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Original Article

Human Oral Microbiota's Response to Antimicrobial Properties of Honey

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Abstract *- The human oral microbiome is important under both healthy and diseased conditions. Temporary intruders, protective bacteria, and opportunistic pathogens of oral cavity niches form the oral microbiome. Most oral microorganisms are commensal and regulate the balance of the oral biological community. However, some microbes are associated with periodontitis and dental caries. Honey applications are known to cure minor (flu or cold) and severe ailments (burns, gastrointestinal, liver, and cardiovascular issues) and exert antimicrobial activities against viruses and bacteria. This study involved microbiological and molecular approaches to evaluate honey's antimicrobial properties against human oral cavity pathogens without disturbing oral microbiota equilibrium. The potential use of honey as a mouthwash was assessed as well. The results revealed the effective antimicrobial potential of Manuka and Sidr honey compared to the control group. The study also confirmed that honey-based natural mouthwash could serve as an efficient alternative to chemical mouthwash.*

Keywords *- Honey, Antimicrobial activity, Oral microbiome, Mouthwash, Dental caries, and Periodontitis.*

1. Introduction

Antoine Van Leeuwenhoek discovered the microorganisms in the eighteenth century by examining dental plaque samples under the microscope. He named them "Dierking", referring to small, lively objects. However, Joshua Lederberg devised the term "microbiome" in the twentieth century to represent all microorganisms inside the human body [1]. The presence of approximately a hundred trillion symbiotic microbial cells in the human body, which include viruses, bacteria, fungi, and archaea, has been estimated.

Human microbiome studies have rapidly increased in recent years, with more than ten thousand investigations in 2018. Human microbes are commonly detected in the oral cavity, skin, vagina, and gut [2]. The human oral cavity environment is humid with neutral pH and relatively constant temperature (34ºC to 36ºC). The anatomical structure of the oral cavity favors numerous types of microorganisms. The complex oral microbiota, particularly of saliva, has been extensively studied as it contains a range of microorganisms on the tongue and supragingival and subgingival regions [3].

Human oral microbiota differs from other body parts [4]. Saliva's 70% culturable bacterial microbiota is mainly comprised of *streptococcus*, *veillonella*, and *prevotella* genera [5]. Collins and Dawes (1987) have estimated an overall oral cavity surface area of 214.7 ± 12.9 cm2 regardless of gender [6]. The pharynx-connected oral cavity is comprised of the tongue, internal cheeks, and tender and tough palates. These surfaces, gingival crevices, and saliva have specific microenvironments which harbour site-specific microbiota [7]. Oral diseases are mostly linked to opportunistic pathogens, predominantly bacteria. Approximately 500 to 700 common oral bacterial species have been reported, including 50% to 60% culturable species [8]. Molecular approaches have been recently employed to identify the remaining unculturable microorganisms. These molecular techniques mainly include next-generation sequencing (NGS), 16S ribosomal RNA sequencing, and pyrosequencing [9].

Different studies have demonstrated antibacterial properties-based honey's minimal inhibitory effects against dental caries-associated bacteria [10]. Honey's minimum inhibitory concentrations (MICs) of 8–12% and 7.5–8.5% have been reported against *lactobacillus caseii Strep. mitis* and *Strep. sobrinus*, respectively [11]. During an investigation, 10% honey concentration effectively reduced the acid production in *Streptococci* and *l. Caseii* by 75–80% and 30%, respectively, compared to sucrose-made products. Adding a 10% sucrose-containing medium inhibited the

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sucrose-based dextran production by 75–89%. The oral microbiome has gained more interest in its association with oral infections and periodontal diseases in recent decades. Oral microbiome-induced periodontal infections could lead to gingival sulcular epithelium ulceration, facilitating bacterial entry into the blood circulation system from the sulcus [12]. This study evaluates the antimicrobial potential of two types of honey mouthwashes (New Zealand's Manuka honey and Sidr honey from Albaha, Saudi Arabia) against oral bacteria while maintaining the oral microbiome equilibrium.

2. Materials and Methods

2.1. Collection of Salivary Samples

Human Research Ethics Committees of King Abdulaziz University approved this study. Salivary samples were collected from nine healthy individuals with their consent. It was ensured that none of the individuals had undergone antibiotic treatment within the last three months. Saliva samples were collected daily for 15 days. The subjects were randomly divided into three groups (Control, Manuka Honey, and Sidr Honey group) labeled as groups A, B, and C. Three volunteers were assigned to each group. The participants of the negative control group (A) (without honey mouthwash) washed their mouths with bottled water (10 ml) for 20 seconds and spit it in a sterile Falcon tube (15 mL). Positive control group (B) used 10 ml of Manuka honey as mouthwash (50% honey and 50% water) for 20 seconds, followed by rinsing with 10 ml of bottled water after 5 minutes and spitting it into the Falcon tube. The mouthwash of group (C) contained Sidr honey (50% honey and 50% water). The participants used 10 ml of this mouthwash for 20 seconds, followed by rinsing it with 10 ml of bottled water after 5 minutes and spitting it into the Falcon tube. Before eating, drinking, or oral hygiene procedures, these saliva samples were collected in the morning (10-11 am). The honey mouthwashes (Manuka and Sidr honey) were prepared by weighing 0.5 gm of honey according to the groups (B and C), whereas water was added right before usage.

2.2. Serial Dilution Method for Bacterial Culturing and Screening

The serial dilution (10 folds) of the salivary samples was performed by following the methodology of Kavanagh (1963) [13]. Diluted samples were inoculated in 5ml of Nutrient Broth.

2.3. Optical Density-Based Measurement of Microbial Load

Optical density measurement at 570 nm is commonly used to assess microbial growth in solutions. This method is based on absorbance detection, which measures the portion of light passing through the microbial suspension.

2.4. Bacterial Identification

Gram staining differentiated the bacteria based on their negative and positive reactions.

2.5. Molecular Characterization

A slightly modified procedure of Azcárate-Peril and Raya (2001) was adopted for total genomic DNA extraction [14]. 1 ml of NA-grown (overnight) pure culture of bacteria was added to a 1.5 ml tube followed by centrifugation (4°C, 10000 rpm) for 5 min. 200 µl of TES buffer was added after discarding the supernatant, followed by vortexing. 20 µl of lysozyme (10 mg/ml) was added to the mixture, vortexed, and incubated for 1 hour at 37°C in a water bath. Then, 20 µl of Proteinase K (10 mg/ml) was added and vortexed. The step was again followed by incubation for 1 hour at 37°C, cooling (5 min), and adding 250 µl sodium acetate. Then, centrifugation was carried out (4°C, 8000 rpm) for 5 min, and the supernatant was transferred to a new tube. 250 µl of Chloroform: isoamyl mixture (24:1) was added to the supernatant, stirred between fingers, and centrifuged (8000 rpm) for 5 min at 4°C. The transfer of the aqueous phase to a new tube was followed by adding isopropanol and overnight storage at -20°C. Then, centrifugation (10000 rpm, 5 min) was carried out, and the liquid zone was removed. The DNA pellet was dried for 10 min at room temperature and re-suspended in distilled water (50 µl). DNA quality was assessed through gel electrophoresis at 120 V. DNA ladder (1 kb) was added into the first lane, whereas DNA samples were loaded to other gel wells. Bacterial DNA was examined under UV light.

The universal Primers (27-F and 519-R) were used for 16S rRNA gene amplification using the bacterial DNA template. PCR mixture (50 µl) contained DNA template (2µl), primers (2µl of each), Master Mix (25µl) (Promega 2X), and water. A 96-Well Thermal Cycler (Applied Biosystems™ Veriti™) was used for the amplification as initial denaturation for 5 min at 94°C (1 cycle), 35 cycles (30 sec at 94°C, 30 sec at 58°C, and 1.30 min at 70°C), and final elongation for 10 min at 70°C. PCR products were compared with a DNA ladder (100 bp) by subjecting them to gel electrophoresis. A gel documentation system was used to examine target bands under UV light. The isolated strains were identified by sequencing the PCR products from Macrogen. The sequence data analysis was performed by employing MEGA (Molecular Evolutionary Genetics Analysis) software, whereas sequences were compared using the NCBI database.

2.6. Statistical Analysis

Three replicates of fresh culture were used for each experiment. The results were expressed as replicates' means, standard deviations, and standard errors. SPSS Version 25 (Statistical Package for Social Science) was used to perform One Way ANOVA whereas Tukey's test and Independent-samples T-Test compared the means.

3. Results and Discussion

Bacterial isolates were identified phenotypically and morphologically (color, elevation, form, shape, and margin

of the colonies) in addition to gram staining. The isolates' colony morphology ranged from circular to irregular, flat to raised, and entire to undulate. They were smoothly contoured and filamentous, whereas the colour varied from white to cream and yellowish to orange. After gram staining, the bacterial samples were visualized under an Olympus BX51M upright inspection and research microscope (Olympus Korea Co. Ltd).

Figure 1 presents the most dominant bacterial species in the saliva samples of the control group. Overall, there were forty-seven gram-negative and gram-positive bacterial species in the control group samples. Gram-negative bacteria dominated the salivary microbiome of the control group with 27 species, whereas 20 species were found to be gram-positive. *Bacillus cereus* strain occupied most of the salivary microbiome of the control group, followed by *Enterobacter cloacae* and *Serratia marcescens*. The other microbes mainly included *Bacillus* sp, *Enterococcus faecalis*, and *Staphylococcus aureus*.

Figure 2 depicts bacterial diversity in the saliva samples of group B (Manuka Honey mouthwash). Bacterial species mainly included *Serratia marcescens*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Pantoea agglomerans*, *Serratia nematodiphila*, *Enterobacter ludwigii*, *Enterococcus faecalis*, and *Pseudogracilibacillus auburnensis*. Overall, forty-four bacterial species were isolated from the salivary samples of group B, where twelve bacterial species were gram-positive, and thirty-two species were gram-negative.

Figure 3 illustrates bacterial diversity in the saliva samples of group C (Sidr Honey mouthwash). The oral microbiome of this group was dominated by *Serratia marcescens* and *Staphylococcus aureus,* followed by *Enterococcus faecalis* and *Enterobacter cloacae*. Seventeen bacterial species were identified in the salivary samples of group C, where five species were gram-positive, and twelve species were gram-negative.

The statistical analysis (Independent-Samples-T TEST) revealed that OD readings of salivary samples did not significantly differentiate in the control group during seven experimental days. Contrarily, the microbial load significantly varied in the salivary samples of group B (Manuka Honey mouthwash). Significant differences were noted on days 3, 4, 5, and 7, which confirmed the antimicrobial effects of Manuka Honey on the oral microbiome. The alleviated overall microbial load could lead to a minimized population density of pathogenic bacteria in the oral cavity. The microbial load significantly differentiated in group C (Sidr honey mouthwash) on days 3, 4, 5, and 6.

One-way ANOVA compared the OD readings of all three groups (A, B, and C) and demonstrated significant differences between all the groups from 2nd to 6th day. However, higher values on the 7th day proved honey-based reduction in salivary microbial load compared to the control group. Figure 4 demonstrates the differences in the microbial load of all the groups (A, B, and C) throughout the experimental period (day 1 to day 7). The highest microbial load was noted in the Control group, followed by the Sidr honey group and the Manuka honey group. The microbial load increased in the Control group, decreased in the Manuka honey group, and remained constant in the Sidr honey group on the 2nd day. The data of 3rd day did not vary much from the previous day except for a rise in the microbial load of the Sidr honey group. Microbial load was significantly reduced in the Sider honey group on the 4th day, whereas there was a slight increase in the other two groups. On the fifth day, microbial load decreased in the Manuka honey group and Control group, whereas a slight increase was noted in the Sidr honey group. The microbial load was alleviated in the Sidr honey group on the 6th day, whereas it slightly increased in the Manuka honey group. Finally, the Manuka honey group mitigated the microbial load more than the Sidr honey group on the 7th day.

Overall, Sidr honey mouthwash reduced the salivary microbial load on the 4th and 6th day, whereas Manuka honey mouthwash reduced the salivary microbial load on the 2nd, 3rd, 5th, and 7th. Sidr honey mouthwash presented higher microbial-reducing effects as compared to Manuka honey mouthwash, but it sustained for a shorter period than Manuka honey mouthwash.

The oral microbiome has three types: indigenous or resident flora, supplemental flora, and transient flora. Indigenous or resident flora are always present in the oral cavity in high numbers (>1% of the total viable count), including *Neisseria*, *Streptococci*, and *Actinomyces*. Supplemental flora of the oral cavity is also permanent but comparatively in low numbers (<1% of total viable count), including *P. gingivalis* III and *Lactobacilli*. Transient flora refers to organisms that pass through the host at any given time. Transient flora is not limited to a specific oral microbiome species [9]. Moreover, a wide variety of tooth-surface or tissue microbes could also appear in the salivary samples. *Streptococcus species*, particularly *Streptococcus salivarius* and Streptococcus oralis, have often been reported in human saliva [15]. During this study, salivary samples were cultured in Nutrient Agar Media, a general-purpose nutrient medium for non-fastidious microorganisms. Nutrient agar can efficiently grow a broad range of fungi and bacteria [16]. Thus, Nutrient agar facilitated the growth of genetically close taxa, as represented by the phylogenetic tree (Figure 5). A classical culturing method was adopted during this study to obtain bacterial species, whereas bacterial species requiring specific nutrient and oxygen levels were unable to grow.

Amer (2019) identified various bacterial phyla, including *Spirochaetes*, *Actinobacteria*, *Firmicutes*,

Bacteroidetes, *Proteobacteria*, and *Fusobacteria,* from the oral samples [2]. They employed culture-independent 16s rRNA and whole-genome sequencing methods to retrieve a large number of bacterial phyla. The classical culturing methods have certain limitations (nutrients and oxygen requirements), which hinder the detection of a broad range of oral microbiomes.

Excessive, unnecessary, and widespread antibiotic usage has complicated bacterial infection treatments as it favors pathogenic resistance to antibiotics, leading to serious public health concerns and financial burdens [17]. Honey's antibacterial features were first recognized by Van Ketel in 1892 [18]. The results of this study indicated that rinsing with Manuka and Sidr honey solutions (50%) affected oral bacterial growth. Taormina et al. (2001) investigated the antimicrobial activity of six types of honey from five floral sources (Dutch Gold Honey) against foodborne bacteria [19].

The mouth is the major bacterial entrance into humans, which helps their persistence in saliva. Honey presented excellent antimicrobial activity against some bacterial species (*Shigella sonnei*, *Staphylococcus aureus*, and *Salmonella typhimurium*). However, honey could not impact the *B. cereus* growth and remained least affected against all types of honey [19]. These results contradict the current study's findings, as Manuka honey exhibited effective antimicrobial activity against *B. cereus* in this study. Manuka honey-treated group did not contain any strain of *B. cereus,* whereas multiple *B. cereus* isolates were detected in the control group. Alqurashi et al. (2013) revealed effective in vitro growth inhibition of *K. pneumoniae* in response to local Sidr honey treatment.

Similarly, Sidr honey treatment also presented efficient antimicrobial activity against K. pneumoniae during this study [20]. Multiple studies have reported Manuka honey's antimicrobial activity against different bacterial species [18]. These investigations have established the therapeutic role of Manuka honey in treating periodontal and gingivitis diseases. Manuka honey mouthwash is also known to significantly reduce plaque formation.

The findings of Singhal et al. (2018) are in line with the results of this study, which reported that rinsing with raw and Manuka honey exerted promising antimicrobial effects and significantly mitigated the dental caries, gingival, and plaque scores [21]. These findings prove that honey is a potent antibacterial agent, and it could replace synthetic antibiotics if properly standardized to avoid antibiotic resistance among synthetic drugs. However, further understanding of honey's chemical and therapeutic properties is needed for clinical applications against periodontal disease and oral microbiome.

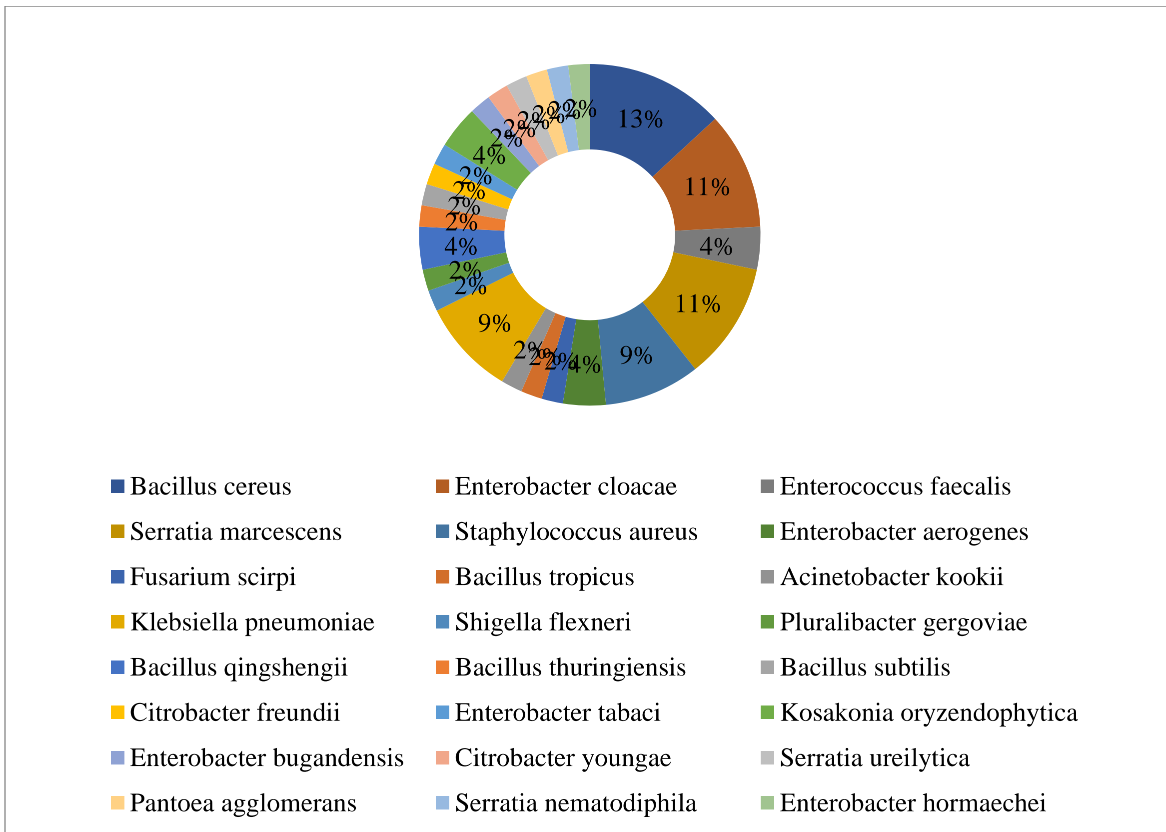

Fig. 1 Bacterial strains in human saliva of the control group

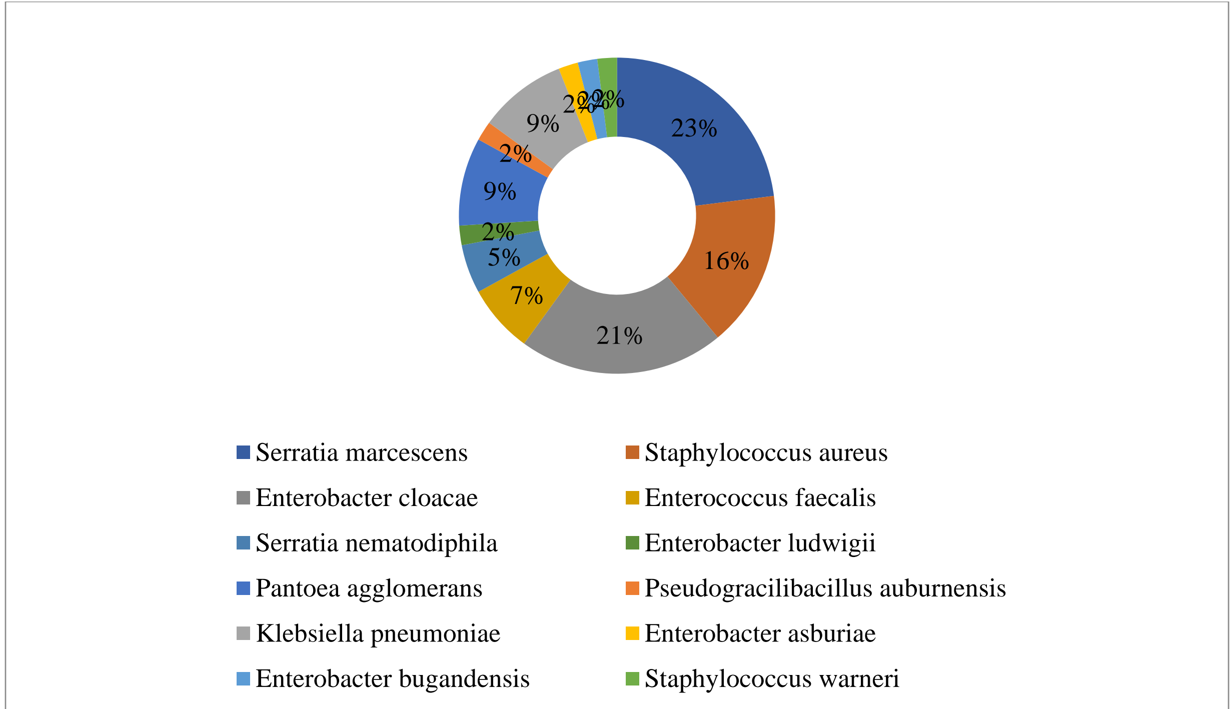

Fig. 2 Bacterial strains in human saliva of the Manuka honey mouthwash treated group.

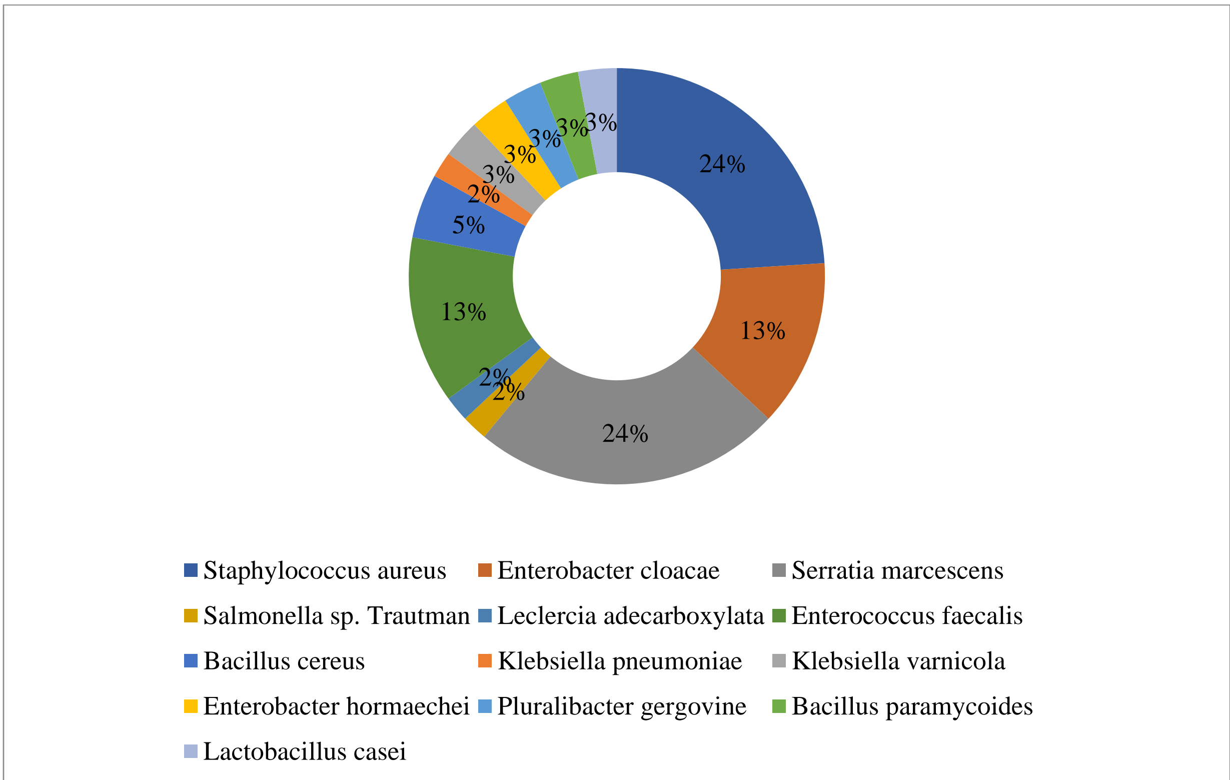

Fig. 3 Bacterial strains in human saliva of the Sidr Honey mouthwash treated group.

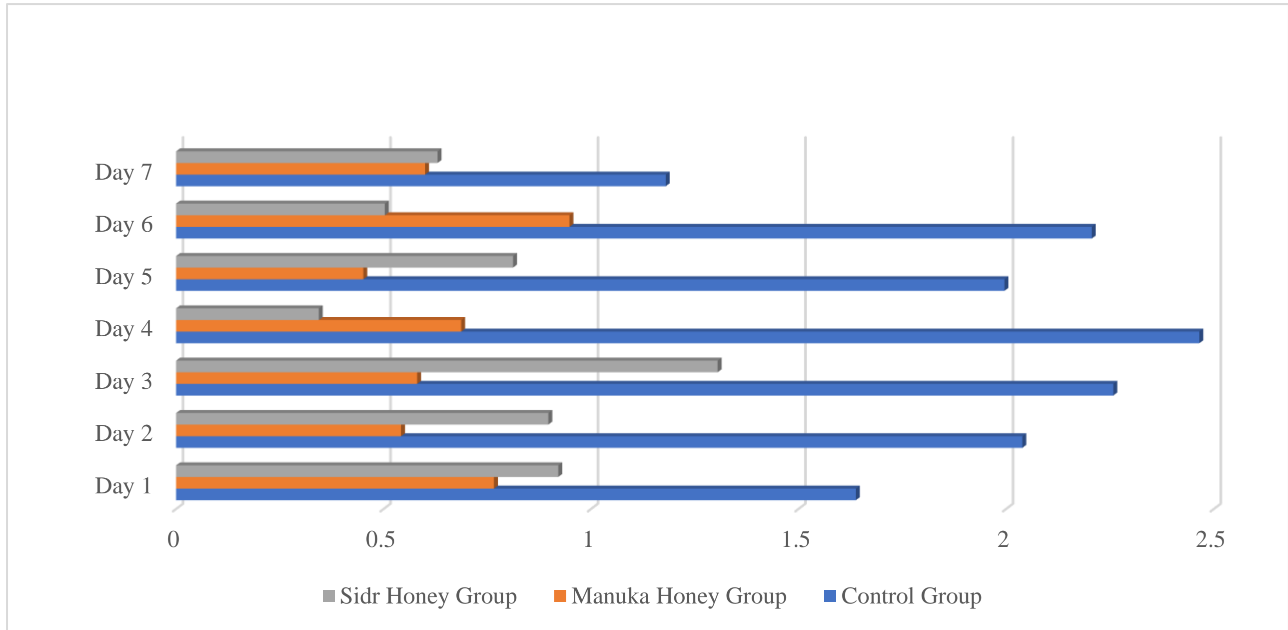

Fig. 4 Microbial Load in Saliva

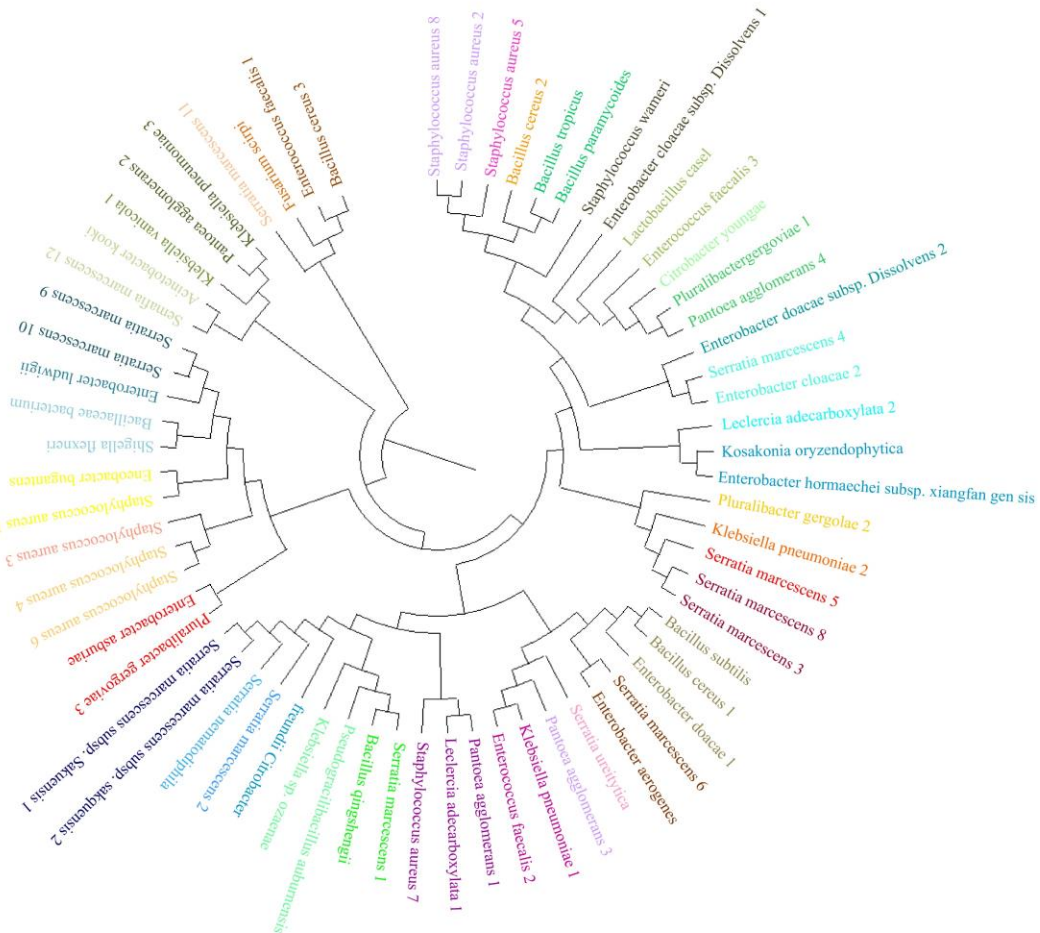

Fig. 5 Phylogenetic tree representing the evolutionary relationships of the taxa

4. Conclusion

The current study revealed bacterial diversity in human saliva and examined the antibacterial effects of Sidr and Manuka honey. Manuka and Sidr honey demonstrated promising antimicrobial potential against isolated oral bacteria compared to the control group. Thus, honey mouthwash could effectively replace chemical mouthwash products.

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