 120 min | 0 | 0 | $1.7 \times 10^7 \pm 0.2$ | $1.7 \times 10^2 \pm 0.3$ | $1.7 \times 10^5 \pm 0.3$ | 0 | $1.7 \times 10^7 \pm 0.2$ | $1.9 \times 10^2 \pm 0.3$ |

SGC, simulated gastric conditions; SIC, simulated intestinal conditions.

**Table 4**
Susceptibility of *Shigella sonnei* to Manuka and Talah honey in simulated gastric conditions (SGC) and simulated intestinal conditions (SIC) without a food source.

| % age dilution of honey (v/v) | Manuka | | | Talah | | |
|---|---|---|---|---|---|---|
| | SGC | SIC | | SGC | SIC | |
| | 60 and 120 min | 60 min | 120 min | 60 and 120 min | 60 min | 120 min |
| 5 | 0 | $1.2 \times 10^4 \pm 0.2$ | $1 \times 10^3 \pm 0.3$ | 0 | $1.2 \times 10^5 \pm 0.4$ | $2.2 \times 10^5 \pm 0.3$ |
| 10 | 0 | $1.4 \times 10^2 \pm 0.4$ | $1.6 \times 10^2 \pm 0.1$ | 0 | $1.4 \times 10^3 \pm 0.3$ | $2.3 \times 10^3 \pm 0.6$ |
| 15 | 0 | 0 | 0 | 0 | $1.7 \times 10^2 \pm 0.2$ | $1.2 \times 10^2 \pm 0.4$ |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 |

SGC, simulated gastric conditions; SIC, simulated intestinal conditions.

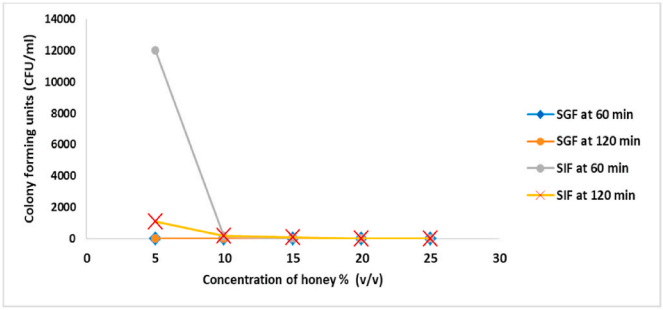


**Fig. 1.** Susceptibility of *Shigella sonnei* to Manuka honey in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) without a food source.

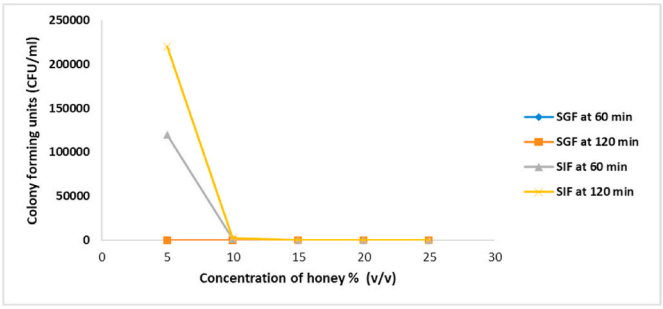


**Fig. 2.** Susceptibility of *Shigella sonnei* to Talah honey in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) without a food source.

Talah honey and 5%–10% v/v dilutions of Manuka honey. Manuka honey completely inhibited the growth of *Shigella sonnei* at a dilution of 15%, whereas Talah honey inhibited the growth of *Shigella sonnei* at a dilution of 25% in SIF without a food source. In comparison with the MIC results obtained in standard medium, Manuka honey inhibited the growth of *Shigella sonnei* at a dilution of $9 \pm 0.57$ (% v/v), and Talah honey inhibited the growth of *Shigella sonnei* at a dilution of $20 \pm 0.5$ (% v/v) without the addition of catalase. The addition of catalase to standard medium decreased the antibacterial potential of both honeys against *Shigella sonnei* because catalase neutralizes hydrogen peroxide, which is an important antibacterial factor in honey. There was a statistically significant difference between the MICs of Manuka honey and Talah honey against *Shigella sonnei* with and without catalase. There was also a significant difference between the MICs of the honey samples against *Shigella sonnei* in SGC and standard medium.

*Shigella sonnei* inoculated on ground beef survived in GSF with Manuka honey at a dilution of 5% v/v and Talah honey at dilutions up to 15% v/v after 60 and 120 min of incubation (Table 5, Figs. 3 and 4). However, the viability of the bacteria was lost in the presence of Manuka honey at a dilution of 10% and Talah honey at a dilution of 20% in the presence of acidic pH and pepsin. In comparison, the bacteria did not survive in SGF against Manuka and Talah honey in all tested dilutions without food sources. On the other hand, the bacteria survived in SIF with Manuka honey at a dilution of up to 15% and Talah honey at a dilution of up to 25%.

## 5. Discussion

MDR *Shigella sonnei* strains are quite common and have been reported in many parts of the world [4,27]. Multiple factors have contributed to the increase in the number of MDR isolates [28]. One study identified three large clonal groups of *Shigella sonnei*, which are widely distributed on five continents [29]. In countries neighbouring Saudi Arabia, the resistance of Shigella serogroups to commonly used antibiotics is also quite high and variable [30]. A study from Iran by Hosseini et al. (2007) showed that the resistance of Shigella species to tetracycline, trimethoprim-sulfamethoxazole, cephalexin and ampicillin is greater than 50% [31]. Another worrisome development is the emergence of carbapenemase-producing and pan-resistant Enterobacteriaceae in many parts of the world, including Saudi Arabia [32,33]. Honey offers the best possible alternative to treat these pathogens because it inhibits the growth of sensitive, MDR or pan-resistant pathogenic bacteria at almost the same concentration, unlike antibiotics, indicating that honey has a distinct mechanism of action [34]. Bacterial resistance to honey has not been documented thus far anywhere in the world [35]. Treatment of infectious diarrhoea with antibiotics disrupts the balance among beneficial members of the gut microbiota, increasing susceptibility to secondary infections, increasing the chance of developing allergic disorders, decreasing the efficacy outcomes of antibiotic therapies, and inducing and spreading antibiotic resistance [36]. Therefore, new strategies to replace or complement the use of antibiotic treatments are necessary.

### 5.1. Antibacterial activity of honey

Tremendous variation in the level of antibacterial activity against *Shigella sonnei* was observed in the tested honey samples by agar well diffusion assay (Table 2). This variation could be caused by differences in the honey bee flora, honey processing, soil composition and climatic conditions of the area from which the honey samples were collected [12,37]. Some previous studies have also shown the same pattern of variability in the antibacterial activity of different honey samples [38,39]. In the microbroth dilution assay, the addition of catalase to honey samples reduced the antibacterial activity of both Talah and Manuka honeys but did not affect the antibacterial activity of artificial honey. This means that hydrogen peroxide has an important role in the total antibacterial activity of both Manuka and Talah honeys, whereas the artificial honey did not possess hydrogen peroxide-related antibacterial activity. Moreover, both Talah and Manuka honeys inhibited *Shigella sonnei* at lower concentrations than the artificial honey samples even after the addition of catalase, meaning that both Talah and Manuka honeys contain non-peroxide factors in addition to hydrogen peroxide. The results show that the antibacterial activity of honey not only is related to high osmolarity but also originates from hydrogen peroxide and non-peroxide

**Table 5**
Susceptibility of *Shigella sonnei* to Manuka and Talah honey under simulated gastric conditions (SGC) and simulated intestinal conditions (SIC) with a food source.

| % age dilution of honey (v/v) | Manuka | | | | Talah | | | |
|---|---|---|---|---|---|---|---|---|
| | SGC | | SIC | | SGC | | SIC | |
| | 60 min | 120 min | 60 min | 120 min | 60 min | 120 min | 60 min | 120 min |
| 5 | $1.2 \times 10^3 \pm 0.2$ | $1.5 \times 10^3 \pm 0.2$ | $1.9 \times 10^3 \pm 0.2$ | $1 \times 10^3 \pm 0.3$ | $1.2 \times 10^6 \pm 0.4$ | $1.6 \times 10^5 \pm 0.3$ | $1.3 \times 10^5 \pm 0.4$ | $1.2 \times 10^5 \pm 0.3$ |
| 10 | 0 | 0 | $1.3 \times 10^2 \pm 0.2$ | $1.6 \times 10^2 \pm 0.4$ | $1.5 \times 10^4 \pm 0.5$ | $1.3 \times 10^3 \pm 0.5$ | $1.6 \times 10^3 \pm 0.3$ | $1.3 \times 10^3 \pm 0.6$ |
| 15 | 0 | 0 | $70 \pm 13$ | $65 \pm 15$ | $1.9 \times 10^2 \pm 0.5$ | $90 \pm 20$ | $1.9 \times 10^2 \pm 0.2$ | $1.7 \times 10^2 \pm 0.4$ |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | $35 \pm 10$ | $40 \pm 15$ |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

SGC, simulated gastric conditions; SIC, simulated intestinal conditions.

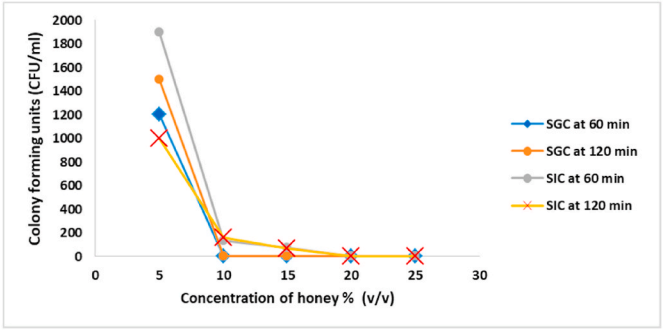


**Fig. 3.** Susceptibility of *Shigella sonnei* to Manuka honey under simulated gastric conditions (SGC) and simulated intestinal conditions (SIC) with a food source.

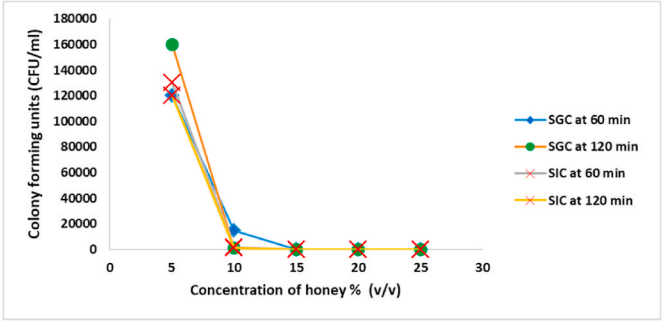


**Fig. 4.** Susceptibility of *Shigella sonnei* to Talah honey under simulated gastric conditions (SGC) and simulated intestinal conditions (SIC) with a food source.

factors. These results are in accordance with previous studies [18,37,38]. However, interestingly, in the agar well diffusion assay, none of the local honey samples displayed antibacterial activity in catalase solution (Table 2), whereas Talah honey showed non-peroxide activity in the microdilution method. This demonstrates that the agar well diffusion assay is a less sensitive technique and could not be used to detect non-peroxide antibacterial activity in Talah honey. The reason could be low concentrations of non-peroxide factors or the presence of larger antibacterial substances present in Talah honey that were unable to diffuse in the agar assay. It is important to identify plant-derived antibacterial substances in Talah honey in future studies.

There are over 300 floral species associated with bees in Saudi Arabia, including shrubs, herbs, vines and trees. Nevertheless, the most common bee flora in the Al-Baha, Taif, and Aseer regions are Sidr (*Ziziphus spina-christi*), Sumra (*Acacia tortilis*), Talah (*Acacia origena*), Lavendula and Dahiana (*Acacia asak*) [40]. Ziziphus and Acacia are both heat- and drought-tolerant species located mainly in tropical and subtropical regions of Saudi Arabia [41]. Sidr honey is dark brown in colour and, due to its unique fragrance and taste, is the most popular and expensive honey in Saudi Arabia. Because of its medicinal and nutritional properties, honey derived from Acacia species is widely consumed. Talah honey is pale yellow, and Sumra honey is dark brown [42]. In Saudi Arabia, honey is not only used as a popular food but also consumed as a therapeutic agent for the treatment of a number of diseases, including diarrhoea, because its healing properties are mentioned in the sacred book of Muslims, the Holy Quran, and in Prophetic Medicine [43].

### 5.2. Survival of Shigella sonnei under simulated gastrointestinal conditions

*Shigella sonnei* was able to survive in the acidic environment of the stomach when inoculated on ground beef, although these isolates could not survive without food when tested under the same conditions. The protective role of certain foods has also been shown in previous studies [44,45]. One study has demonstrated that food containing high protein content may protect bacteria against the killing effects of gastric acid [46]. Recently, it has been demonstrated that the glutamate-dependent acid resistance (GDAR) pathway is an effective acid resistance system in both pathogenic and commensal bacteria [47]. This pathway requires the activity of the PLP-dependent enzyme glutamate decarboxylase (GadB) and a cognate antiporter (GadC) [48]. Both HdeA and HdeB are essential chaperone systems that work in the special periplasmic space of enteric gram-negative bacteria and are responsible for acid resistance [49]. In many pathogenic bacteria, including *Shigella* species, the existence and expression of the GadB and GadC genes has been strongly linked to bacterial survival under highly acidic conditions, such as those found in phagosomes and the human stomach [50]. Beneficial gut flora, such as lactic acid bacteria, also have a similar mechanism of acid resistance and are currently being used as probiotics in a number of gastrointestinal disorders, including infectious diarrhoea [51,52]. Recently, we isolated lactic acid bacteria from five fresh honey samples collected from the Baha and Aseer regions of Saudi Arabia (under publication). These beneficial bacteria are believed to originate from the bee gut [53].

Interestingly, *Shigella sonnei* did not survive in the gastric environment at $1 \times 10^2$ CFU, even in the presence of food. This indicates that the survival of *Shigella sonnei* also depends on inoculum size. *Shigella sonnei* survived on ground beef when inoculated at a concentration of $1 \times 10^7$ CFU. The infective dose (ID) of various enteric pathogens is consistent with their relative ability to resist acid killing. The IDs of *Shigella flexneri*, non-typhi *Salmonella* species and *Vibrio cholerae* are approximately $10^2$, $10^5$ and $10^9$, respectively [54]. *Shigella sonnei* survived at alkaline pH in the presence of bile salts under SIC without any food matrix.

### 5.3. Susceptibility of Shigella sonnei to honey in gastrointestinal conditions

Both Manuka and Talah honeys inhibited the growth of *Shigella sonnei* in the gastrointestinal environment (Tables 4 and 5, Figs. 1–4), as they did in the microbroth dilution assay. This means that honey given orally can effectively inhibit the growth of *Shigella sonnei* in infectious

diarrhoea. However, the percentage of honey required to completely inhibit the growth of *Shigella sonnei* in the gastrointestinal environment was variable and depended on multiple factors, such as the pH, presence of food, inoculum size, contact duration of honey with *Shigella sonnei*, and bile. The most important factor is pH, and *Shigella sonnei* did not survive in the acidic environment of the stomach; however, it survived and grew when inoculated on ground beef. Manuka honey at a dilution of 10% v/v inhibited the growth of *Shigella sonnei* inoculated on ground beef in a stomach environment. The percentage of Manuka honey required to inhibit the growth of *Shigella sonnei* in the acidic environment of the stomach was almost the same as that observed in standard media (9 ± 0.57% v/v). However, the concentration of Manuka honey needed to inhibit *Shigella sonnei* was lower in the intestinal environment (15% v/v) without a food source than that with a food source (20% v/v) (Tables 4 and 5, Figs. 1–4). The reason for the lower MIC of Manuka honey in the intestinal environment may be related to the deleterious effect of bile and ground beef on the antibacterial substances in Manuka honey. Previous studies have shown that the concentration of methylglyoxal, which is the main contributor of non-peroxide antibacterial activity in Manuka honey, is reduced in the intestinal environment. However, other markers of Manuka honey, including leptosin and methyl syringate, were not altered under the same conditions [55,56]. One previous study also revealed that the antibacterial activity of Manuka honey was reduced up to 50% under the alkaline conditions (pH 7.5) of the intestine in the presence of bile and pancreatin [57]. This study, as well as the abovementioned studies, suggests that bile in the intestine may negatively affect antibacterial activity if Manuka honey is orally administered for the treatment of infectious diarrhoea. Similarly, our results have also shown that the antibacterial activity of Talah honey against *Shigella sonnei* is reduced in the intestinal environment. However, the percentage inhibition is less than that of Manuka honey.

Whether it is possible to achieve and maintain such honey concentrations (20% for Manuka and 25% for Talah with food) *in vivo* for approximately one to 2 h remains an important question that needs further research. Honey given orally may be diluted by saliva, food and gastrointestinal secretions; therefore, these factors must be kept in mind when administering honey orally. However, this study has shown that hydrochloric acid and pepsin had no deleterious effects on the antibacterial capability of honey; rather, the acidic pH of the stomach inhibits pathogenic bacteria, which in fact augments the antibacterial activity of honey. However, bile reduced the antibacterial activity of both Manuka and Talah honeys in the intestinal environment.

A lack of acidity in the stomach due to certain diseases or acid neutralizing drugs used for acid peptic disease can increase the rate of enteric infections [58]. Similarly, the use of antibiotics inhibits the growth of gut commensals, resulting in an increased risk of disease, secondary infections and spread of drug-resistant pathogens [36]. Therefore, it is important to develop therapies that replace or complement antibiotic use and aim to selectively target pathogenic bacteria without disturbing the growth of gut microbiota. In this context, honey could be more effective *in vivo* than *in vitro* because fresh honey contains a number of beneficial bacteria, such as lactic acid and bifidobacteria, known as probiotics, which are derived from the stomach of the honey bee [59,60]. The role of probiotics in the treatment of infectious diarrhoea and other gastrointestinal disorders has been well established [61]. We also isolated and identified lactic acid bacteria from fresh honey samples collected from the Al-Baha and Aseer regions of Saudi Arabia (sent for publication). In addition, honey contains prebiotics, which actually promote the growth of beneficial gut flora [7]. Moreover, honey contains a number of important minerals, especially zinc, which has been used in the treatment of paediatric diarrhoea [6]. In summary, honey can serve as a potential therapeutic agent for the prevention and treatment of diarrhoea. Some clinical trials have already shown the effectiveness of honey in the treatment of diarrhoea, but these trials have certain limitations and thus are not widely convincing to physicians [62,63]. Therefore, there is a need for a well-designed controlled clinical trial with standardized honey that provides sufficient evidence for the widespread use of honey in the treatment of diarrhoea and other infective conditions of the gastrointestinal tract.

### 5.4. Study limitations

One of the important gastrointestinal factors that could influence the growth of *Shigella sonnei* is beneficial gut flora, such as bifidobacteria and lactobacilli. In this study, the impact of beneficial gut flora on the growth of *Shigella sonnei* and consequently the changes in the antibacterial activity of honey were not studied due to time constraints.

## 6. Conclusion

Both Manuka and Talah honeys effectively inhibited the growth of *Shigella sonnei* in standard medium as well as under simulated gastrointestinal conditions. Hydrochloric acid and pepsin had no deleterious effects on the antibacterial capability of honey; rather, the acidic pH of the stomach was inhibitory for *Shigella sonnei*, which in fact augmented the antibacterial activity of honey. However, bile salts in the intestine and the presence of food reduced the antibacterial activity of both honeys. The efficacy of Manuka and Talah honeys in treating infectious diarrhoea caused by MDR-resistant *Shigella sonnei* needs to be evaluated in well-controlled clinical trials in future studies.

## Ethics approval and consent to participate

Not applicable.

## CRediT authorship contribution statement

**Saad B. Al-Masaudi:** and. **Muhammad Barkaat Hussain:** and, and. **Saleh M. Al-Maaqar:** designed the study, performed the experiments. **Soad Al Jaouni:** and. **Steve Harakeh:** drafted the manuscript. All the authors approved the final manuscript.

## Declaration of competing interest

The authors declare that they have no competing interests.

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## References

[1] S. Hussen, G. Mulatu, Kassa ZY Prevalence of Shigella species and its drug resistance pattern in Ethiopia: a systematic review and meta-analysis, Ann. Clin. Microbiol. Antimicrob. 18 (1) (2019) 22.
[2] K.L. Kotloff, M.S. Riddle, J.A. Platts-Mills, P. Pavlinac, Zaidi AK shigellosis, The Lancet. 391 (10122) (2018) 801–812.
[3] Y. Zhang, L. Zhang, M. Du, H. Yi, C. Guo, Y. Tuo, et al., Antimicrobial activity against Shigella sonnei and probiotic properties of wild lactobacilli from fermented food, Microbiol. Res. 167 (1) (2011) 27–31.
[4] A.I. Ud-Din, S.U. Wahid, H.A. Latif, M. Shahnaij, M. Akter, I.J. Azmi, et al., Changing trends in the prevalence of Shigella species: emergence of multi-drug resistant Shigella sonnei biotype g in Bangladesh, PloS One 8 (12) (2013), e82601.
[5] K.D. Tickell, R.L. Brander, H.E. Atlas, J.M. Pernica, J.L. Walson, P.B. Pavlinac, Identification and management of Shigella infection in children with diarrhoea: a systematic review and meta-analysis, Lancet Glob Health. 5 (12) (2017) e1235–e1248.
[6] M.B. Hussain, Role of honey in topical and systemic bacterial infections, J. Altern. Complement. Med. 24 (1) (2018) 15–24.
[7] M.L. Sanz, N. Polemis, V. Morales, N. Corzo, A. Drakoularakou, G.R. Gibson, et al., In vitro investigation into the potential prebiotic activity of honey oligosaccharides, J.Agric. Food Chem. 53 (8) (2005) 2914–2921.

[8] M.B. Hussain, A. Hannan, N. Akhtar, G.Q. Fayyaz, M. Imran, S. Saleem, et al., Evaluation of the antibacterial activity of selected Pakistani honeys against multi-drug resistant Salmonella typhi BMC Complement, Altern. Med. 15 (1) (2015) 32.
[9] A. Mohan, S.-Y. Quek, N. Gutierrez-Maddox, Y. Gao, Q. Shu, Effect of honey in improving the gut microbial balance, Food Qual Saf. Now. 1 (2) (2017) 107–115.
[10] A. Hannan, M. Barkaat, M. Usman, W.A. Gilani, W. Sami, In vitro antibacterial activity of honey against clinical isolates of multi-drug resistant typhoidal salmonellae Pakistan, J. Zool. 41 (1) (2009) 1–6.
[11] H.T. Tan, R.A. Rahman, S.H. Gan, A.S. Halim, S. Asma'Hassan, S.A. Sulaiman, et al., The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey BMC Complement, Altern. Med.Med. 9 (1) (2009) 34.
[12] M.B. Hussain, In vitro antibacterial activity of honey against diarrhea causing bacterial isolates, Rawal Med. J. 43 (3) (2018) 498–501.
[13] J.N. Del Hierro, T. Herrera, T. Fornari, G. Reglero, D. Martin, The gastrointestinal behavior of saponins and its significance for their bioavailability and bioactivities, J. Funct. Foods 40 (2018) 484–497.
[14] T. Faye, A. Tamburello, G.E. Vegarud, S. Skeie, Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human gastric and duodenum juice, J.Dairy Sci. 95 (2) (2012) 558–566.
[15] T.D. Brown, K.A. Whitehead, S. Mitragotri, Materials for oral delivery of proteins and peptides, Nat. Rev. Mater. (2019) 1–22.
[16] Y. Alattal, A. AlGhamdi, Impact of temperature extremes on survival of indigenous and exotic honey bee subspecies, Apis mellifera, under desert and semiarid climates Bull. Insectology. 68 (2) (2015) 219–222.
[17] M.B. Hussain, A. Hannan, M. Absar, N. Butt, In-vitro susceptibility of methicillin-resistant Stayphylococcus aureus to honey, Complement Ther. Clin. Pract. 27 (2017) 57–60.
[18] M.B. Hussain, Y.M. Kamel, Z. Ullah, A.A.M. Jiman-Fatani, A.S. Ahmad, In vitro evaluation of methicillin-resistant and methicillin-sensitive Staphylococcus aureus susceptibility to Saudi honeys BMC Complement, Altern. Med. 19 (1) (2019) 185.
[19] Y. Maeda, A. Loughrey, J.P. Earle, B.C. Millar, J.R. Rao, A. Kearns, et al., Antibacterial activity of honey against community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) Complement, Ther. Clin. Pract. 14 (2) (2008) pp.77–82.
[20] V. French, R.A. Cooper, P.C. Molan, The antibacterial activity of honey against coagulase-negative staphylococci, J. AntimicrobChemother. 56 (1) (2005) 228–231.
[21] M. Weinstein, J. Patel, A. Bobenchik, S. Campeau, S. Cullen, M. Galas, Performance standards for antimicrobial susceptibility testing performance standards for antimicrobial susceptibility testing, CLSI Supply Manag. 100 (2019) 1–25.
[22] R.A. Cooper, P.C. Molan, K. Harding, The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds, J.Appl. Microbiol. 93 (5) (2002) 857–863.
[23] K. Brudzynski, R. Lannigan, Mechanism of honey bacteriostatic action against MRSA and VRE involves hydroxyl radicals generated from honey's hydrogen peroxide, Front Microbiol. 3 (2012) 36.
[24] K. Boorn, Y.Y. Khor, E. Sweetman, F. Tan, T. Heard, K. Hammer, Antimicrobial activity of honey from the stingless bee Trigona carbonaria determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology, J. Appl. Microbiol. 108 (5) (2010) 1534–1543.
[25] A.N.F. De Melo, G.T. De Souza, D. Schaffner, T.C.M. De Oliveira, J.F. Maciel, E. L. De Souza, et al., Changes in thermo-tolerance and survival under simulated gastrointestinal conditions of Salmonella Enteritidis PT4 and Salmonella Typhimurium PT4 in chicken breast meat after exposure to sequential stresses, Int. J. Food Microbiol. 251 (2017) 15–23.
[26] A. Madureira, C. Pereira, K. Truszkowska, A. Gomes, M. Pintado, F. Malcata, Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract, Int. Dairy J. 15 (6–9) (2005) 921–927.
[27] A. Madhavan, S. Balakrishnan, J. Vasudevapanicker, Antibiotic susceptibility pattern of Shigella isolates in a tertiary healthcare center, J. Lab. Physicians. 10 (2) (2018) 140.
[28] R. Ranjbar, Farahani A. Shigella, Antibiotic-Resistance Mechanisms And New Horizons For, Treatment infect, Drug Res. 12 (2019) 3137.
[29] I. Filliol-Toutain, C.-S. Chiou, C. Mammina, P. Gerner-Smidt, K.-L. Thong, D. C. Phung, et al., Global distribution of Shigella sonnei clones Emerging Infect, Dis. 17 (10) (2011) p.1910.
[30] R. Ranjbar, M.M. Soltan-Dallal, M.R. Pourshafie, C. Mammina, Antibiotic resistance among Shigella serogroups isolated in Tehran, Iran (2002-2004), J. Infect. Dev. Ctries. 3 (8) (2009) 647–648.
[31] M. Hosseini, R. Ranjbar, H. Ghasemi, H. Jalalian, The prevalence and antibiotic resistance of Shigella sp. recovered from patients admitted to Bouali Hospital, Tehran, Iran during 1999-2001, Pak J. Biol. Sci. 10 (16) (2007) 2778–2780.
[32] R.M. Humphries, J. Hindler, M. Jane Ferraro, A. Mathers, Twenty-first century cures act and antimicrobial susceptibility testing: clinical implications in the era of multidrug resistance, Clin. Inf. Disp. 67 (7) (2018) 1132–1138.
[33] F. Alotaibi, Carbapenem-resistant Enterobacteriaceae: an update narrative review from Saudi Arabia, J. Infect. Public Health. 12 (4) (2019) 465–471.
[34] N.M. George, Cutting KF Antibacterial honey (Medihoney™): in-vitro activity against clinical isolates of MRSA, VRE, and other multiresistant gram-negative organisms including Pseudomonas aeruginosa, Wounds 19 (9) (2007) 231.
[35] R. Cooper, L. Jenkins, A. Henriques, R. Duggan, N. Burton, Absence of bacterial resistance to medical-grade manuka honey, Eur J. Clin. Microbiol. Infect. Dis. 29 (10) (2010) 1237–1241.
[36] S. Becattini, Y. Taur, E.G. Pamer, Antibiotic-induced changes in the intestinal microbiota and disease, Trends Mol. Med. 22 (6) (2016) 458–478.
[37] M.B. Hussain, Evaluation of selected Pakistani honeys in comparison with manuka honey against Vibrio cholerae RADS, J.Biol. Res. & Appl. Sci. 10 (2) (2019) 54–62.
[38] N. Roshan, T. Rippers, C. Locher, K.A. Hammer, Antibacterial activity and chemical characteristics of several Western Australian honeys compared to manuka honey and pasture honey, Arch. Microbiol. 199 (2) (2017) 347–355.
[39] P. Combarros-Fuertes, L.M. Estevinho, L.G. Dias, J.M. Castro, F.A. Tomás-Barberán, M.E. Tornadijo, et al., Bioactive components and antioxidant and antibacterial activities of different varieties of honey: a screening prior to clinical application, J. Agric. Food Chem. 67 (2) (2018) 688–698.
[40] A. Al-Ghamdi, A. Nuru, Beekeeping in the Kingdom of Saudi Arabia opportunities and challenges, Bee World. 90 (3) (2013) 54–57.
[41] N. Adgaba, A. Al-Ghamdi, Y. Tadesse, A. Getachew, A.M. Awad, M.J. Ansari, et al., Nectar secretion dynamics and honey production potentials of some major honey plants in, Saudi Arabia Saudi J Biol Sci. 24 (1) (2017) 180–191.
[42] M.M.E. Ahamed, A. Abdallah, A. Abdalaziz, E. Serag, Atallah A-bEH some physiochemical properties of Acacia honey from different altitudes of the asir region in southern Saudi Arabia Czech, J. Food Sci. (N. Y.) 35 (4) (2017) 321–327.
[43] N.A. Alrowais, N.A. Alyousefi, The prevalence extent of Complementary and Alternative Medicine (CAM) use among Saudis Saudi, Pharm. J. 25 (3) (2017) 306–318.
[44] T. Birk, K. Kristensen, A. Harboe, T.B. Hansen, H. Ingmer, R. De Jonge, et al., Dietary proteins extend the survival of Salmonella Dublin in a gastric acid environment, J.Food Prot. 75 (2) (2012) 353–358.
[45] J.L. Smith, The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions, J. Food Prot. 66 (7) (2003) 1292–1303.
[46] A. Singh, Barnard TG Surviving the acid barrier: responses of pathogenic Vibrio cholerae to simulated gastric fluid, Appl Microbiol. Biotechnol. 100 (2) (2016) 815–824.
[47] D. De Biase, E. Pennacchietti, Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: function, distribution and biomedical implications of the gadBC operon Mol. Microbiol. 86 (4) (2012) 770–786.
[48] C.-J. Lyu, J.-Y. Fei, J.-P. Yan, Q.-C. Xu, J.-Q. Mei, H.-Y. Yue, et al., Improvement of γ-aminobutyrate biosynthesis by genetically engineered Lactococcus lactis, Biochem Eng. J. 157 (2020) p.107525.
[49] S. Zhang, D. He, Y. Yang, S. Lin, M. Zhang, S. Dai, et al., Comparative proteomics reveal distinct chaperone–client interactions in supporting bacterial acid resistance, Proc Natl. Acad. Sci. U.S.A. 113 (39) (2016) 10872–10877.
[50] C. Niu, D. Wang, X. Liu, H. Liu, X. Liu, E. Feng, et al., An H-NS family protein, Sfh, regulates acid resistance by inhibition of glutamate decarboxylase expression in Shigella flexneri 2457T Front, Microbiol. 8 (2017) 1923.
[51] J.Z. Goldenberg, C. Yap, L. Lytvyn, C.K.F. Lo, J. Beardsley, D. Mertz, et al., Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children, Cochrane Database Syst. Rev. (12) (2017).
[52] C. Wang, Y. Cui, X. Qu, Mechanisms and improvement of acid resistance in lactic acid bacteria, Arch. Microbiol. 200 (2) (2018) 195–201.
[53] C. Chandran, T. Keerthi, Probiotic potency of Lactobacillus plantarum KX519413 and KX519414 isolated from honey bee gut FEMS, Microbiol. Lett. 365 (4) (2018) p. fnx285.
[54] J.L. Smith, Infectious Dose and an Aging Population: Susceptibility of the Aged to Foodborne Pathogens Foodborne Pathogens: Springer, 2017, pp. 451–468.
[55] L. Mannina, A.P. Sobolev, E. Coppo, A. Di Lorenzo, S.M. Nabavi, A. Marchese, et al., Antistaphylococcal activity and metabolite profiling of manuka honey (Leptospermum scoparium L.) after in vitro simulated digestion, Food Funct. 7 (3) (2016) 1664–1670.
[56] M. Daglia, D. Ferrari, S. Collina, V. Curti, Influence of in vitro simulated gastroduodenal digestion on methylglyoxal concentration of manuka (Lectospermum scoparium) honey, J.Agric. Food Chem. 61 (9) (2013) 2140–2145.
[57] S.-M.S. Lin, The Effect of Manuka Honey on Enterobacteria: the University of Waikato, PhD thesis, 2010. available at, https://researchcommons.waikato.ac.nz/bitstream/handle/10289/3972/thesis.pdf?sequence=1&isAllowed=y. Accessed 09-07-2020.
[58] C. Bavishi, Dupont H Systematic review: the use of proton pump inhibitors and increased susceptibility to enteric infection, Aliment Pharmacol. Ther. 34 (11–12) (2011) 1269–1281.
[59] B.Y. Bulgasem, M.N. Lani, Z. Hassan, W.M.W. Yusoff, S.G. Fnaish, Antifungal activity of lactic acid bacteria strains isolated from natural honey against pathogenic Candida species, MYCOBIOLOGY 44 (4) (2016) 302–309.
[60] S.N. Syed Yaacob, F. Huyop, Kamarulzaman Raja Ibrahim R, Wahab RA Identification of Lactobacillus spp. and Fructobacillus spp. isolated from fresh Heterotrigona itama honey and their antagonistic activities against clinical pathogenic bacteria, J. Apic. Res. 57 (3) (2018) 395–405.
[61] Y.-T. Li, H. Xu, J.-Z. Ye, W.-R. Wu, D. Shi, D.-Q. Fang, et al., Efficacy of Lactobacillus rhamnosus GG in treatment of acute pediatric diarrhea: a systematic review with meta-analysis World, J. Gastroenterol. 25 (33) (2019) 4999.
[62] Z.V. Shariatpanahi, F. Jamshidi, J. Nasrollahzadeh, Z. Amiri, H. Teymourian, Effect of honey on diarrhea and fecal microbiotain in critically ill tube-fed patients: a single center randomized controlled study, Anesthesiol. Pain Med. 8 (1) (2018).
[63] R.P. Andayani, N. Nurhaeni, Agustini N the effect of honey with ORS and a honey solution in ORS on reducing the frequency of diarrhea and length of stay for toddlers compr. Child adolesc, Nurs. Times 42 (sup1) (2019) 21–28.