Full Length Research Paper

# Effect of honey and starter culture on growth, acidification, sensory properties and bifidobacteria cell counts in fermented skimmed milk

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Accepted 24 October, 2011

*Bifidobacteria* strains (BLR, BLE, Bbv-1 and Bbv-2) were investigated throughout changes in their growth and acidifying activity in the presence of yogurt starters (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) on reconstituted skimmed milk (10% w/v) supplemented with 5 or 10% (w/v) polyfloral or unifloral honey as a sweetener. A positive association (P>0.05) between *S. thermophilus* and each one of the four *Bifidobacterium* strains was observed and acidity of milk containing (5 or 10%) polyfloral or unifloral honey was acceptable (P>0.05). In associated cultures between one *Bifidobacterium* strain and *Lactobacillus bulgaricus*, only 10% of honey stimulated (P>0.05) growth and acid production of both organisms. However, the level of 5% has at the same time both stimulatory (P>0.05) and inhibitory effect (P>0.05) on *bifidobacteria* and lactobacilli, respectively. A significant (P<0.05) improvement in *bifidobacteria* biomass (1.51 to 9.55%) co-cultivated with both lactic acid bacteria was observed in milk containing 5 or 10% honey. However, only 10% honey seems to stimulate (P<0.05) lactic acid bacteria growth in this co-culture. Viability of all bacteria was improved (P<0.05) in the presence of honey, and acidity of fermented milks during storage was regulated, which is probably the cause of the good sensory properties of all honey-added yogurts.

**Key words:** *Bifidobacteria, Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus,* honey, sensory properties, acidification, viability.

## INTRODUCTION

Consumption of yogurt and low-fat flavoured milk beverages has increased steadily over the past decade. Market response to the increased demand of yogurt has resulted in different styles, different flavours, and reduced-calorie and/or reduced fat products (Granato et al., 2010). Also, there has been continued interest in incorporating honey into foods due to its 'healthy' and natural image (Tamime and Robinson, 1985).

Scientists from the Department of Food Science and Human Nutrition at Michigan State University investigated the growth and viability of commercial *Bifidobacterium*  *bifidum* (Bf-1 and Bf-6) in honey-sweetened reconstituted non-fat dry milk (NDM) containing 5% honey, sucrose, fructose, or glucose. The results revealed that growth promotion and acid production were greatest when Bf-1 and Bf-6 were grown in the presence of honey. Their retention of viability was greatest up to 14 days of refrigerated storage at 4°C when they were grown and stored in the presence of honey compared to the other sweeteners (Chick et al., 2001).

Distinctive characteristics of honey are due to a large number of minor components that come from the nectar and the bees themselves (Kwakman et al., 2011). Honey has been used since ancient times for the treatment of some respiratory diseases and for the healing of skin wounds. It has been proposed that the healing effect of honey could be due to various physical and chemical

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properties (Gómez-Caravaca et al., 2006). The high osmolarity and acidity of honey are among the physical characteristics that contribute to its antibacterial activity. Hydrogen peroxide, volatiles, organic acids, flavonoids, beeswax, nectar, pollen and propolis are important chemical factors that provide antibacterial properties to honey (Molan, 1992). However, honey also contains oligosaccharides in small quantities (Popa and Ustunol, 2011). In a recent work, Shin and Ustunol (2005) related the sugar composition of honeys from different floral sources to the growth stimulation of *bifidobacteria*. Although, various studies have addressed the health effects of yogurt, relatively little is known about the potential of a combined effect of honey-yogurt containing *bifidobacteria*.

The objective of this study was to develop a desirable healthy yogurt using honey as a sweetener in lieu of sucrose and by incorporating *bifidobacteria* strains. Therefore, the aim of the present study was to explore the effect of the incorporation of 5 or 10% (w/v) honey to skimmed milk (10% w/v) fermented by *Bifidobacteria* strains and Lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). Thus, the growth of bacteria and their pH changes in milk were studied until coagulation; in addition viability and post-acidifying activity were monitored at 4°C during 4 weeks storage. The sensory properties of honey-yogurt were therefore also analysed after 21 days of refrigerated storage.

#### MATERIALS AND METHODS

#### Honey origin

West Algerian polyfloral honey (from eucalyptus and greenbrier) used in this study was obtained from local beekeepers. Unifloral honey is a "Lime honey" manufactured in France (Les Apiculteurs Associés, France). The honeys were one year old, dark (local honey) or light-coloured (Lime honey), and had been stored in an air-tight jar in a dark place at room temperature. The pH values were 3.92 and 4.2 for the local and the commercial honeys, respectively. Their microbial quality was acceptable, with yeast and mould not exceeding 2 cfu/g; coliforms and aerobic spores were negative in 10 g.

#### Bacteria and media

The organisms used in this study included four strains of *bifidobacteria* isolated from human faeces (breast fed-infants): two *Bifidobacterium breve* (Bbv-1 and Bbv-2) and one *Bifidobacterium longum* (BLE). They were tested for identity as a member of the genus *Bifidobacterium* based on the following criteria: (1) they were gram positive, pleomorphic rods with characteristic bifurcated *Bifidobacterium* cellular morphology, (2) they were unable to grow under aerobic conditions, (3) they were catalase negative, (4) possessed fructose-6-phosphate phosphoketolase (F6PPK; EC 4.1.2.22) activity as described by Scardovi (1986), and (5) they were positive for the *Bifidobacterium* spp. 16S rDNA genus signature sequence determined by a fluorescence *in situ* hybridization (FISH) method, based on the Microscopic enumeration FISH Kit (FITC or Cy3 labelled; RiboTechnologies, Groningen, Netherlands) specific for the genus *Bifidobacterium* 

(Langendijk et al., 1995). These experimental *bifidobacteria* strains were compared with a reference strain of *Bifidobacterium longum* coded "BLR" (B612 strain, Agrarian institute, Milan- Italy), and all organisms were stored at -60°C in 4 ml screw-capped vials of 50% glycerol and 50% culture grown in MRS broth (De Man et al., 1960). Forty-eight hours prior to the start of each experiment, cultures were revived by a series of two inoculations of 10 ml of MRS-L (MRS with 5% (w/v) lactose, Difco; Detroit, MI, Michigan, USA) incubated at 37°C for 24 h in anaerobic chamber (Coy Laboratory Products; Inc., Ann Arbor, MI, Grass Lake, Michigan, USA). The atmospheric composition in the chamber was 85% nitrogen, 10% carbon dioxide, and 5% hydrogen.

### Starters of yogurt

*Lactobacillus delbrueckii* subsp. *bulgaricus* Lb 340 and *Streptococcus thermophilus* TA 040 were from Danisco (Saint Romain, France) and were transferred twice in MRS broth (Merck, Darmstadt, Germany) at 37°C.

Overnight cultures were collected from MRS or MRS-L broths; harvested by centrifugation, washed twice, and resuspended in skimmed milk (non-fat dry milk "NDM" at 10% w/v tyndallized by steaming repeatedly for 30 min on three successive days) to obtain an approximate final concentration of  $10^6$  or  $10^7$  cfu/ml.

Inocula of 3% of associated cultures (1.5:1.5 one *Bifidobacterium* strain with *L. bulgaricus* or *S. thermophiles*; 3%: 1:1:1 one *Bifidobacterium* strain with both starters) were propagated and mixed individually with 97 ml of sterile reconstituted NDM (control) or NDM with 5 or 10% (w/v, added at the mentioned final concentration in milk) pasteurized honey ( $60^{\circ}C/30$  min) and distributed in test tubes of 10 ml. After that, fermentation done on aerobiosis at 37°C was stopped as soon as milk curdles. The fermented milks were then cooled and stored at refrigerated temperature (+4°C) for 28 days.

#### Microbiological analysis

#### Cell enumeration

Viable counts performed in triplicate three times were done by serial dilution in diluent solution for anaerobes (saline and cysteine-HCl). Of each dilution, 100 µl was taken to determine the number of lactic acid bacteria *bifidobacteria* using the poor plating technique on appropriate medium. Samples were homogenized, for at least 15 s with a vortex (Heidolph; Bioblock Scientific, type REAX 2000, Germany).

In this case of associated cultures bifidobacteria with LAB, selective enumerating medium MRS-LP agar (MRS agar (Oxoid. Ltd., Cambridge, UK) added with lithium chloride (3 g), sodium propionate (2 g), and propionic acid (5ml) (all from Sigma Chemical Co. Merck, France) was used for selective *bifidobacteria* enumeration (Vinderola and Reinheimer, 1999), and the three media: M17 (Difco; Detroit, MI, Michigan, USA) containing 0.5% (w/v) lactose (Terzaghi and Sandine, 1975), acidified MRS at pH 5.4 (ac-MRS) and ST (Streptococcus thermophilus agar) for LAB enumeration (Dave and Shah, 1996). MRS-LP plates were incubated for 48 h at 37°C in the anaerobic chamber (85% N2, 10%CO<sub>2</sub> 5% H<sub>2</sub>). M17 (48h at 37°C), ac-MRS (72h at 43°C) and ST (24h at 37°C) (the ST medium was used to distinguish streptococci from lactobacilli) plates were incubated in aerobic conditions (Dave and Shah, 1996). Appropriate colonies were counted using a Gallenkamp colony counter (Waukegan, UK).

#### Growth kinetic and pH changes

Initially (0 h) and at each 2 h interval, 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 (Wissenschaftlich-Technische Werkstätten wtw pH-meter 330, Weilheim; Germany). Also, one ml of each mixed fermented milk was diluted with 99 ml of sterile 0.1% (w/v) peptone-diluent (Difco; Detroit, MI) and plated on adequate media to determine the number of bacteria. Maximal specific growth rate ( $\mu$  max) for each associated bacterial culture grown in milk with (sample) or without (control) honey was calculated using the Desjardins et al. (1991) equation:

 $\mu$  max = (lnX<sub>2</sub> - lnX<sub>1</sub>) / (t<sub>2</sub> - t<sub>1</sub>)

Where,  $X_1$  and  $X_2$  are cell biomass at time t<sub>1</sub> and t<sub>2</sub> of exponential phase, respectively. Doubling time (t<sub>d</sub>) was calculated as:

 $t_d = \ln 2 / \mu$ 

Maximal acidification rate ( $\Delta pH max/\Delta t$ ) was calculated as:

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

Where pH  $_{1}$  and pH  $_{2}$  are  $\,$  pH values at time t  $_{1}$  and t  $_{2}$  of exponential phase, respectively.

#### Viable cell count and post-acidifying activity

Analysis was done each week and the first one (1d) was done 24 h after the end of fermentation. One gram of, each thoroughly mixed fermented milk was diluted with 99 ml of sterile 0.1% (w/v) peptonediluent. Of each dilution, 100  $\mu$ l was pour plating in triplicate in appropriate medium. Samples were homogenized more than 15 s at least with a vortex (Heidolph; REAX 2000, Schwabach, Germany). Viable cells were calculated as follows (Ustunol and Gandhi, 2001):

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

pH was also determined every seven days using a digital pH-meter (wtw, pH 330, Weilheim; Germany). The whole experimental program was conducted in duplicate, and each experiment was repeated at least three times.

#### **Physical measurements**

The viscosities of the fermented milks were determined at 4°C using a digital Brookfield Viscometer (Model DV-II, Brookfield Engineering Laboratories, Stoughton, MA, USA) (Özer et al., 1997).

#### Sensory assessment

The samples were organoleptically assessed by ten panellists, using a sensory rating scale of 1-10 for flavour and taste, and 1-5 for texture and 1-5 for colour and appearance, as described by Meilgaard et al. (1991).

#### Panelists

Ten trained panellists were recruited from Mostaganem University (Algeria). They were selected on the basis of training and experience in the use and evaluation of plain and flavoured yogurt. Panellists were non-smokers, between the ages of 24 and 56; 5 were female, and 5 were male.

#### Panel training

Trained panellists were used, which made group discussions or additional training sessions unnecessary. There were 30 evaluation sessions and each lasted about 15 min. Panellists evaluated 20 g portions of each fermented milk. Panellists used a quality rating score card for evaluation of flavour and texture of fermented milk samples. All perceived criticisms were marked appropriately.

#### Sensory evaluation procedures

Stored fermented milk samples were evaluated for flavour and texture after the elapse of 21 days. Yogurt flavours of honey were evaluated. A 10-point scale was used to measure flavour, where 1 = poor quality to 10 = excellent quality. A list of common yogurt characteristics and defects was also used to allow the panellists an opportunity to comment on the flavours perceived in each sample. These characteristics included overall dairy, dairy fat, cooked, whey, cardboard, sharp/bite, overall sour, lactic, sour, and bitter. However, the defects included gel too firm, weak, shrunken, atypical (foreign), high acid, low flavoring, lacks fine flavor, lacks freshness, low sweetness, low acid, old ingredient, oxidized (light-activated), rancid, high flavoring, high sweetness, unnatural flavor, and unclean. A five-point scale was used for texture and colour, where 1 = poor quality to 5 = excellent quality.

Panellists were seated in individual booths. Samples and ballots were passed through from an adjoining preparation room. Water was used for rinsing between samples. All fermented milk samples were portioned into individual, plastic-covered coded cups and were presented to panellists on a tray at the beginning of the evaluation. Physical and sensory analyses were carried out after 3 weeks of refrigerated storage.

#### 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

#### Biomass

# One Bifidobacterium strain co-cultivated with one lactic acid strain

All control cultures associating one *Bifidobacterium* strain to *S. thermophilus* TA 040 "Bifid+Strep" allowed the four *bifidobacteria* to grow with maximal growth rates of 0.8 to 0.88 h<sup>-1</sup> and to accumulate higher biomass ranging from 8.06 to 8.43 log cfu/ml (Table 1). *S. thermophilus* also revealed an enhanced growth capacity where its biomass was higher than 9 log cfu/ml resulting from a maximal growth rate of 0.6 to 1.02 h<sup>-1</sup> registered at curdling level.

Honey used at 5 or 10% (w/v) was stimulatory for both *bifidobacteria* and streptococci growth (Table 1). Biomass values registered in presence of honey were 8.53 to 9.30 log cfu/ml for *bifidobacteria* and 9.25 to 10.97 log cfu/ml for streptococci. Maximal growth rates were 0.79 to 0.92 h<sup>-1</sup> for *bifidobacteria* and0.71 to 1.89 h<sup>-1</sup> for *S. thermophilus*.

		B. longum BLR + Strep			B	. longum BLE	+ Strep	E	3. breve Bbv-1 +	⊦ Strep	B. breve Bbv-2 + Strep			
Variable	Time	BLR	Strep	pH of	BLE	Strep	pH of	Bbv-1	Strep	pH of	Bbv-2	Strep	pH of fermented	
		biomass	biomass	fermented milk	Biomass	biomass	fermented milk	biomass	biomass	fermented milk	biomass	biomass	milk	
0% honey	0h	6.99±0.01	6.93±0.05	6.61±0.01	6.96±0.05	6.96±0.02	6.54±0.01	6.98±0.03	6.96±0.02	6.60±0.01	7.08±0.19	6.97±0.02	6.54±0.06	
(control)	Curdling time	8.14±0.20	9.06±0.20	5.02±0.02	8.43±0.38	9.28±0.32	4.94±0.01	8.06±0.20	9.18±0.20	4.98±0.01	8.08±0.18	9.02±0.04	5.03±0.01	
5% polyfloral	0h	6.95±0.07	6.97±0.02	6.6±0.01	6.97±0.03	6.94±0.15	6.59±0.01	6.98±0.02	6.97±0.03	6.49±0.05	7.01±0.02	6.87±0.04	6.49±0.06	
honey	Curdling time	9.14±0.12	10.42±0.2	5.03±0.01	9.26±0.15	10.97±0.05	5.09±0.11	9.17±0.17	10.18±0.25	5.04±0.01	9.20±0.17	10.25±0.15	5.00±0.01	
5% unifloral	0h	6.97±0.03	6.99±0.01	6.61±0.01	7.01±0.08	6.96±0.04	6.60±0.01	6.99±0.01	6.96±0.05	6.58±0.31	6.99±0.01	6.98±0.02	6.50±0.01	
honey	Curdling time	8.76±0.04	9.31±0.15	4.99±0.11	8.58±0.20	9.42±0.12	4.98±0.10	8.59±0.11	9.62±0.12	4.97±0.04	8.53±0.39	9.25±0.16	4.99±0.22	
10% polyfloral	0h	6.97±0.07	6.95±0.03	6.38±0.11	6.98±0.02	6.98±0.02	6.37±0.07	6.89±0.22	6.98±0.02	6.35±0.31	6.99±0.01	6.96±0.06	6.44±0.05	
honey	Curdling time	9.28±0.19	9.60±0.09	5.06±0.05	9.30±0.31	9.94±0.04	4.96±0.06	8.70±0.10	9.91±0.07	4.99±0.11	9.12±0.11	9.46±0.15	5.03±0.01	
10% unifloral	0h	6.97±0.05	6.97±0.05	6.47±0.01	6.95±0.01	6.98±0.21	6.47±0.01	6.97±0.05	6.97±0.05	6.45±0.01	6.95±0.07	6.97±0.32	6.47±0.02	
honey	Curdling time	8.75±0.09	9.42±0.05	5.05±0.04	8.92±0.06	9.77±0.07	4.92±0.04	8.77±0.07	9.77±0.07	5.12±0.07	8.78±0.09	9.40±0.07	5.18±0.01	

Table 1. Maximal biomass levels (log CFU/mL) and pH registered at the curdling time of associated cultures of bifidobacteria strains and S. thermophilus (Strep).

Values were determined three times in triplicate and represent means ± SD for all treatments.

Table 2. Maximal biomass levels (log CFU/mL) and pH registered at the curdling time of associated cultures bifidobacteria strains and L. bulgaricus (Lact).

		B. longum BLR + Lact			B	. longum BLI	E + Lact	E	8. breve Bbv-1	+ Lact	B. breve Bbv-2 + Lact			
Variable	Time	BLR	Lact	pH of	BLE	Lact	pH of	Bbv-1	Lact	pH of	Bbv-2	Lact	pH of	
		biomass	biomass	fermented milk	biomass	biomass	fermented milk	biomass	biomass	fermented milk	biomass	biomass	fermented milk	
0% honey	0h	6.99±0.01	6.94±0.04	6.51±0.02	6.86±0.02	6.96±0.05	6.49±0.01	6.96±0.08	6.97±0.04	6.51±0.01	6.99±0.01	6.97±0.03	6.58±0.02	
(control)	Curdling time	8.93±0.02	8.96±0.04	5.05±0.01	8.91±0.07	8.95±0.08	5.02±0.01	8.88±0.03	9.08±0.18	5.07±0.01	8.94±0.04	8.96±0.05	5.04±0.01	
5% polyfloral	0h	6.98±0.12	6.97±0.13	6.56±0.06	6.96±0.03	6.96±0.10	6.49±0.01	6.95±0.05	6.97±0.03	6.52±0.04	6.97±0.01	6.97±0.02	6.52±0.01	
honey	Curdling time	9.10±0.17	8.64±0.06	5.03±0.01	9.17±0.05	8.44±0.19	5.00±0.23	9.15±0.19	8.62±0.12	5.05±0.01	9.16±0.22	8.39±0.22	5.03±0.01	
5% unifloral	0h	6.96±0.07	6.95±0.05	6.55±0.01	7.00±0.09	6.98±0.01	6.55±0.02	6.98±0.06	6.97±0.03	6.59±0.04	6.98±0.08	6.96±0.03	6.57±0.05	
honey	Curdling time	9.13±0.24	8.37±0.18	5.16±0.01	9.14±0.15	8.55±0.05	5.05±0.09	9.12±0.21	8.63±0.05	5.04±0.07	9.08±0.19	8.48±0.11	5.17±0.02	
10% plyfloral	0h	6.97±0.03	6.94±0.05	6.39±0.05	6.98±0.01	6.97±0.03	6.35±0.11	6.97±0.03	6.87±0.02	6.40±0.01	6.88±0.11	6.97±0.02	6.38±0.01	
honey	Curdling time	9.18±0.12	9.22±0.06	5.05±0.01	9.19±0.17	9.16±0.05	5.02±0.14	9.20±0.10	9.23±0.21	5.09±0.05	9.14±0.14	9.18±0.20	5.04±0.05	
10% unifloral	0h	6.87±0.03	6.99±0.12	6.50±0.01	6.92±0.01	6.79±0.07	6.49±0.01	6.98±0.05	6.87±0.07	6.47±0.05	7.00±0.20	6.97±0.03	6.45±0.05	
honey	Curdling time	9.14±0.19	9.27±0.10	5.23±0.05	9.18±0.03	9.18±0.16	5.03±0.11	9.17±0.10	9.12±0.05	5.01±0.04	9.17±0.05	9.29±0.18	5.24±0.01	

Values were determined three times in triplicate and represent means ± SD for all treatments.

In control associated cultures, growth of one *Bifidobacterium* strain with *L. delbrueckii* subsp. *bulgaricus* Lb340 "Bifid+Lact" was also supported. Biomass ranged from 8.88 to 8.94 log cfu/ml for *bifidobacteria* and 8.95 to 9.08 log cfu/ml for lactobacilli (Table 2) with specific maximal growth rates of 0.73 to 0.85 h<sup>-1</sup> and 0.74 to 0.91 h<sup>-1</sup>,

respectively.

Compositional analysis of the two honeys showed unifloral and polyfloral honey to contain 26-35% and 26-33% glucose, and 30-47% and 13-30% fructose, respectively (data not shown).

In contrast to observations noted with the first combination (one *Bifidobacterium* strain+

streptococci), we monitored that unifloral or polyfloral honey at 10% level was stimulatory (P<0.05) to both *bifidobacteria* (2.23 to 3.60%) and lactobacilli (2.35 to 3.26%). However, 5% of honey was at the same time stimulatory to *bifidobacteria* (1.9 to 3.04% with polyfloral honey; 1.56 to 3.82% with unifloral honey) (P<0.05) and

inhibitory towards L. bulgaricus (-3.6 to -6.4% with polyfloral honey; -4.5 to -6.6% with unifloral honey) (P<0.05). Biomass of *bifidobacteria* registered at the end of fermentation (coagulation) in milks containing 5 and 10% polyfloral honey changes respectively from 9.10 to 9.17 log cfu/ml and 9.14 to 9.20 log cfu/ml (Table 2). Their related maximal growth rates ranged from 0.64 to 0.87  $h^{-1}$  and from 0.73 to 0.97  $h^{-1}$ . In the presence of 5 and 10% unifloral honey, it accumulated respectively 9.08 to 9.14 log cfu/ml (P<0.05) and 9.14 to 9.18 log cfu/ml (P < 0.05) which result from growth rates of 0.68 to 0.9 h<sup>-1</sup> and 1.02 to 1.11 h<sup>-1</sup> (Table 2). Biomass of lactobacilli strain varied closely to Bifidobacterium strain cocultivated with it from 8.37 to 8.64 log cfu/ml (P<0.05) in milk supplemented with 5% honey and from 9.12 to 9.29 log cfu/ml (P<0.05) in milk containing 10% (Table 2). Maximal growth rates averaged 0.7 to  $1.08 \text{ h}^{-1}$ .

# Changes in pH

The control combination "Bifid+Strep" allowed a decrease in pH value from 1.51 to 1.62 units, whereas "Bifid+Lact" control associated cultures showed a slight (P < 0.05) decrease in pH (1.44 to 1.54 units) as compared to the first associated cultures (Table 1).

In the presence of 5% honey, pH values of associated cultures "Bifid+Strep" decreased more in a manner similar to that observed in control (Table 1). pH values dropped by 1.47 to 1.53 units ( $\Delta pHmax/\Delta t = 0.34 h^{-1}$ ) and from 1.33 to 1.54 units ( $\Delta pHmax/\Delta t = 0.34 h^{-1}$ ) in milk containing polyfloral and unifloral honey, respectively. However, 10% honey seems to have a mild effect on their acidifying activity, pH values registered with the polyfloral ( $\Delta pHmax/\Delta t = 0.28 h^{-1}$ ) or unifloral honey ( $\Delta pHmax/\Delta t = 0.28 h^{-1}$ ) or unifloral honey ( $\Delta pHmax/\Delta t = 0.27 h^{-1}$ ) were less acidic comparatively to control milk (*P*<0.05) or those supplemented with 5% honey (*P*<0.05).

On the other hand, associated cultures "Bifid+Lact" developed an improved acidity in the presence of honey at the end of fermentation. Variation in pH acidifying rates at coagulation level were with a mean of 0.24 to 0.3 h<sup>-1</sup> in the presence of 5 and 10% honey (Table 2) and only polyfloral honey enhanced acidifying activity of these associated cultures. These improved percentages varied from 1 to 2% (*P*<0.05) as compared to the control milk.

# One Bifidobacterium strain co-cultivated with both LAB

# Biomass

Control associated cultures of one *Bifidobacterium* strain to both LAB at the same time generated an enhanced growth capacity at 37°C which was similar to that observed in associated cultures with each of the two LAB tested.

All bacteria showed a biomass higher than 8.3 log

cfu/ml at coagulation level.

In the presence of 5% honey, biomass quantities of *bifidobacteria* were higher (P<0.05), 8.66 to 9.15 log cfu/ml which resulted from maximal specific growth rates of 0.84 to 0.94 h<sup>-1</sup>, whereas results obtained with unifloral honey were significantly lower (P<0.05) as compared to those obtained with polyfloral honey. Improvement percentages were 1.51 to 5.50% and 4.6 to 8.60%, respectively (Table 3). *S. thermophilus* strain seems to be attenuated by the complex honey composition (Table 3), higher (P<0.05) with the one of unifloral origin than that with the other honey type tested (P<0.05); with the following exception of associated culture with Bbv-1 strain and in the presence of 5% unifloral honey, where we monitored 2% improvement (P<0.05) in streptococci biomass quantity.

For lactobacilli and at 5% honey, biomass registered at the end of fermentation with polyfloral honey was similar (P<0.05) (9.34 to 9.47 log cfu/ml) to that obtained in control milk. Whereas those monitored in milk containing unifloral honey were 0.24 to 0.36 log units lower than control (P<0.05) (9.16 to 9.36 log cfu/ml) with the following exception of associated culture with Bbv-1 strain where honey had no effect on *L. bulgaricus* growth (Table 3).

In the presence of 10% honey, growth of *bifidobacteria* strains was more stimulated (P<0.05) where the biomass ranged from 8.8 to 9.17 log cfu/ml (Table 3) (µmax= 0.87 to 0.91 h<sup>-1</sup>). These results allowed 4.95 to 6.27% and 3.89 to 9.55% improvement in *bifidobacteria* biomass in milk containing polyfloral and unifloral honeys, respectively and as compared to the control.

In contrast to our findings related to the use of 5% honey, 10% of this ingredient seems to be stimulatory to both LAB in co-culture with *bifidobacteria* (P<0.05). At the end of fermentation, streptococci strain exhibited good biomass quantities ranging from 9. 6 to 10.6 log cfu/ml (Table 3) (µmax= 1.2 to 1.54 h<sup>-1</sup>), which reflected 1.2 to 6.3% and 6.5 to 12.5% improvement in *bifidobacteria* biomass in milk containing polyfloral and unifloral honeys, respectively.

*L.* bulgaricus strain also showed an improved accumulated biomass in the presence of 10% honey; from 9.71 to 10.57 log cfu/ml (Table 3) ( $\mu$ max= 0.92 to 1.31 h<sup>-1</sup>), which reflected 3 to 9% and 5.5 to 12.7% improvement in milk containing polyfloral and unifloral honeys, respectively.

Control associated culture of one *Bifidobacterium* strain with both LAB at the same time generated a good acidifying activity (Table 3). pH values decreased from 6.54 at the beginning of fermentation to 5.05 on average at the end of the process for associated cultures with BLR, BLE, Bbv-1 or Bbv-2. This activity was correlated to a fall of 1.49 pH units ( $\Delta$ pH max/ $\Delta$ t= 0.32 h<sup>-1</sup>) and was similar to that observed in associated cultures of one *Bifidobacterium* strain with lactobacilli alone (Table 3).

In milk containing 5% polyfloral honey, pH values decreased from 6.47 at the beginning of fermentation to

		B. longum BLR + Strep+ Lact					B. longum BLE + Strep+ Lact				B. breve Bbv-1 + Strep + Lact				B. breve Bbv-2 + Strep+ Lact			
Variable	Time	BLR biomass	Strep biomass	Lact biomass	pH of fermented milk	BLE biomass	Strep biomass	Lact biomass	pH of fermented milk	Bbv-1 biomass	Strep biomass	Lact biomass	pH of fermented milk	Bbv-2 biomass	Strep biomass	Lact biomass	pH of fermented milk	
0% honey	0h	6.98±0.06	5.93±0.05	5.94±0.04	6.59±0.01	6.97±0.02	5.96±0.02	5.96±0.05	6.53±0.01	6.99±0.08	5.96±0.02	5.96±0.02	6.54±0.01	7.02±0.04	5.97±0.02	5.97±0.03	6.54±0.05	
(control)	Curdling time	8.45±0.21	9.42±0.16	9.42±0.10	5.10±0.01	8.56±0.06	9.59±0.11	9.50±0.19	5.09±0.05	8.47±0.17	9.18±0.25	9.36±0.10	5.01±0.05	8.37±0.09	9.39±0.19	9.38±0.20	5.02±0.05	
5% polyfloral honey	0h Curdling time	6.97±0.02 9.07±0.11	5.97±0.02 9.36±0.16	5.97±0.03 9.47±0.15	6.47±0.05 5.08±0.01	7.00±0.02 9.15±0.05	5.96±0.5 9.59±0.17	5.98±0.01 9.46±0.22	6.49±0.01 4.95±0.05	6.99±0.01 8.86±0.06	5.97±0.03 9.18±0.22	5.97±0.03 9.34±0.22	6.44±0.01 5.05±0.01	7.01±0.02 9.09±0.10	5.97±0.02 9.25±0.15	5.97±0.04 9.39±0.10	6.51±0.05 5.07±0.01	
5% unifloral honev	0h Curdling time	6.97±0.02 8 79+0 10	5.99±0.02 9 25+0 22	5.95±0.05 9 16+0 27	6.49±0.01	7.03±0.04	5.96±0.03 9.33+0.09	5.98±0.09 9.36+0.25	6.51±0.01 4 97+0 05	6.98±0.02	5.96±0.05 9.37+0.12	5.96±0.05 9.35+0.12	6.47±0.01 4 96+0 05	7.06±0.03 8 83+0 09	5.98±0.02 9 14+0 15	5.96±0.02 9.30+0.16	6.54±0.02	
10% polyfloral honey	Oh Curdling time	6.97±0.03 8.98±0.05	5.95±0.03 10.60±0.09	5.92±0.05 10.47±0.15	6.33±0.01 5.12±0.02	6.98±0.02 9.03±0.03	5.98±0.02 10.50±0.8	5.96±0.03 10.54±0.2	6.34±0.01 5.08±0.01	6.98±0.01 8.89±0.05	5.98±0.02 9.78±0.16	5.98±0.02 9.78±0.16	6.34±0.05 5.11±0.01	6.99±0.01 8.86±0.15	5.96±0.06 10.47±0.15	5.97±0.02 10.57±0.22	6.37±0.05 5.17±0.08	
10% unifloral honey	0h Curdling time	7.00±0.03 9.15±0.10	5.97±0.03 9.61±0.16	5.94±0.01 9.96±0.05	6.37±0.06 5.18±0.06	6.99±0.01 8.94±0.10	5.98±0.01 9.71±0.10	5.98±0.07 9.71±0.16	6.41±0.11 5.02±0.01	6.97±0.02 8.80±0.03	5.97±0.03 9.76±0.14	5.97±0.03 9.76±0.14	6.41±0.01 5.03±0.05	6.98±0.01 9.17±0.12	5.97±0.02 9.69±0.02	5.94±0.03 10.23±0.21	6.45±0.01 5.19±0.01	

Table 3. Maximal biomass levels (log CFU/mL) and pH registered at the curdling time of associated cultures of *bifidobacteria* strains and both starters of yogurt *S. thermophilus* (Strep) and *L. bulgaricus* (Lact).

Values were determined three times in triplicate and represent means ± SD for all treatments.

5.06 on average at the end of the process for associated cultures with BLR, Bbv-1 or Bbv-2. With the following exception of the culture with BLE, where pH reached a value of 4.5 ( $\Delta$ pH max/ $\Delta$ t= 0.34 h<sup>-1</sup>) (Table 3). With the 5% unifloral honey, pH values decreased from 6.5 at the beginning of fermentation to 5.07 and 5.09 at coagulation for associated cultures with BLR and Bbv-2 strains, respectively, while cultures of BLE or Bbv-1 showed more acid values of 4.97 and 4.96 ( $\Delta$ pH max/ $\Delta$ t= 0.33 and 0.32 h<sup>-1</sup>), respectively.

In milks with 10% honey, acidification was a little marked and we monitored a decrease of 1.21 to 1.25 ( $\Delta$ pH max/ $\Delta$ t= 0.25 to 0.27 h<sup>-1</sup>) and of 1.19 to 1.39 pH units ( $\Delta$ pH max/ $\Delta$ t= 0.24 to 0.28 h<sup>-1</sup>) in milks containing polyfloral and unifloral honeys,

respectively (Table 3).

Viability and post-acidifying activity during storage Associated cultures "bifidobacteria+ streptococci"

In control milk, one *Bifidobacterium* strain associated with streptococci generated a positive effect on cell viability. At the end of the storage period, loss in *bifidobacteria* biomass quantities was 2.4 to 3.4 log cycles, and the remained biomasses were less than 6 log cfu/g for BLE and Bbv-1 (Figure 1). At that level of the storage period, viable cell counts of streptococci exceeded 6 log cfu/g in all milks with experimental *Bifidobacterium* strains (Bbv-1 or Bbv-2). However, with BLE strain, the biomass did not achieve 5.6 log cfu/g.

In the presence of honey, bacteria were less susceptible to biomass losses (P<0.05) as compared to control. At the end of refrigerated storage, loss in *bifidobacteria* biomass was 14.2 to 22.35% in fermented milks containing 5% polyfloral honey and higher than 20.6% when that type of honey was used at 10% level (Figure 1a). For streptococci, the highest loss (22 to 30%) was registered in co-cultures with BLE or Bbv-1 strains (Figure 1b).

In all associated cultures, the decrease in pH between the first and the 28th day of storage at 4°C was lower (P < 0.05) than that in control, that is from 0.39 to 0.64 in fermented milk containing 5% unifloral or polyfloral honey (final pH values were



**Figure 1.** Viability of bifidobacteria strains. (a) (schematized in this order respectively: BLR, BLE, Bbv-1 or Bb-v2) and *S. thermophilus,* (b) "Strep" in associated cultures "Bifid+Strep" during four weeks storage at 4°C. Each bar represents the mean values from at least three independent experiments.

on average of 4.5). As compared to their control (P<0.05), the post-acidifying activity of associated cultures with BLR or Bbv-2 containing 5% unifloral or polyfloral honey decreased by half (Table 4).

10% of polyfloral honey was stimulatory of the postacidifying activity of the associated culture "Strep+Bbv-1". However, the other associated cultures showed a lower activity as compared to their related control cultures. With the following exception of the associated culture containing BLR *Bifidobacterium* reference strain, where we monitored a similar activity as that registered in its control associated culture (0.88 pH units).

On the other hand, 10% unifloral honey had a mild effect (P<0.05) on post-acidifying activity of co-cultures with BLR or Bbv-1 *bifidobacteria* strains where final pH values were 4.67 and 4.7, respectively (Table 4).

### Associated cultures "bifidobacteria+ lactobacilli"

In control milk, association of *L. bulgaricus* to one *Bifidobacterium* strain has reflected a positive effect on their viability. At the end of storage, decrease in *bifidobacteria* counts was 2.2 to 3.2 log cycles and the remaining biomass quantities were 5.71 to 6.64 log cfu/g (Figure 2a). For lactobacilli, the loss in biomass was 2.4 to 3.4 log cycles (Figure 2b). The remaining cell counts were on average 6.5 log cfu/g in co-cultures with BLR, BLE or Bbv-2, and 5.5 log cfu/g in that with Bbv-1 strain.

Cell viability of *bifidobacteria* was significantly (P<0.05) improved in presence of honey and as compared to the control. In all fermented milks containing 5% honey, the viable counts decreased by 0.5 to 2.4 log cycles (Figure 2a). However, the loss in viability was not significantly (P<0.05) different in milks containing 10% poly- or unifloral honey, 10.5 to 20.8% and 11.3 to 20.7%, respectively.

Post-acidification of fermented milk made by control culture "Bifid+Lact" was pronounced and milk was inconsumable since the 21st day of storage, reaching final values of 4.38 to 4.28 (Table 4).

In the presence of 5% unifloral or polyfloral honey, milk acidity was acceptable and reflected a mild effect and regular production of organic acids by *bifidobacteria*. Final pH values ranged from 4.6 to 4.7 in all associated cultures. However, milk acidity containing 10% honey was similar to that of the control (P<0.05), 4.3 to 4.36 at the end of

storage (Table 4).

# Associated cultures "bifidobacteria + both LAB"

In control fermented milk "one *Bifidobacterium* strain with both LAB" and by the absence of honey, bacteria were more susceptible to lose their viability at 4°C. However, in the presence of honey, viability of *Bifidobacterium* strain BLR was improved in a higher manner (P<0.05) in

Variablo	Time	"one Bifidobacterium Strain + Strep"					"one Bifidobacterium Strain + Lact"					"one Bifidobacterium Strain + Strep+ Lact"				
Vallable		1 day	7 days	14 days	21 days	28 days	1 day	7 days	14 days	21 days	28 days	1 day	7 days	14 days	21 days	28 days
Co. outuroo	0%	5.01±0.01	5.05±0.01	4.84±0.05	4.66±0.05	4.41±0.01	5.02±0.01	4.83±0.01	4.64±0.06	4.48±0.01	4.38±0.01	4.97±0.10	4.87±0.11	4.51±0.08	4.22±0.05	4.24±0.01
with	5%PH	5.08±0.05	4.92±0.01	4.77±0.01	4.54±0.05	4.54±0.01	5.05±0.05	5.04±0.01	4.79±0.05	4.70±0.01	4.67±0.20	4.94±0.05	4.83±0.05	4.71±0.05	4.66±0.05	4.47±0.01
B longum	5%UH	5.09±0.05	4.87±0.05	4.67±0.05	4.57±0.05	4.57±0.05	5.09±0.05	5.01±0.01	4.79±0.05	4.71±0.01	4.68±0.02	4.97±0.01	4.87±0.01	4.74±0.01	4.70±0.01	4.60±0.01
BI R	10%PH	5.05±0.05	5.02±0.05	4.99±0.05	4.80±0.05	4.48±0.01	5.10±0.07	4.88±0.03	4.64±0.06	4.50±0.07	4.30±0.01	5.04±0.01	4.91±0.17	4.42±0.01	4.15±0.01	4.07±0.02
DEIX	10%UH	5.04±0.05	5.07±0.05	5.03±0.04	4.90±0.05	4.67±0.07	5.14±0.05	5.05±0.01	4.76±0.05	4.50±0.06	4.33±0.02	5.09±0.04	4.94±0.05	4.54±0.04	4.33±0.03	4.19±0.01
	0%	4 91+0 06	5 01+0 21	4 76+0 01	4 55+0 05	4 30+0 01	5 00+0 07	4 79+0 05	4 60+0 01	4 50+0 05	4 36+0 01	4 95+0 05	4 86+0 05	4 58+0 01	4 22+0 03	4 29+0 02
Co-cultures	5%PH	4.96±0.01	4.8±0.05	4.74±0.05	4.53±0.05	4.52±0.05	4.96±0.06	4.96±0.04	4.73±0.01	$4.64 \pm 0.07$	4.61±0.01	4.92±0.01	4.85±0.01	4.72±0.01	4.67±0.01	4.49±0.01
with	5%UH	4.96±0.01	4.78±0.01	4.70±0.05	4.55±0.05	4.53±0.01	4.96±0.04	4.98±0.05	4.83±0.01	4.71±0.01	4.67±0.02	4.92±0.01	4.85±0.11	4.71±0.12	4.67±0.21	4.49±0.07
B. longum	10%PH	5.00±0.05	5.01±0.01	4.97±0.05	4.76±0.10	4.41±0.01	4.98±0.03	4.79±0.03	4.68±0.02	4.51±0.01	4.29±0.01	4.93±0.04	4.79±0.01	4.45±0.01	4.10±0.01	4.05±0.06
BLE	10%UH	5.04±0.06	5.04±0.01	4.85±0.01	4.64±0.05	4.35±0.05	5.05±0.01	4.92±0.05	4.78±0.05	4.55±0.01	4.36±0.02	5.03±0.05	4.94±0.05	4.46±0.06	4.28±0.05	4.20±0.05
Co-cultures	0%	4.91±0.03	5.01±0.01	4.87±0.02	4.65±0.01	4.48±0.02	5.01±0.06	4.88±0.14	4.63±0.01	4.43±0.01	4.28±0.11	4.96±0.04	4.86±0.02	4.65±0.01	4.32±0.01	4.21±0.02
with	5%PH	5.02±0.05	4.86±0.01	4.62±0.01	4.48±0.05	4.49±0.05	5.03±0.02	5.02±0.01	4.81±0.01	4.//±0.0/	4.72±0.04	4.91±0.04	4.83±0.03	4.65±0.20	4.63±0.21	4.43±0.06
B. breve	5%UH	4.97±0.01	4.83±0.05	4.65±0.01	4.50±0.01	4.51±0.01	4.97±0.01	4.96±0.01	4.70±0.05	4.67±0.05	4.61±0.01	4.91±0.01	4.83±0.01	4.68±0.05	4.64±0.01	4.55±0.05
Bbv-1	10%PH	4.97±0.05	5.06±0.05	4.88±0.05	4.65±0.01	4.32±0.05	5.10±0.02	5.01±0.02	4.97±0.01	4.56±0.01	4.35±0.05	5.01±0.03	4.87±0.02	4.43±0.06	4.19±0.06	4.08±0.14
	10%UH	5.09±0.05	5.10±0.01	5.04±0.05	4.97±0.07	4.70±0.05	5.02±0.11	4.75±0.05	4.70±0.01	4.54±0.01	4.34±0.05	5.05±0.03	4.97±0.02	4.52±0.05	4.34±0.05	4.16±0.05
	0%	5.02±0.01	5.00±0.01	4.87±0.05	4.64±0.01	4.31±0.01	5.04±0.01	4.84±0.05	4.62±0.04	4.5±0.01	4.34±0.01	5.01±0.05	4.92±0.02	4.54±0.02	4.22±0.05	4.23±0.06
Co-cultures	5%PH	5.06±0.31	4.91±0.05	4.76±0.15	$4.52 \pm 0.06$	4.57±0.01	5.02±0.05	5.01±0.05	4.77±0.04	4.62±0.05	4.58±0.01	4.93±0.07	4.84±0.05	4.66±0.05	4.64±0.01	4.46±0.01
with	5%UH	$4.98 \pm 0.06$	4.88±0.01	4.7±0.01	4.59±0.01	$4.59 \pm 0.05$	5.09±0.05	5.04±0.01	4.71±0.01	$4.69 \pm 0.02$	$4.65 \pm 0.04$	4.95±0.01	4.85±0.11	$4.69 \pm 0.02$	4.66±0.03	$4.50 \pm 0.02$
B. breve	10%PH	5.01±0.01	5.02±0.05	4.97±0.01	4.75±0.05	4.43±0.01	5.02±0.02	4.90±0.04	4.66±0.05	4.51±0.05	4.31±0.05	5.03±0.01	4.90±0.01	4.43±0.05	4.13±0.08	4.04±0.04
Bbv-2	10%UH	5.15±0.01	5.17±0.05	5.01±0.06	4.87±0.01	4.56±0.01	5.15±0.01	5.04±0.06	4.77±0.02	4.53±0.02	4.35±0.01	5.08±0.20	4.95±0.21	4.51±0.02	4.30±0.01	4.20±0.05

Table 4. pH changes in poly-(PH) or uni-(UH) floral honey sweetened yogurt containing *bifidobacteria* during 28 days of refrigerated storage.

Values were determined three times in triplicate and represent means ± SD for all treatments.

fermented milks containing 5% honey than those supplemented with 10% (Figure 3a). Remaining biomasses ranged from 7.2 to 8.6 log cfu/g in all honey-enriched milks that generated 33 to 57.8% improvement in BLR viability as compared to control.

The decrease in BLE biomass was less than that of BLR strain (P<0.05) in control fermented milk. After the 14th day, the biomass of BLE strain continued to decrease significantly (P<0.05) during the remaining storage days, in control and 10% honey-sweetened fermented milks (Figure 1c). At the end of the storage period, the remaining biomass ranged from 7.55 to 7.87 log cfu/g in all fermented milks with honey (P<0.05) and reflected 13.8 to 18.7% improvement in BLE cell viability (P<0.05) as compared to control (6.63 log cfu/g).

The second experimental *Bifidobacterium* strain Bbv-1 exhibited, as BLE strain, higher survival capacity in stored fermented milk (Figure 3c). After 4 weeks of refrigerated storage, remaining biomass of Bbv-1 were 5.47 log cfu/g in control milk, 7.4 log cfu/g on average in milks containing polyfloral honey, and more than 7.8 log cfu/g in those with unifloral honey (Figure 3c). Higher viable biomass registered with honey and allowed 36.3 to 52.4% improvements in Bbv-1 cell viability. Cell count diminution of the fourth strain Bbv-2 was significant (P<0.05) after the first storage day (Figure 3c) in fermented milks containing 10% unifloral honey as well as in control. However, after 28 days viable count of Bbv-2 strain was more than 8.35 log cfu/g in all fermented milks containing honey, reflecting a higher (P<0.05) survival rate of 55.68 to 58% as compared to its control culture where only 5.37 log cfu/g of cells, survived (Figure 3c).

Viability of LAB was also monitored (Figure 3b and c). At the end of the refrigerated period and in the presence of honey, *L. bulgaricus* viable biomasses were higher than 7 log cfu/g, with the following exception of 5% unifloral honey-sweetened fermented milk with Bbv-1 strain



**Figure 2.** Viability of bifidobacteria strains. (a) (schematized in this order respectively: BLR, BLE, Bbv-1 or Bb-v2) and *L. bulgaricus* "Lact", (b) in associated cultures "Bifid+Lact" during four weeks storage at 4°C. Each bar represents the mean values from at least three independent experiments.

which showed a similar (P < 0.05) viable count to that of the control (Figure 3b).

S. thermophilus showed a good (*P*<0.05) survival capacity as compared to lactobacilli in the case of associated cultures with BLR or Bbv-2 strains (Figure 3c). At the end of 4 weeks of refrigerated storage, the remaining biomass was a little above 6 log cfu/g in all control cultures, and between 7.32 to 8.68 log cfu/g in those supplemented with honey. These biomasses of streptococci registered in fermented milks with honey reflected an improved survival rate of 15.23 to 35.62% as compared to the control.

pH changes of control associated-cultures "one *Bifidobacterium* strain and both LAB" during refrigerated storage are shown in Table 4. pH values decreased from 4.92-5.09 at the first day of storage to 4.31-4.21 at the 21st day (P<0.05) which correspond to a fall of 0.61 to 0.88 pH orders. At the end of storage, pH values seemed to increase slightly (P<0.05) by 0.02, 0.07 and 0.01 in associated cultures with BLR, BLE or Bbv-2, respectively; whereas pH of associated culture with Bbv-1 continued to decrease to 4.21.

In the presence of honey (Table 4), all associated cultures showed a mild post-acidifying activity (P<0.05) lower in 5% honey sweetened-fermented milks as compared to those containing 10%; and lowest (P<0.05)

in all unifloral honey-sweetened fermented milks.

With a 5% level, pH values decreased slightly from 4.9 in the first day of storage to 4.4-4.5 after the 28th day in fermented milks containing polyfloral and unifloral honey, respectively and was significant compared to the control (P<0.05). These results reflect a moderate acidity, where the lowering in pH was 0.36 to 0.48 orders.

pH values in 10% honey-sweetened fermented milks were very acid (P<0.05), 4.07 and 4.19 in associated culture with BLR, 4.05 and 4.2 for that containing BLE, 4.08 and 4.16 for culture with Bbv-1, 4.04 and 4.2 for associated culture with Bbv-2. These pH values are registered respectively: in the presence of polyfloral and unifloral honey (Table 4).

## Sensory evaluation

The sensory scores of honey-sweetened and control samples (yogurts containing Bifid+Strep+Lact) after 21 days of refrigerated storage are given in Table 5. The texture was slightly improved during the storage period progress.

As for sensory properties, the product formulation with the highest concentration of honey (that is, 10% w/v) was too sweet and was evaluated as strong in honey flavour. However, the yogurt samples containing 5% (w/v)



**Figure 3.** Viability of bifidobacteria strains. (a) (schematized in this order respectively: BLR, BLE, Bbv-1 or Bbv2) and starters of yogurt *S. thermophilus* "Strep", (b) and *L. bulgaricus* "Lact" (c) in associated cultures "Bifid+Strep+Lact" during four weeks storage at 4°C. Each bar represents the mean values from at least three independent experiments.

of honey were found to have optimum sweetness. The points allocated for colour, body-texture and taste showed that an increase in honey content brought about an improvement in the texture, flavour and aroma of the products (P<0.05). The addition of honey had a good effect on sensory properties of fermented milk with *bifidobacteria* (P<0.05), and a particular noticeable yogurt or probiotic flavour was found. All the samples gave a good total impression, were medium sour and did not have any marked off-flavour during the storage period. None of the sweetened fermented milks were judged to be weak.

# DISCUSSION

The development of functional foods is an opportunity to contribute to an improvement in their quality, in addition to boosting consumer health and well-being (Granato et al., 2010). Moreover, over the years, functional foods hold great promise for human nutrition, but relatively little objective evidence exists for their beneficial effects (Nagai and Inoue, 2004). Most probiotic foods are derived from milk fermentation and the possibility of using other substrates such as honey to make such foods has not been adequately better considered. The advantage of using an associated culture containing *bifidobacteria* and yogurt bacteria is not only the reduction of fermentation time, but also the avoidance of other defects that fermented products containing only *bifidobacteria* may have, such as whey separation, sandy or slimy texture, too mild taste, yeasty or vinegary taste or too little aroma (Rasic and Kurmann, 1983). Also, the addition of honey may improve the dietetic value of such fermented milk.

From the results obtained of associated cultures between one *Bifidobacterium* strain and *S. thermophilus* and with regard to all tested *bifidobacteria*, BLE strain was the best stimulated (P<0.05). The biomass quantities registered with 5 and 10% polyfloral honey allowed the following improvement percentages of 9.84 to 13.86% and 7.94 to 14% for *bifidobacteria*, 13.5 to 18.2% and 4.6to 9.4% for streptococci, respectively. Improvement percentages in *Bifidobacterium* biomass noted with unifloral honey were lower (P<0.05) than those obtained with polyfloral honey, and we calculated 1.78 to 7.61% and 5.81 to 8.80% registered in milk supplemented with 5 and 10% unifloral honey, respectively.

Samplas	Colour and appearance	Body and texture	Taste and flavour	Total appreciation						
Samples	(1-5)	(1-5)	(1-10)	(1-20)						
	Organole	ptic properties/score	es							
Control (0% honey)										
+BLR	$4.22 \pm 0.20^{e}$	$3.80 \pm 0.32^{11}$	8.26± 0.51 <sup>g</sup>	16.28± 0.04 <sup>1</sup>						
+BLE	$4.33 \pm 0.01^{d}$	$4.28 \pm 0.09^{t}$	8.30± 0.02 <sup>g</sup>	16.91± 0.01 <sup>g</sup>						
+Bbv-1	$4.28 \pm 0.55^{e}$	$4.03 \pm 0.04^{h}$	8.27± 0.22 <sup>g</sup>	16.58± 0.07 <sup>h</sup>						
+Bbv-2	$4.26 \pm 0.22^{e}$	3.71± 0.33 <sup>′</sup>	$8.21 \pm 0.82^{h}$	16.18± 0.08 <sup>′</sup>						
5% polyfloral honey										
+BLR	$4.28 \pm 0.03^{\circ}$	$4.40 \pm 0.02^{d}$	$8.95 \pm 0.07^{d}$	17.63± 0.01 <sup>d</sup>						
+BLE	$4.27 \pm 0.05^{e}$	$4.61 \pm 0.07^{a}$	9.15± 0.07 <sup>b</sup>	18.03± 0.01 <sup>b</sup>						
+Bbv-1	$4.22 \pm 0.08^{e}$	$4.58 \pm 0.24^{b}$	$9.0 \pm 0.12^{\circ}$	17.80± 0.02 <sup>cd</sup>						
+Bbv-2	$4.30 \pm 0.01^{\circ}$	$4.20 \pm 0.06^{9}$	$8.97 \pm 0.06^{\circ}$	17.47± 0.01 <sup>e</sup>						
5% unifloral honey +BLR +BLE +Bbv-1 +Bbv-2	$4.23 \pm 0.14^{e}$ $4.36 \pm 0.03^{d}$ $4.41 \pm 0.07^{c}$ $4.29 \pm 0.06^{de}$	$4.31 \pm 0.28^{et}$ $4.48 \pm 0.11^{c}$ $4.60 \pm 0.02^{a}$ $4.30 \pm 0.31^{t}$	$8.51 \pm 0.02^{t}$ $9.20 \pm 0.04^{b}$ $9.11 \pm 0.03^{b}$ $9.00 \pm 0.41^{c}$	$17.05 \pm 0.03^{t}$ $18.04 \pm 0.01^{b}$ $18.12 \pm 0.01^{a}$ $17.59 \pm 0.05^{e}$						
10% polyfloral honey +BLR +BLE +Bbv-1 +Bbv-2	$4.60 \pm 0.30^{a}$ $4.44 \pm 0.21^{c}$ $4.38 \pm 0.34^{cd}$ $4.29 \pm 0.04^{de}$	$4.13 \pm 0.31^{9}$ $4.38 \pm 0.05^{d}$ $4.34 \pm 0.08^{e}$ $4.20 \pm 0.11^{9}$	$9.10 \pm 0.24^{b}$ $9.04 \pm 0.04^{c}$ $9.14 \pm 0.08^{b}$ $9.13 \pm 0.07^{b}$	$17.83 \pm 0.01^{\circ}$ $17.86 \pm 0.02^{\circ}$ $17.86 \pm 0.04^{\circ}$ $17.62 \pm 0.01^{de}$						
10% unifloral honey +BLR +BLE +Bbv-1 +Bhy-2	$4.51 \pm 0.07^{b}$ $4.44 \pm 0.03^{c}$ $4.33 \pm 0.22^{d}$ $4.35 \pm 0.06^{d}$	$4.30 \pm 0.20^{t}$ $4.40 \pm 0.06^{d}$ $4.38 \pm 0.25^{d}$ $4.27 \pm 0.01^{t}$	$9.36 \pm 0.06^{a}$ $9.02 \pm 0.02^{c}$ $9.05 \pm 0.14^{c}$ $8.88 \pm 0.09^{e}$	18.17± 0.02 <sup>a</sup> 17.86± 0.01 <sup>c</sup> 17.76± 0.01 <sup>d</sup> 17.50± 0.01 <sup>e</sup>						

**Table 5.** Organoleptic properties<sup>†</sup> of *bifidobacteria* yogurt (one *Bifidobacterium* strain: "BLR, BLE, Bbv-1 or Bbv-2"+ Strep+Lact) after 21 days of refrigerated storage.

a-i, Means in the same column followed by different letters were significantly different (P< 0.01);<sup>†</sup>, Mean values from 10 panellists.

were insignificant differences (P < 0.05) in improvement percentages for streptococci in the presence of 5 or 10% unifloral honey, and we calculated 1.4 to 6.2% and 2.6 to 5.3%, respectively.

S. thermophilus (facultative anaerobe) oxygen consumption can help *bifidobacteria's* growth (strict anaerobe). In addition, the proteolytic activity of S. *thermophilus* releases amino acids which serve as essential nutriment for *bifidobacteria* growth. Our findings suggests that the microbial behaviour of the tested bacteria in this type of association was species dependent with the increasing of honey concentration (from 5 to 10%) in milk. This increase in honey level was found to influence biomass quantity of proteolytic lactic acid strain and stimulate that of *bifidobacteria*, that is, the case of BLE *Bifidobacterium* strain.

Honey seems to be of interest as a prebiotic material because it contains many oligosaccharides and low molecular weight polysaccharides likely to resist degradation by host enzymes, and thus be available as a nutrient source for the microflora in the large bowel. Sanz et al. (2005), Jan Mei et al. (2010), and Nagpal and Kaur (2011) concluded that honey contained oligosaccharides

which would function well as prebiotics for probiotics.

In the type of association between one *Bifidobacterium* strain and *L. bulgaricus* "Bifid+Lact", Bbv-1 showed the best capacity of growing in presence of honey. Consequently, curdling time was 30 to 45 min shorter. There were no antagonist effects towards *bifidobacteria* by *L. bulgaricus*. The curd was aromatic but with a weak consistency. Biomass of lactobacilli varied in response to the type of added-honey and/or *Bifidobacterium* strain co-cultivated with it. Sodini et al. (2002) monitored that *bifidobacteria* co-cultivated with proteolytic strains brought out peptides in sufficient amounts which have a growth promoting effect on probiotic bacteria.

All the published articles about the influence of honey on *Bifidobacterium* strains underlined its stimulatory effect or that of its separated fructo-oligosacchrides fraction on growth and acid production of *bifidobacteria* (Shamala et al., 2000; Chick et al., 2001; Ustunol and Gandhi, 2001; Kajiwara et al., 2002; Sanz et al., 2005; Shin and Ustunol, 2005; Jan Mei et al., 2010; Riazi and Ziar, 2010; Popa and Ustunol, 2011). However, there are no published data –until today- describing the behavior of associated cultures between *bifidobacteria* and lactic acid bacteria in sweetened-honey milk. In the present study, the inhibitory effect of 5% honey on L. bulgaricus growth in associated culture with one Bifidobacerium strain could be explained by the disruption in the established bacterial symbiotic relationship by the presence of honey. In a previous work (Riazi and Ziar, 2008), we reported that the growth of both yogurt starters in associated culture was not inhibited by the presence of the same types of assayed honeys at the level of 5%. Despite the higher decrease in biomass, 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. Antibacterial and/or bactericidal effects of honey are lacking (Kwakman et al., 2011), and thus make unclear the differences in bacterial behavior in response to the level and the type of added honev.

Association of one starter of yogurt to *bifidobacteria* improved significantly (P<0.05) their acidifying activity as compared to that obtained in each *Bifidobacterium* monoculture (Riazi and Ziar, 2010). Improvement percentages in the acidifying activity in milks were 55% for "Bifid+Strep" and 22.72% for "Bifid+Lact" as compared to the control LAB monocultures (Riazi and Ziar, 2008). On the other hand, associated cultures "Bifid+Lact" developed an improved acidity in the presence of honey at the end of fermentation and this improvement seemed to be honey level and floral origin dependent (P<0.05).

In associated culture with both LAB, there were no differences in curdling time of cultures supplemented with 10% honey as compared to the control (data not shown). However, curdling time of milk in the presence of 5% honey was 1 h 20 min to 1 h 50 min shorter. Furthermore, all tested *Bifidobacterium* strains showed improved and symbiotic growth capacities. On the other hand, inhibitory honey effect was lower on lactic acid bacteria as demonstrated here and comparatively to their monocultures (Riazi and Ziar, 2008). In general, associated culture with BLE *Bifidobacterium* strain gave the best results comparatively to the other associated cultures and it is in this culture, where the 5% inhibitory honey effect on LAB was the lowest.

In the present work, losses in biomass for *S*. *thermophilus* in co-cultures with *bifidobacteria* and lactobacilli were higher (27.7 to 40.2%) than those observed in streptococci monocultures (P<0.05) (Riaziand Ziar, 2008). These differences in streptococci biomass survival might be largely correlated to the *Bifidobacterium* strain co-cultivated with it. Our conclusion is similar to the findings of Shah's (2000), who studied the behaviour of LAB in associated culture with *bifidobacteria*.

In the presence of honey, pH decrease in associated cultures between one *Bifidobacterium* strain and both starters of yogurt was higher in milks supplemented with honey at a 5% level than in those with a 10% concentration. Our findings allowed 53 to 69% (P<0.05) improvement in

acidifying rates of *bifidobacteria* as compared to results obtained with their monocultures (Riazi and Ziar, 2010). Floral origin has no effect on pH changes but the symbiotic relationship between each *Bifidobacterium* strain and both LAB seems to regulate organic acids synthesis. Varga (2006) reported that honey has the ability to decrease solutions sourness. This property might serve to increase consumer acceptability to acidic products such as yogurt.

It is very important to note that there is no published data – at our knowledge- until today on viability and postacidifying activity of associated culture "*bifidobacteria* with LAB" in the presence of honey.

During refrigerated storage, the loss in streptococci viability in the type of association "Bifid+Strep" was *bifidobacteria* type dependent and not influenced by honey level. The decrease in counts observed in control fermented milk might be due to metabolic activity of starter culture during refrigerated storage as previously demonstrated by Godward et al. (2000). Vinderola et al. (2002) showed that *B. bifidum* strain losses 1.6 to 4 log cycles in its viability at 5°C when associated to LAB. This diminution was LAB type dependent.

Associated culture of *L. bulgaricus* with Bbv-2 *Bifidobacterium* strain in the presence of honey gave the best viability result. The remaining biomasses of Bbv-2 were 8.5 to 8.7 log cfu/g, and 7.1 to 8.3 log cfu/g for lactobacilli. This reflected improvement percentages of 35 to 49% and from 9 to 27%, respectively. The little decrease in cell viability registered in associated cultures with honey might reflect a protective effect on microbial viability.

In a recent study, Nagpal and Kaur (2011) reported that honey added at the level of 5% improved the viability of lactobacilli pure cultures after 5 weeks storage and that improvement might be strain dependent.

On the whole, our results demonstrated that 10% honey seems to be a protective ingredient towards microbial viability of both *bifidobacteria* and lactic acid bacteria during four weeks of refrigerated storage and where more than 7 log cfu/g of cells, survived.

Finally, the good sensory properties of honey sweetened fermented milk suggested in this study (containing Bifid+Strep+Lact) may result from a higher proteolytic activity and the exopolysaccharides synthesis ability of the starter cultures incorporated in milk. This improvement might also be partially related to honey FOS fraction. Accordingly to Cáceres et al. (2004), short chain fructo-oligosaccharides have a positive effect on the sensory properties of conventional and reduced-fat cooked meat sausages.

## Conclusion

The results of this study demonstrated that both assayed honeys stimulated the growth, acidity and viability of *bifidobacteria* strains in associated cultures with lactic acid bacteria. Yogurt with 5% honey and containing one *Bifidobacterium* strain and both yogurt starters seems to be the best combination for commercial production. Finally, honey could be used as a sweetener and prebiotic in order to improve fermentative aptitudes of *bifidobacteria* in desirable flavour mix probiotic product and with a relatively stable shelf life.

### ACKNOWLEDGEMENTS

Authors thank Michelle Christensen (Chicago, USA) for final English revision of the manuscript. This work was financially supported by the Algerian ministry of Scientific Research (CNEPRU project N°: F 022 20070100).

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