The Effect of Honey on the Growth of Bifidobacteria

Summary of a research project funded by the National Honey Board and conducted at Michigan State University.
Investigator: Z. Ustunol, Ph.D.

Background

Bifidobacteria are part of a group of bacteria considered important to the health of the gastro-intestinal tract (GI). Clinical studies have associated other beneficial effects such as immune enhancement and anti-carcinogenicity with the presence of bifidobacteria in the GI tract.

One approach for ensuring or increasing the presence of healthful colonic bacteria is to provide them as a probiotic. A probiotic is a live microbial feed supplement, which beneficially affects the host organism by improving its intestinal microbial balance.[1]

Dairy products have been the preferred medium to reintroduce viable populations of lactic acid bacteria and bifidobacteria into the GI tract of both children and adults. Bifidobacteria must remain viable in large numbers in the carrier food to be used with confidence as a dietary adjunct. However, maintaining the viability of bifidobacteria during processing and refrigerated storage has been a challenge to dairy processors.

Another approach to increasing the numbers of bifidobacteria in the GI tract is the incorporation of prebiotics in the diet. A prebiotic is a non-digestible dietary supplement that modifies the balance of the intestinal microflora stimulating the growth and/or activity of the beneficial organisms and suppressing potentially deleterious bacteria.[1] Currently, the most common prebiotics are nondigestible oligosaccharides, such as fructooligosaccharides (FOS) and inulin.

Growth and viability of bifidobacteria in fermented milk can be enhanced significantly by the incorporation of FOS and galacto-oligosaccharides (GOS) in milk prior to fermentation. Honey contains a variety of oligosaccharides varying in their degree of polymerization. The unique composition of honey suggests that it could enhance the growth, activity and viability of

bifidobacteria in milk and thus, fermented dairy products. To evaluate this hypothesis, the following study on growth-promoting and prebiotic activity of honey on bifidobacteria was conducted.

Objectives

The objectives of this research project were:

1. To determine the effect of honey on growth and activity of bifidobacteria in milk and determine its effect on viability of bifidobacteria during refrigerated storage of the fermented milk.
2. To investigate the effect of honey on growth, activity and viability of additional commercially available bifidobacteria strains in milk.
3. To identify the compound(s) in honey that possess bifidobacteria growth-promoting activity.
4. To determine the prebiotic activity of honey and the compound(s) identified above on bifidobacteria.
5. To compare the growth-promoting and prebiotic effects of honey on bifidobacteria to that of commercially available oligosaccharides.

Objective 1. Effect of Honey on Growth, Activity and Viability of Two Strains of Bifidobacteria

Materials and Methods

Bifidobacterial Growth

Samples were prepared by adding 1, 3 or 5 percent of tempered Grade A honey, sucrose, fructose or glucose to 12 percent (w/v) reconstituted nonfat dry milk (NDM). A control of NDM without sweetener added was also prepared. Samples were pasteurized at 70 °C for 30 minutes and cooled to 37 °C. Each sample was divided into two portions and inoculated at a 5 percent level with either *Bifidobacterium* Bf-1 or *Bifidobacterium* Bf-6 (Systems Bio-Industries) propagated in an MRS medium with 5 percent lactose added (MRSL) (Difco Laboratories, Detroit, MI). Both Bf-1 and Bf-6 are commercial bifidobacteria strains.

Inoculated milk samples were incubated at 37 °C for 48 hours. A sample was taken every 12 hours and diluted (1:10, v/v) with 0.2 percent EDTA (pH 12) and turbidity was measured at 640 nm as described by Hughes and Hoover (1995).[2] Uninoculated NDM was used as the blank. The specific growth rate (μ) for each sample was calculated according to the following equation (Desjardin *et al*, 1991[3]):

$$\mu = (\text{Ln } X_2 - \text{Ln } X_1)/(t_2 - t_1)$$

where X_2 and X_1 are the cell densities at time t_2 and t_1, respectively. Mean doubling time (T_d) for bifidobacteria was calculated as follows:

$$T_d = \text{Ln}2 / \mu$$

The pH of the samples was also monitored.

Bifidobacterial Activity

To determine culture activity, NDM containing 5 percent (w/v) honey, sucrose, fructose, or glucose and fermented with the two strains of bifidobacteria were prepared for High Performance Liquid Chromatography (HPLC) analysis using the method described by Dubey and Mistry (1996).[4] One hundred μL of 15.8 N HNO_3 and 14.9 mL of 0.009 N H_2SO_4 was added to 1.5 mL of sample. The samples were centrifuged at 5000 x g for 10 minutes. The supernatant was filtered using 0.45 μm membrane filter and was eluted through a reverse phase Superclean tube and stored in HPLC vials at -20 °C until the HPLC analysis.

Culture activity was determined by measuring the end products of fermentation (lactic acid and acetic acid) using HPLC. An Aminex HPX-87H Column (300mm x 7.8mm) and guard column with disposable cartridges H^+ maintained at 65 °C was used for analysis.

Bifidobacterial Viability
Each strain of bifidobacteria was cultured in the presence of 5 percent (w/v) honey or other sweetener. All samples were fermented at 37 °C for 36 hours and stored for four weeks at 4 °C. Viability of each culture in the fermented milk was determined at one-week intervals by taking 1 mL of each sample and diluting it with 99 mL of 0.1 percent (w/v) Bactopeptone and making the subsequent dilutions and enumerating using MRSL agar. The inoculated plates were incubated anaerobically at 37 °C for 48 hours using Gas Pak® (Becton Dickinson Microbiology Systems, Cockeysville, MD). The colonies were counted and percent viability was determined.

Results

Bifidobacterial Growth
The effect of honey on specific growth rate of both bifidobacteria strains was dependent on the strain of the bifidobacteria and concentration of honey in the growth medium. Overall, specific growth rate of both Bf-1 and Bf-6 was enhanced with an increase in the concentration of honey in NDM. Concentrations of 3 percent and 5 percent were most effective in enhancing growth of either strain compared to the NDM control and other sweeteners. Five percent honey was the most effective on Bf-1. Sweetener concentration other than honey did not appear to have an effect on stimulating bifidobacteria growth.

Bifidobacterial Activity
Activity of Bf-6 was greatly enhanced when this organism was grown in the presence of honey as evidenced by acetic acid and lactic acid production and their ratio. The effect on acetic acid production was more pronounced. However, results with Bf-1 did not confirm these results because no acetic acid was produced. (Note: Acetic acid production is not desirable in milk fermentation.) These results suggest that the effect of honey on activity of bifidobacteria may be strain-specific.

Bifidobacterial Viability
As expected, bifidobacteria counts in the 12 percent NDM declined during the four weeks of refrigerated storage. Although bifidobacteria counts declined steadily, in the case of Bf-1, higher cell numbers were maintained in the presence of 5 percent honey compared to the control or other sweeteners. In the case of Bf-6, higher cell numbers and higher viability was observed within the first week of refrigerated storage. However, these differences were not significant after the second week and are likely strain-specific.

In order to confirm these results and to explore the possible strain-specific differences, these experiments were repeated with the most common commercial strains of bifidobacteria.

Objective 2. Effect of Honey on Growth, Activity and Viability of Six Commercial Bifidobacteria Strains

Materials and Methods

All the commercially available bifidobacteria strains from three major dairy suppliers (*Bifidobacterium infantis*, *Bifidobacterium bifidum, and Bifidobacterium longum* from Chr. Hansen's Laboratories Inc., Milwaukee, WI; *Bifidobacterium* ssp Bf-1 and Bf-6 from Systems Bio-Industries (SBI), Waukesha, WI; *Bifidobacterium infantis* from Rhone Poulenc Inc, Madison, WI) were obtained. Tempered Grade A honey, sucrose, fructose or glucose was added at a 5 percent (w/v) level to 12 percent (w/v) reconstituted nonfat dry milk (NDM). A concentration of 5 percent was selected because this was determined to be the optimum amount in promoting bifidobacteria growth in previous studies. Samples were pasteurized at 70 °C for 30 minutes and cooled to 37 °C. Next, each sample was divided into the appropriate number

of portions and inoculated at a 5 percent level with each of the commercial bifidobacteria strains previously propagated in MRSL (MRS medium with 5 percent lactose added). Inoculated samples were incubated at 37 °C for 48 hours. A sample was taken every 12 hours and diluted (1:10, v/v) with 0.2 percent EDTA (pH 12) and turbidity was measured at 640 nm as described by Hughes and Hoover (1995).[2] Uninoculated NDM was used as the blank. Mean doubling times were calculated as described above. The pH was also determined.

Growth and viability determination of different bifidobacteria strains in the presence of various sweeteners during refrigerated storage was conducted over four weeks. Fermented milk samples were prepared as described above. All samples were then stored for four weeks at 4 °C. Viability of each bifidobacteria strain in the fermented milk was determined at one week intervals by taking 1 mL of each sample and diluting it with 99 mL of 0.01 percent (w/v) Bactopeptone and making the subsequent dilutions and enumerating using MSRL agar. The inoculated plates were incubated anaerobically at 37 °C for 48 hours using Gas Pak®. The colonies were counted and percent viability was determined.

Results

Mean doubling times (T_d) were significantly ($p<0.05$) reduced compared to other sweeteners when *Bifidobacterium* Bf-1, Bf-6 and *Bifidobacterium longum* were grown in the presence of honey. In the case of *Bifidobacterium infantis* (Rhone Poulenc), although the mean doubling time in the presence of honey was significantly reduced ($p<0.05$) compared to sucrose and glucose, it was similar to the mean doubling time observed in the presence of fructose. Since fructose is a main component of honey, this reduction in mean doubling time may be due to fructose present in honey. In the case of *Bifidobacterium infantis* (Chr. Hansen) and *Bifidobacterium bifidum* (Chr. Hansen), the mean doubling times were increased compared to other sweeteners suggesting strain-specific utilization of different sweeteners by these organisms.

The lactic and acetic acid production by bifidobacteria in the presence of various sweeteners was determined by HPLC analysis. Lactic acid production was higher ($p<0.05$) with Bf-1 and Bf-6 strains when grown in the presence of honey. Acetic acid

production was not as significantly affected. These two organisms also showed the shortest mean doubling time in the presence of honey, confirming enhancement of both growth and activity of these organisms in the presence of honey. Mean doubling times data did not support the results for lactic and acetic acid production by other strains.

Results of the storage study show that the counts for all strains except *B. longum* were higher or similar to other sweeteners when grown in the presence of honey over 28 days of refrigerated storage. Viability of Bf-6 and Bf-1 was significantly enhanced when grown in the presence of honey up to 14 days and up to 21 days, respectively. In the case of *B. infantis* (Rhone Poulenc) viability was maintained significantly higher ($p<0.05$) during 14 through 28 days of refrigerated storage in the presence of honey. The other strains did not show any specific trends.

Table 1 summarizes the results from the experiments in objectives 1 and 2.

Table 1. Effect of Honey on Bifidobacteria Growth Rate, Activity and Viability.

STRAIN	GROWTH RATE (↓ doubling time at 37 °C for 48 hours)	ACTIVITY (↑ lactic and acetic acid production at 37 °C for 36 hours)	VIABILITY (↑ cell count during 28 days refrigerated storage)
OBJECTIVE 1 STUDIES			
Bifidobacterium Bf-1 (Systems Bio-Industries)	Enhanced (3 percent and 5 percent honey)	No acetic acid production	Higher than control or other sweeteners (5 percent honey)
Bifidobacterium Bf-6 (Systems Bio-Industries)	Enhanced (3 percent and 5 percent honey)	Enhanced (5 percent honey)	Higher than control or other sweeteners week 1 only (not significant)
OBJECTIVE 2 STUDIES (Note: all sweeteners added at 5 percent w/v)			
Bifidobacterium Bf-1 (Systems Bio-Industries)	Enhanced compared to other sweeteners	Lactic acid production significantly higher compared to other sweeteners	Higher or similar to other sweeteners (significantly enhanced up to 21 days storage)
Bifidobacterium Bf-6 (Systems Bio-Industries)	Enhanced compared to other sweeteners	Lactic acid production significantly higher compared to other sweeteners	Higher or similar to other sweeteners (significantly enhanced up to 14 days storage)
Bifidobacterium infantis (Rhone Poulenc Inc.)	Enhanced compared to sucrose and glucose; similar to fructose		Higher or similar to other sweeteners (significantly higher after 14 days through 28 days storage)
Bifidobacterium infantis (Chr. Hansen's Laboratories Inc.)	Decreased compared to other sweeteners		Higher or similar to other sweeteners
Bifidobacterium longum (Chr. Hansen's Laboratories Inc.)	Enhanced compared to other sweeteners		Similar to fructose and higher than sucrose but less than glucose
Bifidobacterium bifidum (Chr. Hansen's Laboratories Inc.)	Decreased compared to other sweeteners		Higher or similar to other sweeteners

Objective 3. Compound(s) in Honey that Possess Bifidobacteria Growth-Promoting Activity

Materials and Methods

Purified versions of the major sugar fractions of honey (maltose, panose, erlose, isomaltotriose, turanose, melezitose, maltrotriose) were obtained from Sigma (St. Louis, MO). Honey and sucrose were the controls. Twelve percent nonfat dry milk (NDM) was divided into 1.5 mL aliquots to which 75 mg of one of the sugars was added. All samples were pasteurized (70 °C, 30 minutes), cooled to 37 °C and inoculated at a 5 percent (v/v) level with Bf-1, Bf-6 or *B. longum*. These strains were selected because they were shown to exhibit the highest growth rate in the presence of honey in the results from the experiments performed in objective 2. After mixing the cultures, all tubes were incubated at 37 °C for 48 hours. A sample was taken every 12 hours to determine the effect of the different honey fractions on growth rate and mean doubling time of each bifidobacteria stain as described previously. All experiments were replicated three times.

Results

Honey was the most effective in stimulating the growth of Bf-6 and *B. longum* compared to sucrose and the individual fractions of honey. There appears to be a synergistic effect among the different sugar components of honey in enhancing bifidobacteria growth. Panose was the component of honey that enhanced the growth of Bf-1. This effect was more significant ($p<0.05$) than the effect of honey and the different fractions of honey except for maltose.

Objective 4. Prebiotic Activity of Honey and Relevant Components on Bifidobacteria

Materials and Methods

Prebiotic activity of honey was determined in vitro using strains of bifidobacteria that have been reported to make up the microflora of the human GI tract. Reinforced Clostridial Medium (RCM) containing honey, glucose, sucrose, or fructose at 5 percent (w/v) level was filter sterilized and each sample was divided into two. Blanks had no sweetener added. Autoclaved RCM with or without oxgall (bile salts) was added to each of the above solutions to give final concentrations of 0 or 2 percent as described by Clark and Martin (1994)[5]. Next, each solution was divided into five aliquots and inoculated with one of the strains of bifidobacteria reported to make up the microflora of the human GI tract [*B. longum* (American Type Culture Collection 15707), *B. adolescentis* (ATCC 15705), *B. breve* (ATCC 15700), *B. bifidum* (ATCC 129521) and *B. infantis* (ATCC 15697)] at 5 percent (v/v) level. All samples were incubated anaerobically at 37 °C for 48 hours. Growth rate of each strain was determined every 12 hours over the 48-hour incubation. Mean doubling times were calculated as described previously. All experiments were replicated three times.

The ability of other microbial strains that are predominant in the gut to ferment honey was also investigated. Sucrose or Grade A clover honey was added to thioglycollate medium and sterilized. Each batch was divided into six portions and inoculated at a 5 percent (v/v) level with *Enterococcus faecalis* (ATCC 27274), *Clostridium perfringens* (ATTCC 12919), *Eubacterium aerofaciens* (ATCC 25986), *Ruminococcus productus* (ATCC 27340), *Bactriodes thetaiotamicron* (ATCC 29148) or *Bifidobacterium longum* (ATCC 15707). Inoculated samples were incubated at 35 °C for 48 hours. Growth of the culture was measured spectrophotometrically

every 12 hours at 610 nm. Specific growth rate (μ) and mean doubling time (T_d) were calculated as described previously.

Results

In the presence of 2 percent oxgall, growth of all bifidobacteria strains was inhibited regardless of the type of sweetener present. The only exception was *B. breve* whose growth was exclusively enhanced in the presence of honey when oxgall was present. In the absence of oxgall, all bifidobacteria strains showed the highest growth rate during the first 12 hours of incubation when grown in the presence of honey. After 12 hours, this effect was only apparent with *B. infantis* otherwise there were no differences in the growth rate of different bifidobacteria strains in the presence of different sweeteners. This was also apparent with mean doubling times of these strains.

Measurements of the specific growth rates and mean doubling times of the other predominant gut bacteria grown with honey and sucrose indicate that there were no differences in their growth when averaged over 48 hours.

Objective 5. Comparison of the Growth-Promoting and Prebiotic Effects of Honey and Commercially Available Oligosaccharides on Bifidobacteria

Materials and Methods

Twelve percent NDM was reconstituted and divided into four portions. Honey, inulin (trade name Frutafit from Imperial Suiker Unie, Sugarland, TX), GOS (trade name Galactooligosucrose L500 from Samyang Genex Co., Ltd. Seoul, Korea) or FOS (trade name Raftilose from Orafti, Malvern, PA) was added at 5 percent (w/v) level. The mixtures were pasteurized (70 °C, 30 minutes) divided into six portions and inoculated with one of the six commercial strains of bifidobacteria used in objective 2 experiments. The inoculated samples were incubated anaerobically at 37 °C for 48 hours. A sample was taken every 12 hours to determine the effect of the different commercial oligosaccharides and honey on the growth rate and mean doubling time of each commercial strain of bifidobacteria as described above. This same experiment was repeated a second time using the five bifidobacteria strains (ATCC cultures) used in objective 4. All experiments were replicated three times.

Results

Honey appeared to enhance the growth rate of Bf-1 compared to other commercial oligosaccharides. However, this difference was not statistically significant when mean doubling times were compared. In the case of ATCC cultures, honey enhanced the growth of *B. longum, B. breve*, and *B. infantis* better than inulin or FOS but not as well as GOS. In the case of *B. bifidum*, honey was not as effective as inulin but was more effective than FOS or GOS. The effect of honey on *B. adolescentis* appeared to be similar to that of inulin.

Table 2 summarizes the results from the experiments in objectives 4 and 5.

Table 2. Growth-promoting and Prebiotic Activity of Honey and Oligosaccharides

Growth-promotion • Honey was more effective than individual components in two (Bf-6 and *B. longum*) of the three strains tested • No statistical difference in growth of the six bifidobacteria strains in the presence of honey and commercial oligosaccharides (FOS, GOS and inulin) Prebiotic Activity • Growth of *B. longum, B. breve* and *B. infantis* enhanced more with honey than with inulin or FOS but not as well as GOS • Growth of *B. bifidum* enhanced more with honey than with FOS and GOS but not as well as inulin • Growth of *B. adolescentis* with honey or inulin appears to be similar

Conclusions

This research project has a number of key findings:

- Honey enhanced the growth, activity and viability of commercial strains of bifidobacteria typically used in the manufacture of fermented dairy products. However, this effect was strain-specific.
- There was a synergistic effect among the carbohydrate components of honey in promoting growth and activity of bifidobacteria.
- The effect of honey on the growth and activity of intestinal *Bifidobacterium* spp was similar to that of commercial oligosaccharides (FOS, GOS, and inulin).

This research provides promising results on the growth-promoting and prebiotic activity of honey on bifidobacteria.

References

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