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Full Length Research Paper

Growth and viability of yogurt starter organisms in honey-sweetened skimmed milk

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Lactic acid bacteria (LAB): *Streptococcus thermophilus* TA 040 and *Lactobacillus delbrueckii* spp. *bulgaricus* Lb 340, were cultured in reconstituted (10%, w/v) skimmed milk with 5 or 10% (w/v) polyfloral or unifloral honey. Inoculated samples were incubated aerobically at 42°C until milk coagulation. Samples were collected at 2 h intervals and examined for biomass and pH changes. Cell viability and post-acidifying activity of both strains during 28 days of storage at 4°C were also measured. A higher increase (P<0.05) in growth and acidifying activity of *S. thermophilus* monocultures was observed when 10% honey was added. However, *L. bulgaricus* did not show such a marked increase in its growth capacity. In associated cultures, LAB growth was slightly inhibited, whereas curdling time was prolonged by an hour when 10% honey was added and yogurt acidity was moderate. Cell viability improved by 5 to 6.6% for *S. thermophilus* and 10% for *L. bulgaricus* in pure honey-sweetened cultures over 28 days of refrigerated storage. This protective effect of honey on LAB cell viability was also observed in associated cultures (10 to 12% comparatively to the control).

Key words: *Streptococcus thermophilus*, *Lactobacillus bulgaricus,* honey, growth, viability.

INTRODUCTION

A greater concern in the use of natural and healthy new substances as food additives and prebiotics has been recently raised (Kneifel and Pacher, 1993). Incorporation of sweeteners into fermented dairy products, in order to improve micro-organism growth and viability, has been of much interest in the dairy industry (Tamime and Robinson, 1985).

Lactic acid bacteria (LAB) are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. These bacteria are broadly used in the production of fermented food products, such as yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), cheese (*Lactococcus* spp.), sauerkraut (*Leuconostoc* spp.) and sausage (Daly et al., 1998). Lactic acid production owing to food carbohydrate consumption is one of the most desirable effects of LAB metabolic activity, inducing an important decrease in pH which may drop to as low as 4, low enough to inhibit the growth of a wide variety of microorganisms including the most common human pathogens, thus allowing extended shelf life in such food (Sanders, 1988; Salminen et al., 1998). The acidity changes the texture of the food due to protein precipitation. Also, the biochemical conversions involved in growth enhance the flavour. The fermentation process (including microbial growth) could be self-limited because of LAB sensitivity towards such low pH.

Honey is a syrup containing primarily fructose (38.5%) and glucose (31.3%) (Ustunol and Gandhi, 2001) and is considered a natural sweetener giving rise to many benefits (Bansal et al., 2005). Antioxidants such as hydrogen peroxide and non peroxide components of honey inhibit growth of *Shigella, Listeria monocytogenes*, and *Staphylococcus aureus* helping in food preservation (Molan, 1992). However, *Clostridium botulinum* may be present in small amounts in honey (Nevas et al., 2005).

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Honey could also be used as a potential natural source of antioxidants to reduce negative effects of polyphenol oxidase browning in fruits and vegetables (Chen et al., 2000). It is also found to lower bacterial counts in refrigerated poultry and fish products (Nagai et al., 2006).

Various non-milk additives are used in the dairy Industry in order to improve the positive sensory properties of dairy products. Honey, which is becoming a popular ingredient in dairy products (Tamime and Robinson, 1985), has the ability to reduce the sourness of solutions which can enhance consumer acceptability of acidic products such as yogurt (Varga, 2006). In spite of previously reported results on the inhibitory effects of honey against LAB (Čurda and Plocková, 1995; Roumyan et al., 1996), recent reports suggest that it could be used as a sweetener and a good cell protective agent in fermented milk products (Chick et al., 2001; Varga, 2006). Thus, when used at suitable levels, honey does not inhibit the growth of common bacteria such as *Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus delbrueckii* and *Bifidobacterium bifidum* which contribute to maintain a healthy gastrointestinal tract (Sanz et al., 2005; Ezz El-Arab et al., 2006).

Yogurt starter organisms lack the ability to grow on honey-enriched medium although some reports show the contrary. Therefore, an attempt is made here by investigating the ability of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus,* to ferment milk in the presence of 5 and 10% (w/v) polyfloral or unifloral honey, and to determine the effect of honey as a protective agent of cell viability during refrigerated storage. Polyfloral honey was used here because it is much more common than unifloral honey in Algeria.

MATERIALS AND METHODS

Honey origin

Polyfloral honey (dark-coloured, harvested from eucalyptus and greenbrier in West Algeria) used in this study was obtained from local beekeepers. Unifloral honey (light-coloured) is a “lime honey” manufactured in France (Les Apiculteurs Associés, France). The honeys were one year old and had been stored in an air-tight jar in a dark place at room temperature. pH values were 3.92 and 4.2 for polyfloral and unifloral honeys, respectively. Their microbial quality was acceptable, and on average, total micro-organisms, yeast and mould did not exceed 2 CFU/g. Coliforms and aerobic spores were negative in 10 g.

Bacteria and media

Lactic acid bacteria, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (Lb 340 and TA 040; εZAL® Groupe Rhône Poulenc; Rhodia, France) were transferred at least twice for 24 h at 37°C in lactobacilli MRS (de Mann, Rogosa and Sharpe) broth (Merck, Germany) (De Man et al., 1960). Overnight cultures were collected from MRS broth, harvested by centrifugation, washed twice, and re-suspended in skimmed dry milk (10% w/v; tyndallized by steaming repeatedly for 30 min on three successive days) to obtain an approximate final concentration of 10^6 CFU/ml.

Inocula (5 or 3% for each pure or associated-bacterial culture, respectively) were propagated and mixed individually with sterile reconstituted skimmed milk in the absence (control) or in the presence (sample) of 5 or 10% (w/v) pasteurized honey (60°C/30 min) and distributed in 10 ml test tubes incubated at 42°C.

The fermentation is stopped as soon as milk curdles which depend on the micro-organisms involved in the process and the presence or the absence of honey. Because it was not possible to reach pH 4.7 and in order to compare the acidifying activity of all cultures, the fermentation time to reach pH 5 was chosen as the end point of the fermentation. Then, samples were stored at 4°C for viability and post-acidifying determination. All experiments were performed in triplicate three times.

Cell enumeration

Viable counts were done by serial dilutions with 0.1% peptone-water and serially diluted 10-fold. Samples were homogenized for at least 15 s with a vortex mixer and 100 µL of each sample was taken in triplicate on suitable medium to determine the viable cell counts using the pour plating technique. *S. thermophilus* and *L. bulgaricus* were enumerated on M17 media (Difco laboratories, Detroit, MI), containing 0.5% (w/v) lactose (Terzaghi and Sandine, 1975) and acidified MRS at pH 5.4, respectively. Plates were incubated at 37°C for 48 h and 43°C for 72 h, using an anaerobic jar in the case of each pure culture or aerobically in the case of associated LAB culture. ST medium was used to differenciate lactic acid bacteria and incubation was carried out for 24 h at 37°C (Dave and Shah, 1996). Appropriate colonies were counted using a GALLENKAMP colony counter (UK).

Growth and variation in pH

Viable cell counts and pH values were assessed at 2 h intervals. One ml of each thoroughly pure or associated fermented milk was diluted with 99 ml of sterile 0.1% (w/v) peptone-diluent and plated on adequate media to determine numbers of bacteria. Maximal specific growth rate (µmax) for each pure or associated LAB culture was calculated using the Desjardins et al. (1991) equation:

$$\mu \max = (\ln X_2 - \ln X_1) / (t_2 - t_1)$$

Where X_2 and X_1 are the cell biomass at time t_2 and t_1 of exponential phase, respectively. Consequently, doubling time (t_d) was calculated as:

$$t_d = \ln 2 / \mu$$

A sample was taken for pH determination using a digital pH-meter with combined glass electrode standardized with pH 4 and 7 standard buffer solutions (wtw, pH 330, Weilheim; Germany). Maximal acidification rate (ΔpH max/Δt) was calculated as follows:

$$\Delta pH \max/\Delta t = (pH_1 - pH_2) / (t_2 - t_1);$$

Where pH_2 and pH_1 are the pH values at time t_2 and t_1 of exponential phase, respectively.

Bacteria viability and changes in pH in yogurt during storage at +4°C

Samples of different yogurts were assessed for viability and pH at 7 day intervals for 28 days of refrigerated storage. One gram of each sample was diluted with 99 ml of sterile (0.1% w/v) peptone diluent (Difco laboratories, Detroit, MI) and subsequent serial dilutions were made. An aliquot of 100 µl of each sample was taken in order to enumerate the viable cell counts. Cell counts, performed in triplicate, were calculated from the colonies on appropriate medium plates after suitable incubation, and thus expressed as colony-forming units per gram (CFU/g). The first analysis (1 d) was done 24 h after the fermentation was completed. Viable cells were calculated as follows:

% Viability = (CFU at n week (s) of storage / initial CFU) x 100

(Ustunol and Gandhi, 2001).

Statistical analysis

Statistical analysis was conducted using ANOVA analysis (StatBox logiciel, GrimmerSoft; version 6.4, France). Comparisons were made using Student–Newman–Keuls test for multiple comparisons. A $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Biomass

Control (without added honey) monocultures of *S. thermophilus* and *L. bulgaricus* (Table 1) confirmed their well-known high growth ability in milk. *L. delbrueckii* spp. *bulgaricus* Lb 340 is aromatic mild lactobacilli with a low proteolytic activity responsible for the weakest curd consistency. After 6 h of fermentation at 42°C, biomass of this strain markedly increased with a registered maximal specific growth rate of 1 h^{-1} and a doubling time (td) of approximately 40 min. *S. thermophilus* TA 040 exhibited a lower growth rate and a higher biomass level in comparison with *L. bulgaricus*. However, control LAB populations were not significantly different ($P>0.05$) from those obtained in the presence of 5% polyfloral or unifloral honey (Table 1). Doubling time of *L. bulgaricus* was 11 and 13% shorter in the presence of polyfloral and unifloral honeys ($P<0.05$), respectively (data not shown). A similar kinetic growth of *S. thermophilus* was observed in the presence of 5% of both honeys resulting in a curdling time of 7 h of fermentation. Registered growth rates and biomass were about 0.9.h^{-1} and 8.9 log CFU/ml ($P<0.05$), respectively. The fermentation process is 40% shorter when 5% honey is added to milk. Such observations are in agreement with those of Chick et al. (2001), who reported that pasteurized clover honey (70°C, 15 min), sucrose or fructose at the level of 5% (w/w), generates similar improved growth of *S. thermophilus, L. delbrueckii* spp. *bulgaricus* and *L. acidophilus*, and that honey did not exert an inhibitory effect at this level.

Shamala et al. (2000) measured the number of *L. acidophilus* and *L. plantarum* under *in vitro* conditions and found that both species grew well in the medium containing 0.5% glucose and lactose. Similar growth was observed with 1% honey which was 10 to 100 fold higher compared to sucrose. However, under *in vivo* conditions, the honey-fed rats showed a significant ($P<0.05$) increase in micro-organisms in comparison with the control and the sucrose-fed rats.

In the present experiment, Lb 340 strain was inhibited ($P<0.05$) in the presence of 10% honey in milk (Table 1) since biomass decreased by 0.5 to 0.88 log in comparison with the control, which represents a 6.5 and 9.8% decrease in the presence of polyfloral and unifloral honey in milk, respectively. These results are in accordance with those of Čurda and Plocková (1995) who reported that unheated honey at the level of 10% (w/v) may have an inhibitory effect on mesophilic starters. On the other hand, the same level (10% w/v) of both honeys enhance the growth of *S. thermophilus* TA 040 (Table 1) ($P<0.05$). The curdling time was reduced up to 2 h comparatively to the control (data not shown). The population counts were higher than 9 log CFU /ml in all 10% added-honey milk.

In a previous work (Riazi and Ziar, unpublished data), we measured the growth kinetic and acid production by the same strains in presence of 5% and 10% D-glucose or D-fructose in order to bring out the role of the fructo-oligo-saccharide (FOS) honey fraction. D-Glucose slightly stimulated the growth of *S. thermophilus* reaching a biomass level of 9.1 to 9.8 log CFU/ml at the end of the fermentation (8 h) (μ= 0.78 to 0.80 h^{-1}) in the presence of 5 and 10% of this sugar, respectively. *S. thermophilus* also metabolized D-fructose reaching a biomass level of 9.9 log CFU/ml at coagulation which occurred after 10 h of fermentation. It is very likely that D-fructose did not check the biomass production since the registered growth rates at 5 or 10% of this sugar were very similar (0.79 and 0.80h^{-1}).

According to Hutkins and Morris (1987) and Giraffa et al. (2001), *S. thermophilus* only uses a few sugars, and most of the strains prefer saccharose and lactose, while glucose and fructose are more slowly fermented. Additionally, in a study of Amoroso et al. (1989) significant differences in growth response to glucose, galactose, fructose, lactose and sucrose were found among these strains in pure and mixed cultures. While *S. thermophilus* showed a preference for lactose, glucose consumption was more rapid in *L. delbrueckii* spp. *bulgaricus* than with fructose or lactose. Furthermore, both micro-organisms (pure cultures) grow actively in fructose + glucose medium than in fructose alone. Although, lactose remains the best carbon substrate for Streptococci, the most likely glucose and fructose uptake by LAB (*S. thermophilus* and *L. bulgaricus*) in honey-sweetened skimmed milk could be suggested. The presence of glucose in the growth medium excludes the consumption of other sugars by *L. bulgaricus* and *L. helveticus* (Hickey et al., 1986).

Table 1. Growth of Lactic acid bacteria (log CFU/ml) and pH (in pure and associated cultures) of skimmed milk containing honey.

Honey	Time (h)	Pure cultures				Associated cultures		
		St Biomass	pH of milk	Lb Biomass	pH of milk	St Biomass	Lb Biomass	pH of milk
0% honey (control)	0	5.98±0.09	6.60±0.05	5.96±0.06	6.48±0.01	6.01±0.06	5.96±0.05	6.55±0.05
	2	7.13±0.11	6.53±0.05	7.17±0.03	6.38±0.02	7.57±0.03	7.45±0.04	6.39±0.07
	4	7.76±0.18	6.35±0.05	8.46±0.06	5.70±0.08	7.95±0.05	7.51±0.07	5.77±0.01
	6	8.89±0.04	6.07±0.1	8.98±0.01	5.29±0.01	9.39±0.16	9.15±0.13	5.11±0.02
	8	9.41±0.10	5.87±0.05	ND	ND	ND	ND	ND
5% polyfloral honey (5%PH)	0	6.19±0.17	6.29±0.05	6.12±0.15	6.40±0.05	5.88±0.10	5.89±0.09	6.44±0.01
	2	6.60±0.17	6.16±0.05	6.82±0.07	6.28±0.01	6.97±0.03	7.17±0.11	6.38±0.01
	4	6.98±0.01	5.86±0.01	7.78±0.02	5.68±0.05	8.02±0.04	8.48±0.03	5.98±0.01
	6	6.99±0.02	5.43±0.05	8.85±0.03	5.13±0.01	8.56±0.05	8.73±0.01	5.11±0.05
	8	8.94±0.01	5.27±0.05	ND	ND	ND	ND	ND
5% unifloral honey (5%UH)	0	5.96±0.05	6.32±0.05	5.98±0.02	6.50±0.01	5.92±0.06	5.95±0.04	6.5±0.01
	2	6.54±0.25	6.19±0.01	6.81±0.07	6.26±0.05	6.98±0.02	7.24±0.10	6.38±0.05
	4	7.46±0.15	5.88±0.01	7.56±0.22	5.65±0.01	7.79±0.10	8.31±0.07	6.00±0.05
	6	7.51±0.07	5.36±0.01	8.90±0.03	5.12±0.01	8.54±0.06	8.72±0.02	5.07±0.01
	8	8.94±0.01	5.12±0.01	ND	ND	ND	ND	ND
10% polyfloral honey (10%PH)	0	6.20±0.20	6.26±0.01	6.27±0.04	6.21±0.05	5.94±0.05	5.93±0.05	6.30±0.01
	2	7.48±0.02	6.05±0.05	6.81±0.03	6.14±0.05	5.97±0.02	5.98±0.01	6.27±0.02
	4	8.30±0.03	5.74±0.05	7.35±0.13	5.84±0.01	7.83±0.06	7.13±0.14	6.03±0.05
	6	9.42±0.02	5.40±0.05	8.10±0.17	5.16±0.08	8.47±0.02	8.44±0.02	5.34±0.02
10% unifloral honey (10%UH)	0	5.96±0.05	6.28±0.05	6.06±0.20	6.25±0.01	5.93±0.06	5.97±0.02	6.34±0.05
	2	7.55±0.13	6.03±0.05	6.76±0.15	6.16±0.01	6.09±0.17	6.98±0.01	6.23±0.01
	4	8.36±0.07	5.68±0.05	7.12±0.58	5.87±0.01	7.61±0.15	7.32±0.28	6.00±0.01
	6	9.10±0.27	5.07±0.01	8.39±0.30	5.12±0.02	8.49±0.03	8.42±0.04	5.19±0.02

In all cultures, inoculums were used at final concentration of 1×10^6 CFU /ml.
Values represent means±SD for all treatments.
ND: not determined, because fermentation is stopped as soon as milk curdles.

Yogurts are made from the symbiosis of the two bacteria *S. thermophilus* and *L. delbrueckii* spp. *Bulgaricus*. In associated control culture (without honey addition) (Table 1), the biomass of *S. thermophilus* was not influenced by the presence of lactobacilli strain ($P>0.05$) and its doubling time was shorter ($P>0.05$) than that of *L. bulgaricus* (data not shown). These findings are in accordance with those of Amoroso et al. (1989) who showed that in mixed culture, the growth of both microorganisms is stimulated and that the biomass increase of *S. thermophilus* is higher than that of *L. bulgaricus*.

The growth of both LAB strains was not inhibited by the presence of honey at 5%. Indeed, despite a lower biomass (Table 1), their specific maximal growth rates were not statistically influenced ($P>0.05$) whereas the needed curdling time was similar to that observed in the associated control culture. Levels of biomass decrease were higher with streptococci (7.9 to 8.7%) than those observed with lactobacilli strains (4.6 to 4.7%) and there were no statistical differences in the effects between the two honeys tested. A similar biomass was registered ($P>0.05$) in both 5% unifloral or polyfloral honey-added milks (Table 1).

According to Desmazeaud (1983), the better growth of *S. thermophilus* in milk could be explained by its lower nutritional requirements comparatively to lactobacilli in milk. In fact, *S. thermophilus* requires few amino acids and is able to synthesize branched-chain amino acids (Garault et al., 2000), and its growth can probably be supported by free amino acids and peptides present in milk (Letort et al., 2002). In contrast, *L. bulgaricus* is much more in need from a nutritional point of view than *S. thermophilus* (Letort and Juillard, 2001), and its optimal growth depends on the supply of essential factors

(CO_2, pyruvate, formate) produced by *S. thermophilus* (Tamine and Robinson, 1999; Zourari et al., 1992).

In the present experiment, associated cultures were performed at 42°C, a temperature more suitable for *S. thermophilus*, which has an optimal growth temperature ranging between 40 and 45°C, versus 45 and 50°C for *L. bulgaricus*. Polyfloral or unifloral honey clearly inhibited LAB growth when added at a 10% level in milk ($P<0.05$) (Table 1). This observation is well shown by the decrease of the biomass rates of *S. thermophilus* and *L. bulgaricus,* which are 9.6 and 7.85%, respectively. Milk curdling time is also prolonged up to one hour comparatively to the obtained result with 5% honey. According to Amoroso et al. (1989), in a standard medium with fructose or sucrose there is an inhibition of growth of both micro-organisms in mixed culture. Furthermore, such inhibition could be partially due to the osmotic pressure and honey composition which contains fructo-oligo-saccharide (FOS). These results are in agreement with those of Roumyan et al. (1996), who found a considerable inhibition in the growth of *L. bulgaricus* when testing the influence of honey addition to the starter bacteria of Bulgarian yogurt.

Kaplan and Hutkins (2000) reported that 12 out of 16 *Lactobacillus* tested strains and none of the four *S. thermophilus* strains were able to ferment FOS. Indeed, most of *S. thermophilus* and *L. bulgaricus* strains used in yogurt manufacturing are FOS-non fermenter.

pH variations

The acidifying activity of LAB monocultures (*S. thermophilus* and *L. bulgaricus*) in 5% honey enriched-skimmed milk was improved (Table 1). When the fermentation was over, the pH of milk inoculated with *L. bulgaricus* dropped by 1.28 to 1.38 units ($P<0.05$) as a result of a maximal acidification rate of 0.28 h^{-1}. *S. thermophilus* also showed an increased acidifying activity ($P<0.05$) by 44 and 64.4% in milk containing polyfloral and unifloral honey, respectively as compared to the control. Such observations are similar to those of Chick et al. (2001) who reported that honey enhanced lactic acid production by *S. thermophilus* and *L. bulgaricus* in a similar way ($P>0.05$) to that of sucrose, fructose or control.

A similar range of acidifying activity was registered for *L. bulgaricus* when 10% polyfloral ($\Delta pH/\Delta t = 0.26\ h^{-1}$) or unifloral ($\Delta pH/\Delta t = 0.24\ h^{-1}$) ($P>0.05$) honey was used (Table 1). No significant difference ($P>0.05$) was noted in lactic acid production in all honey-supplemented milk fermented by *S. thermophilus* ($\Delta pH/\Delta t = 0.21\ h^{-1}$ and 0.16 h^{-1} with polyfloral and unifloral honey, respectively) (Table 1).

In a previous work (Riazi and Ziar, unpublished data), acidification kinetic of milk by *S. thermophilus* expressed as pH values fulfilled the registered lactic acid levels. The decline in pH was higher in the presence of 5% D-Glucose (4.81) than in the presence of 10% (5.11) after 8 h of fermentation. In the presence of D-fructose, the pH values registered were 5.1 and 4.7 after 10 h of fermentation and the acidifying rates were almost similar, 0.52 and 0.51 h^{-1} with 5 and 10% D-fructose, respectively.

Acidifying activity of associated culture of *S. thermophilus* and *L. bulgaricus* with 5% polyfloral honey was not significantly different ($P>0.05$) from that observed with 5% unifloral honey or control ($\Delta pH/\Delta t = 0.31$ and 0.32 h^{-1}, respectively) (Table 1). These results corroborate with those of Chick et al. (2001) who reported that lactic acid production was not influenced by sweetener type and was similar in all treatments (fructose, sucrose, control) ($P>0.05$). These observations confirm that honey supported lactic acid production by these organisms in a similar manner to other sweeteners, and was not inhibitory.

Decrease in acidifying activity of such associated cultures has been registered when the level of added honey rose up to 10% in milk ($P>0.05$) ($\Delta pH/\Delta t = 0.23\ h^{-1}$ and 0.26 h^{-1} with polyfloral and unifloral honey, respectively) (Table 1). In fact, Varga (2006) reported that 1, 3 or 5% honey have no effect on lactic acid produced by *S. thermophilus* and *L. bulgaricus* of the associated ferment "YC-350".

Viability

Lactic acid bacteria viability in yogurt stored at 4°C during 4 weeks is reported in Tables 2 and 3.

Pure cultures

Viable counts of *S. thermophilus* with 5% honey did not show significant statistical changes ($P>0.05$) comparatively to the control, and were reduced by 1 to 1.7% after one day and by no more than 17% at the end of the refrigerated storage period (Table 2). After 4 weeks of storage, biomass values remained above 7.4 log CFU/g in both fermented milks. Consequently, both polyfloral and unifloral honeys improved the viability of *S. thermophilus* by 5 to 6.6%, respectively, as compared to control yogurt.

Such results may suggest that honey could have a powerful effect by modulating the survival capacity of lactic acid bacteria, especially when simple sugars as fructose and glucose were almost totally consumed during fermentation. Furthermore, honey might have a barrier role or a micro-encapsulating agent in which the cells are protected and cell loss is reduced.

In spite of the 7.7% highest level of viable biomass registered with 10% level, honey has no significant apparent effect ($P>0.05$) on streptococci viability in fermented milk (Table 2), comparatively to the control. This fermented milk formulation fulfilled the legal requirements in

Table 2. Viability of Lactic acid bacteria (pure cultures) in yogurt containing honey during 28 days of storage at 4°C.

Strain	Storage time (days)	Viability*				
		Control (without honey)	Polyfloral honey		Unifloral honey	
		0%	5%	10%	5%	10%
Lb	1	99.83±0.02^{a} (8.97±0.03)	97.45±0.03^{b} (8.63±0.4)	98.52±0.02^{a} (7.98±0.05)	96.79±0.02^{bc} (8.62±0.03)	96.23±0.07^{c} (8.07±0.12)
	7	96.36±0.02^{a} (8.66±0.18)	97.09±0.04^{a} (8.60±0.13)	91.13±0.30^{b} (7.30±0.07)	94.49±0.05^{a} (8.41±0.11)	89.28±0.42^{b} (7.49±0.18)
	14	77.34±0.04^{c} (6.95±0.48)	82.70±0.06^{b} (7.32±0.12)	77.70±0.01^{c} (6.29±0.01)	84.57±0.20^{a} (7.53±0.07)	72.60±0.16^{d} (6.09±0.09)
	21	76.27±0.04^{c} (6.85±0.42)	80.27±0.10^{b} (7.11±0.05)	64.12±0.16^{d} (5.19±0.06)	80.93±0.1^{ab} (7.21±0.09)	70.01±0.09^{c} (5.87±0.08)
	28	66.44±0.08^{b} (5.97±0.12)	77.13±0.02^{a} (6.83±0.19)	56.81±0.02^{c} (4.60±0.11)	75.90±0.02^{a} (6.76±0.12)	67.33±0.48^{b} (5.56±0.21)
St	1	92.7±0.04^{c} (8.73±0.12)	98.31±0.02^{b} (8.79±0.12)	98.56±0.05^{a} (9.28±0.05)	99.05±0.03^{a} (8.86±0.01)	97.54±0.06^{b} (8.88±0.12)
	7	91.45±0.09^{a} (8.61±0.03)	88.17±0.03^{b} (7.88±0.04)	92.95±0.01^{a} (8.74±0.20)	88.49±0.07^{b} (7.91±0.08)	94.72±0.05^{a} (8.62±0.11)
	14	91.16±0.07^{a} (8.58±0.13)	88.05±0.03^{b} (7.87±0.13)	88.69±0.03^{a} (8.35±0.03)	87.00±0.14^{c} (7.78±0.08)	90.59±0.18^{a} (8.24±0.07)
	21	84.42±0.10^{c} (7.95±0.07)	87.95±0.07^{bc} (7.86±0.08)	88.56±0.02^{a} (8.34±0.05)	86.03±0.08^{c} (7.69±0.07)	80.83±0.10^{d} (7.36±0.09)
	28	78.43±0.10^{b} (7.38±0.06)	85.06±0.02^{a} (7.60±0.22)	77.06±0.24^{b} (7.26±0.11)	83.39±0.15^{ab} (7.46±0.22)	77.72±0.19^{b} (7.07±0.16)

*% viability and (Survival values in terms of log CFU/ml). % viability = (CFU after n week (s) storage/ initial CFU) × 100.
Means with different letters are significantly different (P<0.05). Comparisons are made only within the same row.
Values represent means± SD for all treatments.

terms of viable levels of starter organisms present during the whole refrigerated storage period.

A noticeable positive effect on *L. bulgaricus* viability was observed with 5% honey. Despite the significant (P<0.05) loss in biomass registered after the first day of storage (1.4 and 2.1 log cycles) comparatively to the control, the percentage of survival was almost the same (about 77%) in both fermented milks containing unifloral or polyfloral honey after 28 days of storage, which represents a 10% increase (P<0.05) in lactobacilli viable counts (6.5 log CFU /g).

However, less viability (P<0.05) of this strain in 10% honey-enriched fermented milk was noted in comparison with the control after the first day of storage. At the end of the refrigeration period, viable counts decreased by 43.2% rising the viability level to about 56.8% (P<0.05). Effectiveness of unifloral honey was higher (67.4%) than that of polyfloral honey in maintaining better viability of *L. bulgaricus* in fermented milk stored at 4°C (Table 2).

Associated cultures

With both honeys at 5%, the loss of viability of *S. thermophilus* strain in associated culture was lower than 3.2% after one week of storage and was not significant (P>0.05) comparatively to that of the control (Table 3). On the 14th day, the decrease in biomass was higher (P>0.05) in yogurt containing unifloral honey (18.4%) than that of polyfloral honey (8.9%). At the end of the storage period, the lethal level was similar (P>0.05) in both yogurts supplemented with unifloral or polyfloral honey (35.5 and 34%) and led up to 5.5 and 5.6 log CFU/g of cell survival, respectively. These results show 10 to 12% improvement (P>0.05) in *S. thermophilus* viability (Table 3). *L. bulgaricus* strain showed the same ability to survive in presence of both honeys (P>0.05) since the loss in biomass was only 5% after one week (P>0.05), and less than 30% (P<0.05) after 4 weeks of storage period. The count of persisting lactobacilli cells

Table 3. Viability of Lactic acid bacteria (associated cultures) in yogurt containing honey during 28 days of storage at 4°C.

Strain	Storage time (days)	Viability*				
		Control (without honey)	Polyfloral honey		Unifloral honey	
		0%	5%	10%	5%	10%
Lb	1	98.25±0.01^{a} (8.99±0.11)	97.31±0.02^{ab} (8.50±0.11)	99.75±0.01^{a} (8.42±0.05)	97.14±0.03^{b} (8.47±0.21)	98.23±0.03^{ab} (8.27±0.22)
	7	79.1±0.04^{c} (7.24±0.22)	95.30±0.02^{b} (8.30±0.21)	99.68±0.01^{a} (8.41±0.16)	95.00±0.02^{b} (8.29±0.05)	96.72±0.06^{b} (8.14±0.11)
	14	76.65±0.01^{c} (7.01±0.19)	92.05±0.03^{b} (8.04±0.19)	98.07±0.02^{a} (8.27±0.22)	91.50±0.10^{b} (7.98±0.02)	94.84±0.02^{b} (7.98±0.14)
	21	71.93±0.02^{d} (6.58±0.13)	79.70±0.02^{c} (6.98±0.06)	97.23±0.14^{a} (8.20±0.03)	81.19±0.18^{c} (7.08±0.05)	91.87±0.15^{b} (7.73±0.11)
	28	54.46±0.02^{d} (4.98±0.08)	71.19±0.06^{c} (6.22±0.14)	96.62±0.11^{a} (8.15±0.08)	71.85±0.03^{c} (6.27±0.22)	82.35±0.08^{b} (6.93±0.22)
St	1	97.74±0.31^{a} (9.18±0.03)	98.56±0.05^{a} (8.44±0.14)	99.24±0.05^{a} (8.40±0.16)	97.55±0.03^{a} (8.33±0.02)	98.17±0.05^{a} (8.34±0.09)
	7	83.53±0.57^{b} (7.84±0.05)	97.19±0.02^{a} (8.32±0.11)	98.24±0.05^{a} (8.36±0.18)	96.90±0.09^{a} (8.28±0.08)	97.68±0.08^{a} (8.29±0.06)
	14	75.36±0.01^{d} (7.07±0.19)	91.02±0.10^{c} (7.79±0.27)	96.61±0.04^{ab} (8.18±0.12)	81.54±0.09^{c} (7.65±0.16)	94.30±0.06^{b} (8.01±0.12)
	21	71.5±0.05^{b} (6.71±0.09)	69.88±0.05^{b} (5.98±0.04)	87.26±0.35^{a} (7.39±0.07)	70.02±0.05^{b} (5.98±0.02)	84.77±0.17^{a} (7.20±0.07)
	28	60.61±0.08^{b} (5.69±0.01)	66.20±0.03^{a} (5.67±0.21)	42.72±0.15^{d} (3.62±0.04)	64.45±0.05^{a} (5.50±0.10)	46.12±0.06^{c} (3.91±0.05)

* % viability and (survival values in terms of log CFU/ml). % viability = (CFU after n week (s) storage/ initial CFU) × 100.
Means with different letters are significantly different (P<0.05). Comparisons are made only within the same row.
Values represent means± SD for all treatments.

was 6.2 log CFU/g which corresponds to an increasing viability level of 10.6 to 11.2% (P<0.05) comparatively to the control (Table 3). For yogurt containing 10% honey, viability of *S. thermophilus* cells was about 75% after 3 weeks of storage at 4°C. This means that its viability was improved by 47% comparatively to the control (Table 3). However, if storage is prolonged up to 21 days, the loss in biomass might be significantly (P<0.05) increased to the level of 54 and 57.3% in the presence of polyfloral and unifloral honey, respectively. The remaining biomass did not reach 4 log CFU/g. The data obtained herein seem to be in accordance with those of Dave and Shah (1997) who noticed a higher production of hydrogen peroxide with the starter culture containing *L. bulgaricus* and suspected it to cause a partial injury to the cells of *L. acidophilus*. Varga (2006) reported that the survival population of *S. thermophilus,* in honey yogurt during 42 days of refrigerated storage exceeded the range value of 8.28 - 8.81 log CFU/g, throughout the entire storage period. Although the initial viable counts of *L. bulgaricus* were found to be approximately 0.4 log cycles lower than those of *S. thermophilus*. After 6 weeks of storage, viability retention of lactobacilli in the final product was in the range of 35.3 to 42.3%.

In the present experiment, the strain *L. bulgaricus* exhibited a higher ability (P<0.05) to survive than streptococci in associated cultures, especially when 10% polyfloral honey was present (P<0.05). The viability level of retention was 92.5 and 61.3% with persisting biomass of 8.15 and 6.93 log CFU/g in polyfloral and unifloral honey yogurt, respectively (Table 3).

Post-acidifying activity

The results of pH variation during refrigerated storage are reported in Table 4.

Pure cultures

In the range of 4.5 to 4.65, a decrease in pH of about 0.7 units (P<0.05) was observed in both 5% polyfloral or uni-

Table 4. Post acidifying activity (in terms of pH) of Lactic acid bacteria (pure and associated cultures) in yogurt containing honey during 28 days of storage at 4°C.

Strain	Storage time (days)	pH*				
		Control (without honey)	Polyfloral honey		Unifloral honey	
		0%	5%	10%	5%	10%
Lb pure culture	1	5.29±0.05^{b}	5.21±0.01^{c}	5.38±0.05^{a}	5.09±0.03^{e}	5.19±0.01^{d}
	7	5.35±0.08^{b}	5.10±0.02^{c}	5.38±0.05^{a}	4.97±0.05^{e}	5.08±0.01^{d}
	14	5.25±0.05^{b}	5.24±0.03^{b}	5.30±0.02^{a}	4.78±0.02^{d}	5.11±0.05^{c}
	21	5.21±0.03^{a}	5.18±0.03^{c}	5.23±0.07^{b}	4.65±0.01^{d}	5.23±0.05^{b}
	28	5.31±0.01^{b}	5.07±0.06^{c}	5.33±0.01^{a}	4.52±0.07^{e}	4.88±0.07^{d}
St pure culture	1	5.97±0.01^{a}	5.34±0.05^{c}	5. 57±0.05^{b}	5.21±0.01^{d}	5.09±0.05^{e}
	7	6.08±0.03^{a}	5.18±0.03^{c}	5.40±0.05^{b}	5.07±0.05^{e}	5.12±0.01^{d}
	14	6.03±0.07^{a}	5.05±0.01^{c}	5.20±0.07^{b}	5.06±0.01^{c}	4.99±0.04^{d}
	21	6.05±0.05^{a}	4.76±0.05^{d}	5.16±0.03^{b}	4.64±0.01^{e}	5.01±0.02^{c}
	28	6.04±0.05^{a}	4.65±0.01^{d}	5.05±0.05^{b}	4.51±0.01^{e}	4.89±0.07^{c}
Associated culture St+Lb	1	5.30±0.07^{b}	5.24±0.02^{c}	5.41±0.01^{a}	5.10±0.01^{e}	5.21±0.01^{d}
	7	5.43±0.05^{a}	5.14±0.07^{c}	5.40±.0.02^{b}	5.01±0.01^{d}	4.99±0.04^{e}
	14	5.32±0.02^{a}	4.94±0.01^{d}	5.03±0.05^{b}	4.96±0.01^{c}	5.03±0.04^{b}
	21	5.37±0.03^{a}	4.66±0.04^{e}	4.72±0.07^{b}	4.63±0.01^{c}	4.65±0.03^{c}
	28	5.52±0.07^{a}	4.67±0.07^{b}	4.53±0.06^{d}	4.57±0.05^{c}	4.54±0.01^{d}

Means with different letters are significantly different (P<0.05). Comparisons are made only within the same row.
Values represent means± SD for all treatments.

floral honey-enriched yogurt containing streptococci after 4 weeks of storage at 4°C (Table 4). The persisting post-acidifying activity of *S. thermophilus* could be due to the residual honey sugars. Variations in pH were significant (P<0.05) for lactobacilli in yogurt containing 5% honey comparatively to the control. pH decreased by 0.14 and 0.57 unit in fermented milks containing polyfloral and unifloral honey, respectively (Table 4).

Post-acidifying activity of streptococci was more pronounced with 5% honey than with 10% honey in yogurt (P<0.05). pH decreased by 0.20 and 0.52 unit in yogurt containing polyfloral and unifloral honey, respectively (Table 4).

Post-acidifying activity of *L. bulgaricus* stopped (P>0.05) after 21 days of refrigerated storage with polyfloral honey. However, with unifloral honey, the drop in pH (0.31unit) was significant (P<0.05) throughout the storage period, comparatively to the results obtained with 5% unifloral honey (Table 4).

Associated cultures

In the presence of honey, variation of pH during storage at 4°C showed a very acceptable post-acidifying activity (P<0.05) comparatively to the control. Decrease in pH values was 0.53 and 0.57 unit in yogurt containing unifloral and polyfloral honey, respectively (Table 4). However, these pH values remained less acid (P<0.05) in comparison with those obtained with lactobacilli alone and by the presence of unifloral honey in fermented milk. A stimulatory effect of honey towards acidifying activity of streptococci was observed, comparatively to its pure culture (P<0.05).

10% honey also enhanced lactic acid production in associated culture, and pH decreased significantly by 0.67 and 0.88 unit (P<0.05) in unifloral and polyfloral honey-enriched yogurt, respectively (Table 4). Both honeys showed a pH value of 4.5 at the end of storage which reflected an excessive production of lactic acid and could be the cause of the viability loss of strain *S. thermophilus* observed at the same level (10%).

Conclusion

The present experiment led to the exploration of the growth and viability of *S. thermophilus* and *L. bulgaricus* in honey-sweetened skimmed milk by means of measuring their fermentative characteristics in pure and associated cultures. Given that the LAB viability has been significantly (P<0.05) improved by the presence of honey in skimmed milk, the use of this sweetener could be suggested in yogurt manufacturing for a longer shelf life

of this product. Further studies concerning the sensorial effects of honey on yogurt taste would be useful.

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