Growth and Acid Production by Lactic Acid Bacteria and Bifidobacteria Grown in Skim Milk Containing Honey

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ABSTRACT: Twelve percent nonfat dry milk containing 5% (w/w) honey, fructose, or sucrose were pasteurized and inoculated with *Streptococcus thermophilus, Lactobacillus acidophilus*, *Lactobacillus delbrukeii* subsp *bulgaricus*, or *Bifidobacterium bifidum*. Inoculated tubes were incubated at 37 °C, 24 h. Samples were collected at 0 and 24 h and examined for (a) viability of bacteria, and (b) levels of fermentation end products (lactic and acetic acids) as measured by HPLC. Honey supported growth of all 4 organisms similar to other sweeteners and was not inhibitory. Lactic acid production was similar for all, except for bifidobacteria and was not influenced by sweetener type. Although lactic acid production was enhanced ($p < 0.05$) when bifidobacteria were grown in the presence of honey, acetic acid production was not affected. Various oligosaccharides found in honey may be responsible for enhanced lactic acid production by bifidobacteria.

Key words: bifidobacteria, lactic acid bacteria, milk, honey, sucrose, fructose

Introduction

LACTIC ACID BACTERIA ARE COMMON starter cultures used by the dairy industry to manufacture fermented dairy products. More recently, probiotic organisms such as bifidobacteria also have been incorporated into fermented dairy products due to their reported health benefits. These benefits include inhibition of bacterial pathogens, reduction of colon cancer risk, stimulation of the immune response, and reduction of serum cholesterol levels (Tannock 1999; Salminen and others 1993; Sanders 1993). Due to the perceived prophylactic and therapeutic properties of the live cultures present, consumption of fermented dairy products such as yogurt containing viable cultures continues to increase steadily in the United States (Putnam and Allhouse 1993).

Sucrose and corn syrup have been the traditional and most commonly used sweeteners in the dairy industry. Although honey has been added as a flavoring agent to yogurt and ice cream, it is typically not used to replace sucrose or corn syrup in fermented dairy products (that is, yogurt), since it is believed that honey may be inhibitory to lactic starter cultures (NHB 1996). In recent years, however, there has been increasing interest in the use of "natural" and "healthy" food additives and incorporating health-promoting substances into the diet. Due to its "healthy" and "natural" image (Lagrange and others 1991), honey has been gaining interest as a substitute sweetener in foods such as yogurt. Honey-sweetened products are viewed as value-added and consumers are willing to pay up to 13% more for them compared to products containing other sweeteners (NHB 1996).

Honey is a natural syrup containing primarily fructose (38.5%) and glucose (31.3 %). Other sugars in honey include maltose (7.2%), sucrose (1.5%), and various oligosaccharides (4.2%). The average pH of honey is 3.9. Honey also contains a variety of organic acids such as acetic, butyric, citric, formic, gluconic, lactic, malic, pyroglutamic, and succinic acids (0.17 to 1.17%), which give the product an average pH of 3.9 (NHB 1996).

Inhibitory properties of honey against pathogens such as *Bacillus cereus, Listeria monocytogenes*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Salmonella typhi*, *Salmonella typhimurium, Shigella* sp., *Staphylococcus aureus*, *Vibrio cholera* (Molan 1992a,b) and *Helicobacter pylori* (Somal and others 1994) have been demonstrated. Microbial inhibition of honey has been attributed to its low pH as well as the presence of enzymes such as glucose oxidase, catalase, and lysozyme. Compounds such as 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl- 3,4,5-trimethoxybenzoate, and 3,4,5- trimethoxybenzoic acid and methyl 3,5-dimethoxy-4-hydroxybenzoate (methyl syringate) have been isolated from manuka honey and their antimicrobial properties have been demonstrated by Molan and Russell (1989) and Russell and others (1990). Structurally, these aromatic acids are similar to benzoic and 4-hydroxybenzoic acid that are typically used in foods as preservatives to inhibit bacterial growth. The antibacterial activity of manuka honey is reportedly heat stable and unaffected by exposure to light (Molan and Russell 1989). Information is presently lacking on the ability of lactic acid bacteria and bifidobacteria to metabolize honey and grow in this product. Therefore, the objective of this research was to (a) investigate the ability of lactic acid bacteria and bifidobacteria to grow in the presence of clover honey in comparison to sucrose and fructose, and (b) determine the levels of lactic and acetic acid produced by these organisms when grown in the presence of honey, fructose, and sucrose. Clover honey, a light-colored honey, was selected because of its abundant supply in the United States and its higher commercial value as compared to dark-colored honeys (Wooten and others 1976; White 1978).

Materials and Methods

Culture preparation

Commercial strains of *Streptococcus thermophilus* (St-133) and *Lactobacillus delbrukeii* subsp *bulgaricus* (Lr-78), along with the probiotic organisms *Lactobacillus acidophilus* (La-7) and *Bifidobacterium bifidum* (Bf-13), were obtained from Systems Bio-Industries (Waukesha, Wis., U.S.A.). Each strain underwent 2 successive 24 h/37 °C

transfers in the De Man Rogosa Sharpe (MRS) medium (Difco, Detroit, Mich., U.S.A.) (De Man and others 1960). MRS containing 5% (w/v) lactose (MRSL) was used with *B. bifidum* which was incubated at 37 °C, 24 h, anaerobically using Gas Paks (Becton Dickinson Co., Cockeysville, Md., U.S.A.). All cultures were centrifuged 15 min at 1000 × g at 4 °C and resuspended in 12% w/v nonfat dry milk pasteurized at 63 °C, 30 min (NDM; Difco) to obtain approximately 10^8 CFU/mL.

Growth of lactic acid bacteria and bifidobacteria in the presence of various sweeteners

A 12% (w/v) NDM (Difco) solution was prepared and divided into 4 portions. Sucrose (J.T. Baker, Phillipsburg, N.J., U.S.A.,), fructose (Sigma, St. Louis, Mo., U.S.A.,) or tempered grade A clover honey (W. Stoller's Honey Inc., Latty, Ohio, U.S.A.) were added at a level of 5% (w/w) to each NDM solution. The control was devoid of added sweetener. The sweetened mixtures were further divided into 4 aliquots, pasteurized at 70 °C, 15 min, and cooled to room temperature. Next, the NDM with and without sweetener was inoculated to contain 5% (v/v) *S. thermophilus*, *L. delbrukeii* subsp *bulgaricus, L. acidophilus* and *B. bifidum,* and incubated at 37 °C for 24 h. *B. bifidum* samples were incubated anaerobically using Gas Paks. Initially and after 24 h of incubation, a sample was taken for pH determination and 1 mL of each thoroughly mixed fermented milk sample was diluted with 99 mL of sterile 0.1% (w/v) peptone (Difco) and plated on MRS or MRSL agar containing 1.5% agar (Difco) to determine numbers of lactic acid bacteria and bifidobacteria, respectively. The inoculated plates were incubated aerobically at 37 °C for 48 h. Bifidobacteria plates were incubated anaerobically using Gas Paks (Becton Dickinson, Co) under similar conditions. Appropriate colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, Pa., U.S.A.).

Lactic and acetic acid determination

Levels of lactic and acetic acid produced by lactic acid bacteria and bifidobacteria when grown in NDM containing 5% sucrose, fructose, and honey were determined using the HPLC as described by Dubey and Mistry (1996). One hundred µL of 15.8 N HNO_3 and 14.9 mL of 0.009 N H_2SO_4 were added to each 1.5 mL of thoroughly mixed sample and centrifuged at 5000 x g for 10 min. The supernatant was filtered through Whatman #1 filter paper and a 0.22 µm membrane filter (Millipore Corp., Bedford, Mass., U.S.A.), eluted through a reverse-phase Supelclean tube (Supelco Inc., Bellefonte, Pa., U.S.A.), and stored in HPLC vials at −20 °C until HPLC analysis. The HPLC system (Waters Associates, Inc., Milford, Mass., U.S.A.) consisted of a M-45 solvent delivery system, a 486 UV/Vis tunable absorbance detector, and a 730 data module. An Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and a guard column with disposable cartridges H^+ (Bio-Rad Laboratories) maintained at 65 °C were used for the analysis. The degassed mobile phase of 0.009 N H_2SO_4 filtered through a 0.45 µm membrane filter (Millipore Corp.) was used at a flow rate of 0.6 mL/min. The wavelength of detection was optimized at 220 nm for the organic acids being quantitated (Bouzas and others 1991). Standard solutions of organic acids (lactic and acetic acid; Sigma, St. Louis, Mo., U.S.A.) were prepared to establish elution times and calibration curves.

Statistical Analysis

Each experiment was independently replicated 3 times in a completely randomized design. All analyses and plating were done in triplicate. Statistical analysis was conducted using Sigma Stat 2.0 (Jandel Corp., San Rafael, Calif., U.S.A.). Appropriate comparisons were made using Student-Newman-Keuls test for multiple comparisons. A $p < 0.05$ was considered statistically significant.

Table 1—Growth (log CFU/mL) of lactic acid bacteria and bifidobacteria in skim milk as influenced by sweetener type

Sweetener	*Streptococcus thermophilus*		*Lactobacillus delbrueckii* subsp *bulgaricus*		*Lactobacillus acidophilus*		*Bifidobacterium bifidum*	
	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
Sucrose	7.08	8.57	7.58	8.69	8.18	9.59	7.79	8.36
Fructose	7.00	8.38	7.38	8.62	8.30	9.26	7.67	8.46
Honey	7.08	8.43	7.36	8.60	8.38	9.43	7.92	8.62
Control	7.00	8.59	7.38	8.66	8.32	9.46	7.89	8.56

There were no statistical differences between treatments; comparisons are made only within the same column.
n = 3 for all treatments.

Table 2—The pH of skim milk fermented with lactic acid bacteria as influenced by sweetener type

Sweetener	*Streptococcus thermophilus*		*Lactobacillus delbrueckii* subsp *bulgaricus*		*Lactobacillus acidophilus*	
	0 h	24 h	0 h	24 h	0 h	24 h
Sucrose	6.13±0.06	3.92±0.14	5.88+0.09	3.93±0.06	5.81±0.09	3.92±0.06
Fructose	6.14±0.08	3.91±0.13	5.84+0.12	3.94±0.07	5.83±0.09	3.82±0.03
Honey	6.05±0.03	3.88±0.12	5.90+0.10	3.90±0.06	5.82±0.06	3.79±0.03
Control	6.13±0.09	3.91±0.14	5.93+0.08	3.88±0.04	5.86±0.07	3.94±0.12

There were no statistical differences between treatments; comparisons are made only within the same column.
n = 3 for all treatments.

Results and Discussion

FOLLOWING 24 H OF INCUBATION, LACTIC acid bacteria attained higher populations when grown in NDM containing sucrose and unsupplemented NDM. However, populations were not significantly higher ($p > 0.05$) when compared to populations obtained in NDM supplemented with fructose or honey (Table 1). Consequently, honey, sucrose, and fructose all supported similar growth of these 3 microorganisms, and honey was not inhibitory at the 5% level. In the case of bifidobacteria, although not statistically significant, higher cell numbers were obtained when this organism was grown in the presence of honey. These results are contrary to those of Curda and Plockova (1995), who added either unheated or sterilized honey to skim milk at levels of 0, 1, 3, 5, or 10% (w/v) and monitored growth of both *Lactobacillus acidophilus* and a mesophilic starter culture containing *Lactococcus lactis* subsp *cremoris*, *Lactococcus lactis* subsp *lactis*, and *Lactococcus lactis* subsp *lactis* biovar. *diacetylactis* by measuring imped-

Table 3—The pH of skim milk fermented with *Bifidobacterium bifidum* as influenced by sweetener type

Sweetener	0 h	24 h
Sucrose	6.03±0.08	5.10±0.22
Fructose	6.00±0.04	5.10±0.14
Honey	6.03±0.07	4.55±0.09*
Control	6.09±0.07	5.06±0.05

*Significantly different from other treatments ($p < 0.05$); comparisons are made only within the same column.
n = 3 for all treatments.

Table 4—Lactic acid production in skim milk fermented with lactic acid bacteria as influenced by sweetener type

Sweetener	Lactic acid (mM)					
	Streptococcus thermophilus		*Lactobacillus delbrueckii* subsp *bulgaricus*		*Lactobacillus acidophilus*	
	0 h	24 h	0 h	24 h	0 h	24 h
Sucrose	1.8 ±0.06	55.0±7.4	5.3±1.3	89.4±19.3	7.2±1.1	130.0±17.9
Fructose	1.4±0.08	68.3±7.8	5.1±1.1	115.0±15.9	3.0±1.8	127.0±20.6
Honey	0.0±0.03	70.6±23.6	5.0±1.2	112.0±22.1	5.7±0.6	135.0±32.3
Control	2.2±0.09	72.7±8.2	3.8±0.8	106.0±33.0	4.8±0.7	117.0±25.9

There were no statistical differences between treatments; Comparisons are made only within the same column.
n = 3 for all treatments.

ance changes in the media. *L. acidophilus* was inhibited by honey at levels ≥ 5% regardless of the heat treatment. In contrast, growth of the mesophilic starter culture was inhibited by 10% unheated honey, but not by the sterilized honey at any of the concentrations tested.

These researchers used honey common to the central Bohemian countryside. Antimicrobial characteristics of honey reportedly vary, depending on floral sources of the honey (Molan 1992a). Thus, the honey used in their studies may have different antimicrobial properties than honeys indigenous to the United States. Our results are also contrary to those of Molan (1992a,b), who showed honey to be inhibitory to *Streptococcus salivarius*, *Streptococcus faecalis*, and *Streptococcus pyogenes*. However, the level of honey used in these studies were not specified.

Using the lactic acid bacteria cultures, the pH of skim milk dropped as expected following 24 h incubation and no statistical differences in pH were observed between the different sweeteners, thus indicating that honey supported acid production in a similar manner to other sweeteners and was not inhibitory (Table 2). However, the pH decrease was significantly greater ($p < 0.05$) when *B. bifidum* was grown in the presence of honey compared to sucrose, fructose, or the no-sweetener-added control, suggesting that acid production by *B. bifidum* was enhanced by honey (Table 3). Therefore, further studies were undertaken to determine the influence of these sweeteners on acid production by lactic acid bacteria and bifidobacteria.

Table 4 shows the levels of lactic acid produced when lactic acid bacteria were grown in the presence of sucrose, fructose, and honey, and in the unsupplemented NDM. Lactic acid production was not influenced by sweetener type and was similar in all treatments ($p > 0.05$), confirming that honey supported lactic acid production by these organisms in a similar manner to other sweeteners, and was not inhibitory. In case of *B. bifidum,* however, lactic acid production was significantly enhanced ($p < 0.05$) when *B. bifidum* was grown in the presence of honey with production of lactic acid 2.5 and nearly 4 times greater as compared to sucrose and fructose, respectively (Table 5). The results obtained with fructose exclude the possibility that fructose in honey may be the contributing factor to this enhanced lactic acid production by *B. Bifidum*.

Bifidobacteria is known to be a fastidious organism. Numerous researchers have reported that bifidobacteria grows poorly in milk (Biavati and others 1992; Klaver and others 1993; Shah and others 1995; Kailasapathy and Rybka 1997; Dave and Shah 1997, 1998; Rybka and Fleet 1997), and therefore, requires specific growth factors (Klaver and others 1993; Modler 1994; Poch and Bezkorovainy 1988; Roy and others 1990; Dave and Shah 1998). Enhanced acid production by *B. Bifidum* in the presence of honey was somewhat unexpected. Oligosaccharides previously have been shown to increase growth, activity, and viability of *Bifidobacterium* spp in milk. Fructooligosaccharide (FOS) and galactooligosaccharide (GOS) were more effective than inulin (Shin and others 2000). Hopkins and others (1998) reported that GOS and FOS, having lower degrees of polymerization (DP), were best in supporting growth of bifidobacteria. In contrast, carbohydrates with high DP were poor bifidobacterial substrates. Very little is known about the mechanism of carbohydrate uptake by bifidobacteria; however, it appears likely that the substrate transport systems may be more efficient for dimeric and oligomeric carbohydrates. Honey has a variety of oligosaccharides (Da Costa Leite and others 2000; Swallow and Low 1990; Weston and Brocklebank 1999) with low DP. These oligosaccharides result from the action of honeybee α-D-glucosidase, which catalyzes the transfer of α-D-glucopyranosyl groups from sucrose to an acceptor carbohydrate. These low DP oligosaccharides may be the favored substrate for bifidobacterial support, thereby enhancing lactic acid production as observed in the present study.

Bifidobacteria fermentation also results in the production of acetic acid. Typically, in a synthetic media, 3 moles of acetic acid and 2 moles of lactic acid are produced per 2 moles of glucose (Scordovi and Trovelli 1965). Therefore, the influence of fructose, sucrose, or honey on acetic acid production by *B. Bifidum* was also investigated. In contrast to lactic acid, production of acetic acid by *B. bifidum* was not enhanced in the presence of honey with similar amounts of acetic acid produced regardless of the carbohydrate source (Table 5). Although lactic acid production is essential in fermented dairy foods, high concentration of acetic acid can result in an undesirable vinegar flavor in fermented dairy foods.

In summary, honey was not inhibitory to *S. thermophilus*, *L. delbrueckii* subsp *bulgaricus, L. acidophilus*,or *B. bifidum* when added to NDM at a level of 5% with honey enhancing lactic acid production by bifidobacteria. Honey could be a suitable sweetener for manufacturing fermented dairy products such as yogurt. Furthermore, understanding the substrate preferences of bifidobacteria will facilitate development of probiotics, prebiotics, and synbiotics.

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Table 5—Lactic and acetic acid production in skim milk fermented with *Bifidobacterium bifidum* as influenced by sweetener type

	Lactic acid (mM)		Acetic acid (mM)	
Sweetener	**0 h**	**24 h**	**0 h**	**24h**
Sucrose	0.0	10.4±1.2	0.0	28.4±5.7
Fructose	0.0	7.2±1.6	2.3±2.0	21.4±6.5
Honey	0.0	26.2±4.4*	0.0	28.3±8.3
Control	0.0	4.6±0.9	0.0	15.9±5.6

*Significantly different from other treatments ($p < 0.05$); Comparisons are made only within the same column.
n = 3 for all treatments.

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