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Effect of Manuka Honey and Licorice Root Extract on the Growth of *Porphyromonas gingivalis*: an *In Vitro* Study

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Summary

Chronic bad breath (also known as "halitosis") is very common in general population and nearly 50% of the general population have halitosis. Liquid mouthwashes such as Listerine®, and ACT®, mouth fresheners, and sugar-free gum are commonly used to alleviate bad breath, but these products are not portable, have many side effects, or only temporarily mask the problem. Hence, a lozenge made from natural ingredients would be an excellent alternative to reduce bad breath. We evaluated the effectiveness of natural ingredients such as Manuka Honey and Licorice root extract in reducing growth of *Porphyromonas gingivalis*, one of the main bacteria that cause bad breath. Solutions containing Manuka honey and/or licorice root extract, Listerine®, or ACT® were added to *P. gingivalis* cultures to assess their effect on bacterial growth. After 18 hours, the absorbance at 680nm of each solution was measured as an indicator of growth. We found that Manuka honey is almost as effective as Listerine® and ACT® in reducing *P. gingivalis* growth, while licorice root extract, had a very minor effect. Aliquots from the cultures that were inoculated on to brain heart infusion (BHI) plates showed similar trends on growth inhibition. These data suggest that a natural lozenge made from Manuka honey may be effective in reducing bad breath.

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Introduction

Human breath is composed of highly complex substances with numerous variable odors that can contribute to halitosis. Halitosis is derived from the Latin *halitus* (breathed air) and *osis* (pathologic alteration), and is used to describe any disagreeable bad or unpleasant odor emanating from the breath (1). This undesirable condition is a common complaint of both genders and all age groups, and creates social and psychological disadvantages for individuals that affect their relationships with others (2). Halitosis affects nearly 50% of the general population, and affected individuals generally use liquid mouthwashes such as Listerine®, ACT®, mouth fresheners, or sugar free gum to alleviate bad breath.

While mouthwashes do reduce bad breath, they have some drawbacks. First, bottles of these mouthwashes (even travel size ones) are not easy to carry in your pocket or a small purse, nor are they convenient to use in certain settings. Moreover, these mouthwashes have a large alcohol content (often exceeding 20%), which causes dry mouth, an unpleasant burning sensation when used, and potentially higher risk of oral cancer (3). Mouth fresheners and sugar free gum only temporarily mask bad breath (4). Hence, a lozenge made from natural ingredients could be a safe and more effective alternative to reduce bad breath.

One of the main bacteria that cause bad breath is *Porphyromonas gingivalis* (*P. gingivalis*) (5). Manuka honey has been shown to have some antibacterial effect on *P. gingivalis* (6). While the effect of licorice root extract on certain oral pathogens has been studied (7), its antibacterial effect on *P. gingivalis* has not been studied. We hypothesized that natural ingredients with antibacterial properties such as Manuka honey and licorice root extract may be as effective as commercially available mouthwashes in reducing the growth of *P. gingivalis* bacteria. Thus, we evaluated effectiveness of Manuka honey and licorice root extract, as compared to commercial mouthwashes such as Listerine® and ACT®, to reduce growth of *P. gingivalis* bacteria. We found that Manuka honey is almost as effective as Listerine® and ACT® in reducing *P. gingivalis* bacteria growth, while licorice root extract, had a very minor effect on *P. gingivalis* growth. Our data suggest that a natural lozenge made from Manuka honey may be effective in reducing bad breath.

Results

Our experiments focused on testing the growth of *P. gingivalis* in different oral solutions containing Listerine®,

Absorbance Data of different solutions in the wells of microtiter plate															
Absorbance	0.063	0.051	0.128	1.839	0.509	0.848	0.018	0.013	0.0665	1.2845	0.3365	0.603	0.539	0.014	
"	0.064	0.055	0.149	1.782	0.502	0.83	0.0205	0.0135	0.0785	1.3015	0.3445	0.6515	0.5115	0.0135	
"	0.064	0.059	0.144	1.889	0.488	0.863	0.0205	0.014	0.0715	1.2885	0.3455	0.653	0.5355	0.013	
"	0.06	0.053	0.132	1.616	0.512	0.811	0.019	0.014	0.0695	1.306	0.3385	0.6625	0.511	0.0125	
Average Absorbance	0.06275	0.0545	0.13825	1.7815	0.50275	0.838	0.0195	0.013625	0.0715	1.295125	0.34125	0.6425	0.52425	0.01325	
	Listerine + Bacteri	ACT + Bacteri	Honey (H) + Ba	Licorice (L)+ Bacteria	(75% H, 25% L) + Bacteria	(25% H, 75% L) + Bacteria	Listerine only	ACT only	Honey only	Licorice Only	75% H, 25% L only	25% H, 75% L only	Bacteria only	Water	
													Positive Control	Negative Control	
Δ (Absorbance)	0.481	0.483375	0.4575	0.037875	0.36275	0.32875									

Table 1. Absorbance data of the different solutions in the microtiter plate.

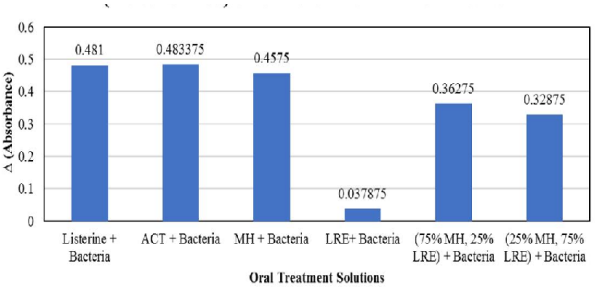

Figure 1. Absorbance of Different Oral Treatments. ΔAbsorbance of oral treatments. Treatment with ACT and Listerine resulted in the greatest ΔAbsorbance values.

ACT®, Manuka honey (MH) or licorice root extract (LRE) in different concentrations by measuring absorbance of each solution and calculating the difference in average absorbance (ΔAbsorbance) between the oral treatment and control bacteria solutions. Using a microtiter plate, we pipetted solutions of Listerine®, ACT®, 100% MH, 75% MH and 25% LRE, 25% MH and 75% LRE, and 100% LRE in replicate and added solutions of *P. gingivalis* to wells of each treatment condition. After 18 hours to incubate the bacteria in anaerobic conditions at body temperature (36.5-37°C), we used a plate reader to measure the absorbance of light by each solution in the well.

A solution containing a higher bacterial count is more turbid than a solution containing lesser number of bacteria. As turbidity of the solution increases, more light is absorbed by the solution at 680nm and the measured absorbance is higher. Thus, a greater reduction in absorbance (higher ΔAbsorbance) implies a greater reduction in the number of *P. gingivalis*. Hence, the oral treatment solution with the highest value of ΔAbsorbance will be most effective in reducing bad breath.

ΔAbsorbance of ACT® was the highest amongst the 6 oral treatment solutions at 0.483, followed by Listerine® at 0.481, and MH at 0.456 (**Table 1 and Figure 1**). ΔAbsorbance value of LRE was minimal at only 0.038, while combination of LRE and MH yielded intermediate ΔAbsorbance values between that of MH and LRE alone.

Hence, it can be surmised that ACT® would be most effective in reducing bad breath amongst the 6 oral treatment solutions, followed by Listerine®, 100% MH, 75% MH and 25% LRE, 25% MH and 75% LRE, and 100% LRE.

In order to establish that the efficacy of the oral treatment solution alone (and not any other unrelated reason) is responsible for affecting bacterial growth, we calculated the difference in absorbance ($R_{absorbance}$) between the treatment only value and (treatment + bacteria) value for each solution and compared it to the absorbance value of the untreated bacteria solution. $R_{absorbance}$ was 0.041 for ACT®, 0.043 for Listerine®, and 0.067 for MH, however the $R_{absorbance}$ value for LRE was substantially higher at 0.49, very close to absorbance value of the untreated bacteria solution (0.543). This analysis further confirms that MH is almost as effective as Listerine® and ACT® in reducing the growth of the *P. gingivalis* bacteria, whereas LRE has a very minimal effect.

In addition, aliquots from the respective treatments were plated on to BHI plates and incubated overnight at 37°C. Visual observations of the colonies showed very similar trends in bacterial growth that confirmed the spectroscopic measurements (data not shown).

Discussion

We investigated the effectiveness of natural ingredients such as Manuka honey (MH) and licorice root extract (LRE) in reducing growth of *P. gingivalis* as compared to widely used commercial mouthwashes such as Listerine® and ACT®. If MH or LRE are close to the commercial mouthwashes in effectiveness, then one or both can be used as the main active ingredients of a lozenge aimed at reducing bad breath. Our study suggests that while the commercial mouthwashes are more effective than the natural ingredients we tested, Manuka honey was nearly as effective. Hence, a lozenge made from MH could be an effective, convenient, and safe solution to treat bad breath. Licorice root extract, however, was minimally effective.

The current solutions to treating halitosis, such as commonly available liquid mouthwashes, come with safety concerns (they present a risk of oral cancer due to high alcohol content) and are not easy to carry, whereas mouth fresheners and sugar free gums mask bad breath but do not treat it. Hence, this problem of halitosis begs for a safer, convenient solution. As a natural ingredient, MH is thought to be safe and our data shows that it is almost as effective in our bacterial growth inhibition

assay as some of the leading commercial mouthwashes. The ability of MH to reduce the growth of *P. gingivalis* may be primarily due to its high osmolarity (8). The high osmolarity draws water out of the bacterial cell, making it difficult for the bacteria to survive. Gram-negative bacteria such as *P. gingivalis* have a thinner cell wall compared to gram-positive bacteria and hence, water can be drawn out more easily. The low pH of Manuka honey could also play a role in reducing growth by inhibiting proteolytic activity in *P. gingivalis* (9).

Our study only focused on *P. gingivalis* bacteria. While *P. gingivalis* is one of the main bacteria responsible for halitosis, there are other oral bacteria such as *Treponema denticola*, *Prevotella melaninogenica*, and *Porhyromonas endodontalis* which also cause bad breath (10). The effectiveness of MH and LRE at reducing growth of these other bacteria needs to be studied and compared to the commercially available mouthwashes. In addition, commercially available MH comes in different grades with different antibacterial activity. The MH used in our study was rated Unique Manuka Factor (UMF) 20+. UMF is a measure of the non-peroxide activity of honey and ranges from 10 to 25. A higher UMF value indicates greater antibacterial activity of the honey (11). It is will also be important to study how the effect on MH on these bad-breath causing bacteria varies based on UMF rating.

Methods

Solution Preparation

Manuka honey (MH) (Kiva UMF 20+, UNSPC#: 50192403) and Licorice Root Extract (LRE) (Nature's Answer, Item# AF98) were weighed using a balance and mixed with equal weight of sterile water to create 1:1 solutions each of MH and LRE. Using a micro-pipette to measure volume precisely, MH and LRE solutions were measured and mixed to create 2 solutions of different concentrations: one solution being 75% MH and 25% LRE (Solution A) and the other being 25% MH and 75% LRE (Solution B).

Experimental Procedure

100 µL each of Listerine® (Johnson & Johnson), ACT® (Chattem), MH, LRE, Solution A, and Solution B were added via a micro-pipette to the first set of rows and columns of a 96 well microtiter plate (**Figures 2 and 3**). After that, 200 µL each of Listerine®, ACT®, MH, LRE, Solution A and Solution B were added to the next set of rows and columns of a 96 well microtiter plate. These were the control solutions. 200 µL of sterile water was added to the one of columns of the wells and this served as the negative control. Next, the microtiter plate was transferred to an anaerobic chamber (glove box with airlock, ock.2X1, catalog number 50040211). Nitrogen at 40 psi was supplied into the chamber for 15 minutes to create an anaerobic environment. Inside the chamber, 100 µL of the *P. gingivalis* bacteria culture (ATCC W-84) was added to each well of the first set of rows and columns only. Four sets of 200 µL of the bacteria culture were then added to a separate group of four wells to create the positive control. The microtiter plate was then removed out of the anaerobic chamber, sealed completely with tape applied all around and put inside an oven, set at body temperature (36.5-37°C) (Fisher Scientific Isotemp Incubator). The microtiter plate was kept in the oven for 18 hours to incubate the bacteria. After 18 hours, the plate was removed and inserted into a plate reader (Synergy microtiter plate reader) to measure the absorbance of 680nm light by each solution in the well. The absorbance values for each well were then recorded.

Figure 2. Photo of Solutions. Solutions were aliquoted separately or mixed together in a 96-well microtiter plate, bacteria were added to the appropriate wells, and the plate was incubated at 37°C in anaerobic. environment for 18 hours.

Columns (stating from left at the top)
Column 1: Listerine® solution (100 µl)
Column 2: ACT® solution ((100 µl)
Column 3: MH solution ((100 µl)
Column 4: LRE solution + Bacteria
Column 5: 75% MH + 25% LRE solution (100 µl)
Column 6: 25% MH + 75% LRE solution (100 µl)
Column 7: Sterile Water (200 µl)
Column 8: Listerine® solution (200 µl)
Column 9: ACT® solution (200 µl)
Column 10: 75% MH + 25% LRE solution (200 µl)
Column 11 & 12: 25% MH + 75% LRE solution (200 µl)
Column 13 (bottom right side, not shown): LRE solution (200 µl)

Figure 3. Assay Well Setup. Representative image from a replicate experiment of layout of samples on 96 well microtiter plate before bacteria addition.

Table 1 Calculations

First, the average absorbance for each solution was calculated from the 4 measured absorbance values. Then, the average absorbance of the bacteria only solution was added to the average absorbance of the oral treatment only solution. Finally, the average absorbance of the (oral treatment + bacteria) solution was subtracted to determine the ΔAbsorbance.

$$\Delta\text{Absorbance} = (\text{Absorbance}_{\text{Bacteria only}}) + (\text{Absorbance}_{\text{Treatment only}}) - (\text{Absorbance}_{\text{Bacteria and treatment together}})$$

The average absorbance for each solution was calculated from the 4 measured absorbance values. Then, the average absorbance of the (oral treatment + bacteria) solution was subtracted to determine the $R_{absorbance}$.

$$R_{absorbance} = (\text{Absorbance}_{\text{Treatment only}}) - \text{Absorbance}_{\text{Bacteria and treatment together}})$$

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