Growth and viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in traditional yoghurt enriched by honey and whey protein concentrate

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Summary

The ability of whey protein concentrate (WPC) (1% w/v) and/or honey (2% and 4% w/v) to improve lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) growth and viability in yoghurt during a 21 day period of storage was investigated. Another focus of this study was to examine fermentation kinetics and post-acidification rates through pH and lactic acid content measurements over the 21 day period. The addition of WPC and acacia honey accelerated fermentation and improved lactic acid bacteria (LAB) growth over the 21 days, but honey proportion did not significantly affect the viability of LAB. Moreover, adding honey and WPC did not support the overproduction of lactic acid, which positively influenced yoghurt stability during the 21 day storage period.

Key words: Honey, LAB, Viability, Whey protein concentrate (WPC), Yoghurt

Introduction

Lactobacillus delbrueckii subsp. *bulgaricus* and *Streptococcus thermophilus* are thermophylic lactic acid bacteria (LAB), which are highly adapted to growing on lactose and converting it into lactic acid. *Lactobacillus bulgaricus* maintains extensive proteolytic and amino acid transport systems that are useful in the protein-rich milk environment (Klaenhammer *et al*., 2008). Due to their high adaptation to milk environments and their ability to resist low pH values (Delley and Germont, 2002), yoghurt is the primary habitat of these species. Milk fermentation success often relies on the synergy between these species. Using this symbiotic action, the desirable acidity of the final yoghurt product can be achieved (Chandan, 2006).

Whey protein concentrate (WPC) is a functional ingredient often used in yoghurt manufacturing to enhance its physical and structural properties. It also improves nutritional values and biological effects of yoghurt on health (Antunes *et al*., 2005; Özer and Kirmaci, 2010). WPC may or may not (Kailasapathy and Supriadi, 1996; Antunes *at al*., 2005) improve culture viability; nevertheless, it can negatively affect yoghurt flavour and consumer satisfaction and preference (Fox, 2001).

Owing to its low pH and variety of beneficial nutritional properties, honey has become a popular natural sweetener in dairy production (Tamime and Robinson, 1999), especially in yoghurt manufacturing (Bogdanov *et al*., 2008). Honey is a syrup primarily containing fructose (38.5%) and glucose (31.3%); maltose (7.2%) and sucrose (1.3%) and various oligosaccharides up to 10.9% (Bogdanov *et al*., 2008). Honey has several well known antimicrobial properties (Molan *et al*., 1997). Previous studies have reported the inhibitory effects of honey against LAB (Čurda and Plockova, 1995; Roumyan *et al*., 1996), but more recent works favour the beneficial effects of honey on LAB and bifidobacteria viability in yoghurt (Varga, 2006; Riazi and Ziar, 2008). Stijepic *et al*. (2012) reported improvements in the sensory properties of probiotic yoghurt samples produced by adding formulations of 1% WPC and 2, 4 or 6% acacia honey.

To the authors' knowledge, no studies have addressed the effect of WPC and acacia honey addition on LAB growth and viability. The purpose of the present study was, therefore, to examine the effect of WPC (1% w/v) and acacia honey (2 and 4% w/v) addition on the growth and viability of lactic acid bacteria counts in yoghurt. Another aim of this study was to examine fermentation kinetics through pH and postacidification rate measurements by determining lactic acid content over a 21 day period of storage.

Materials and Methods

Materials

Homogenized raw milk (1.5% fat, 3.3% proteins, 4.7% lactose), obtained from "Vindija" Varaždin, Croatia, was used for the production of yoghurt samples. VIVOLAC DriSet Yogurt 442: 10% *L. delbrueckii* subsp. *bulgaricus* and 90% *S. thermophilus* (Vivolac Culture Corporation, Indiana, USA) was applied to achieve a concentration of 0.03 gl^{-1} in the yoghurt samples. Added ingredients included whey protein concentrate (Textrion™ PROGEL 800, DMW International BV, Veghel, The Netherlands) containing 80% proteins, 5% ash, 5% fat and 7% lactose; and acacia honey ("BK Kompani", Banja Luka, Bosnia and Herzegovina) containing 16.9% water, 0.08% ash, 82.6% total sugars and 71.1% reducing sugars.

Yoghurt production

Raw and homogenized skim milk supplemented with WPC (1% w/v) was heated at 85°C for 20 min and cooled to 55°C when 2% w/v (1% WPC + 2% H) and 4% w/v (1% WPC + 4% H) acacia honey was added. Control samples were produced without any addition. The milk was cooled to 41°C and inoculated with the chosen yoghurt starter. Incubation at the same temperature was discontinued when the pH reached 4.6. Fermented milk samples were rapidly cooled to 20°C and placed into a cold storage at 4°C ± 1. Each trial was repeated three times.

Chemical analysis

Dry matter content was determined after drying at 105°C and ash after mineralization at 550°C (Caric *et al*., 2000). The pH was measured during fermentation and over 21 days of storage using pH 510/mV Meter (Eutech Instruments, England). Lactic acid content was calculated according to basic titratable acidity (Sabadoš, 1996) every 7 days after storage. Maximal acidification rate (ΔpH max/Δt) was calculated as follows:

$$\Delta pH\ max/\Delta t = (pH_1 - pH_2)/(t_2 - t_1) \quad [1]$$

where
pH_1: The pH value at time t_1 of the exponential phase
pH_2: The pH value at time t_2 of the exponential phase

Enumeration of lactic acid bacteria

To enumerate lactobacilli and streptococci, appropriate dilutions of yoghurt samples were pour-plated into MRS agar (Biolife, Milano, Italy), after 1st and 21st days of storage. The MRS agar pH was adjusted to 6.2. Dilli *et al*. (2010) found that *L. bulgaricus* and *S. thermophilus* showed positive growth on MRS agar medium at pH = 6.3; however, *S. thermophilus* did not grow and a pH of 5.4.

While *S. thermophilus* plates were later incubated at 42°C for 72 h for counting purposes, *L. bulgaricus* plates were incubated at 30°C for 72 h. Total counts from all plates were used to calculate viability.

Viable cells were calculated as follows:

$$\%\ Viability = (CFU\ at\ n\ week(s)\ of\ storage/initial\ CFU) \times 100 \quad [2]$$

Microstructural analysis

The microstructure of fermented milk beverages was analysed by scanning electron microscopy (SEM) technique, using a *Joel*, *JSM-6460LV* Scanning Electrone Microscope (Oxford, Instruments). Samples were prepared by fixation in 2.8% glutaraldehyde, dehydration in different ethanol solution percentages, extraction in chloroform, dehydration in absolute ethanol for 24 h, drying using a "Critical Point Dryer" (CPD 030, BAL-TEC, Leichtenstein) and coating with gold using BAL-TEC, SCD 005, Sputter coater (Sandoval-Castilla *et al*., 2004; Iličić *et al*., 2006). The Voltage used for SEM watching was 25 kV.

Statistical analysis

Data were analyzed using a one-way analysis of variance along with the Holm-Sidak test for comparison of means at a 95% confidence level (SigmaPlot 11.0, Sysstat Software, Inc., USA). Values of different tests were expressed as means ± standard deviations (X ± SD).

Results

Figure 1 shows fermentation kinetics of the experimental yoghurts. Fermentation time for the samples produced with WPC and honey was approximately 2-3 h shorter than that of the controls. Samples with 1% WPC and 1% WPC + 2% H were fermented for 1 h longer compared to the 1% WPC + 4% H sample.

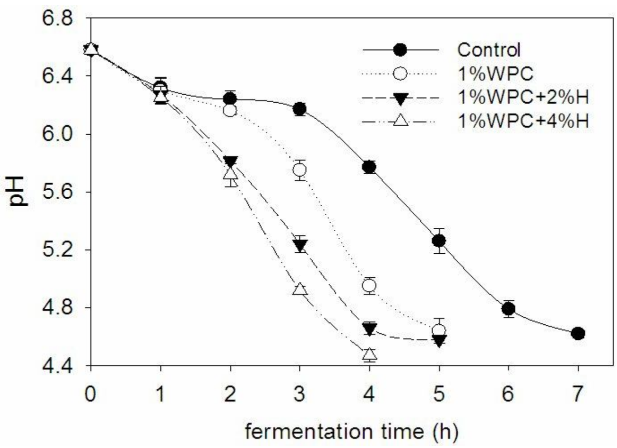


Fig. 1: pH values in the produced yoghurt samples during fermentation time. WPC: Whey protein concentrate additions, and H: Acacia honey additions

Results obtained for the acidifying activity of LAB over fermentation showed the maximal acidification rate for all experimental yoghurts to be 1h before the end of fermentation (Table 1). Maximal acidification rate at the end of fermentation (ΔpH/Δt) was observed in the yoghurt with the highest proportion of honey (0.53 h^{-1}), while minimal acidification rate (0.28h^{-1}) was observed in the control yoghurt.

The pH and lactic acid content (%) of all produced samples are showed in Fig. 2. The decreasing pH value over the first week and the stabilisation during the rest of storage period was common in all yoghurt samples (Fig. 2A).

Table 1: Acidification rate (ΔpH/Δt) during fermentation of yoghurt samples

Hours of fermentation	Yoghurt samples			
	Control	1% WPC	1% WPC + 2% H	1% WPC + 4% H
1st	0.26 ± 0.06 h^{-1}	0.28 ± 0.07 h^{-1}	0.31 ± 0.07 h^{-1}	0.33 ± 0.06 h^{-1}
2nd	0.17 ± 0.03 h^{-1}	0.21 ± 0.01 h^{-1}	0.38 ± 0.02 h^{-1}	0.43 ± 0.03 h^{-1}
3rd	0.14 ± 0.01 h^{-1}	0.28 ± 0.03 h^{-1}	0.45 ± 0.02 h^{-1}	0.55 ± 0.004 h^{-1}
4th	0.20 ± 0.01 h^{-1}	0.41 ± 0.01 h^{-1}	0.48 ± 0.01 h^{-1}	0.53 ± 0.01 h^{-1}
5th	0.26 ± 0.02 h^{-1}	0.39 ± 0.01 h^{-1}	0.40 ± 0.01 h^{-1}	
6th	0.30 ± 0.01 h^{-1}			
7th	0.28 ± 0.006 h^{-1}			

WPC: Whey protein concentrate additions, and H: Acacia honey additions

Table 2: Macro-components of yoghurt samples and viable cell counts (cfu.ml^{-1}) of the cultures on the 1st and 21st days of storage of yoghurts with WPC and honey additions (mean±SD)

Samples	Macro-components (%, w/w)		Viable cell counts (cfu.ml^{-1})	
	Dry matter	Ash	1st day of storage	21st day of storage
Control	9.689 ± 0.124^{a}	0.700 ± 0.071^{a}	$1.0 \times 10^{11} \pm 5 \times 10^{9a}$	$3.0 \times 10^{8} \pm 7 \times 10^{6a*}$
1% WPC	10.387 ± 0.156^{b}	0.744 ± 0.057^{a}	$3.0 \times 10^{11} \pm 4 \times 10^{9b}$	$5.0 \times 10^{8} \pm 4 \times 10^{6a}$
1% WPC + 2% H	11.506 ± 0.018^{c}	0.757 ± 0.028^{a}	$7.0 \times 10^{11} \pm 9 \times 10^{9bc}$	$4.0 \times 10^{9} \pm 9 \times 10^{7b*}$
1% WPC + 4% H	12.491 ± 0.035^{d}	0.805 ± 0.035^{a}	$1.1 \times 10^{12} \pm 10^{10c}$	$3.0 \times 10^{9} \pm 10^{8b}$

WPC: Whey protein concentrate additions, and H: Acacia honey additions. ^{abcd} Means in a column with different letters differ significantly (P≤0.05; * P≤0.001, Holm-Sidak test)

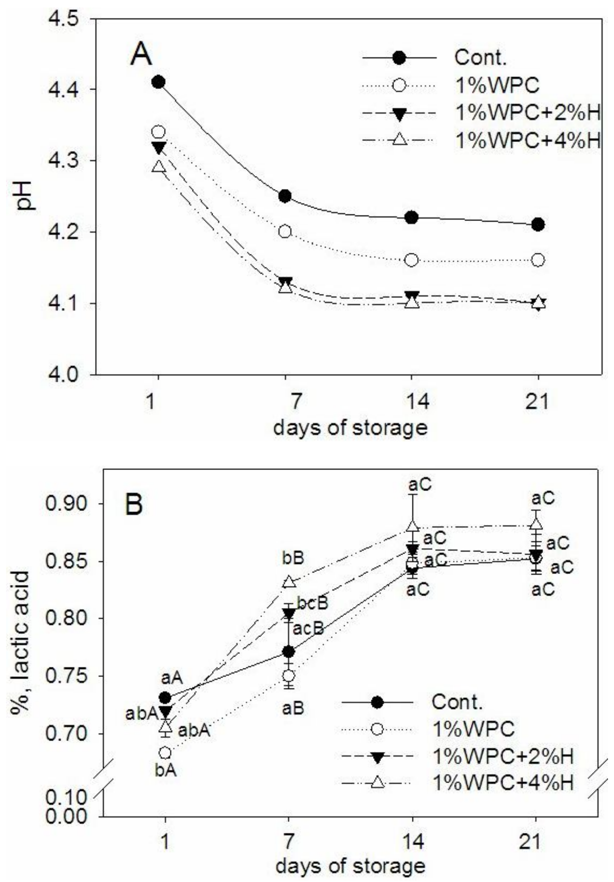


Fig. 2: Change of pH value (**A**) and lactic acid content, v/w (**B**) in the produced yoghurts over 21 storage days. WPC: Whey protein concentrate additions, and H: Acacia honey additions. Values present means of three replicates ± SD. ABC: Different capital letters denote the results within a same treatment over different days of storage (P<0.05, Holm-Sidak test). abc: Different small letters denote significantly different results within a different treatment (WPC: with or without honey addition) over the same day of storage, (P<0.05, Holm-Sidak test)

Lactobacillus bulgaricus produces lactic acid during cold storage. This process is known as post-acidification. Even when the culture ratio was in favour of *S. thermophilus*, the increase of lactic acid content over three weeks of storage was significant (P<0.05) (Fig. 2B). Honey addition did not appear to influence the changes in lactic acid content on the first day of storage compared with other yoghurt samples. However, as Fig. 2B shows, a larger proportion of honey influenced the highest lactic acid content compared to the control and WPC added yoghurt samples on the seventh day of storage (P<0.05). Furthermore, no difference was found in lactic acid content between the produced yoghurts on the 14th and 21st days (P>0.05).

Total solid contents of the yoghurt samples are presented in Table 2.

LAB counts at the 1st and 21st days of storage are presented in Table 2. Compared to the control samples, adding honey to milk significantly enhanced LAB counts on the first day of storage (P<0.05). Additionally, 1% WPC and 1% WPC + 2% H formulations induced similar LAB counts (P>0.05), while control yoghurt had the lowest counts (P<0.05). The higher proportion of honey induced the highest LAB counts in yoghurt at the 1st day of storage.

As Table 2 shows, no significant differences were found between LAB counts in the control and WPC alone samples at the 21st day (P>0.05). As seen from Fig. 3, adding 1% WPC did not improve the viability of LAB over storage. Viable LAB counts were the largest in the sample containing 1% WPC + 2% honey at the end of storage (>10^{9} cfu.ml^{-1}), and differed significantly from the 1% WPC and control samples at P<0.05 and P<0.001, respectively. A stimulatory effect of acacia honey on the growth of LAB over 21 days of storage was observed (Table 2 and Fig. 3), but honey proportions did not significantly influence the viability of LAB as demonstrated by similar LAB counts in the 1% WPC +

2% H and 1% WPC + 4% H formulations (P>0.05). As Table 2 shows, higher proportions of honey improved viable LAB counts in yoghurt as compared to the control and the 1% WPC samples (P<0.05); however, as shown in Fig. 3, the addition of honey did not significantly affect the viability of the LAB bacteria, compared to the control (P>0.05).

Microstructures of the produced yoghurts were observed by scanning electron microscopy (Fig. 4).

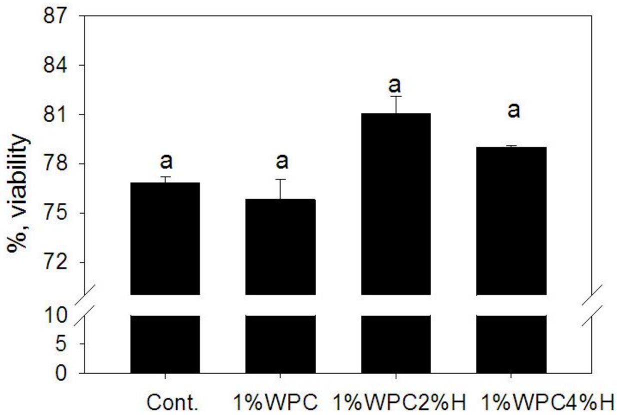
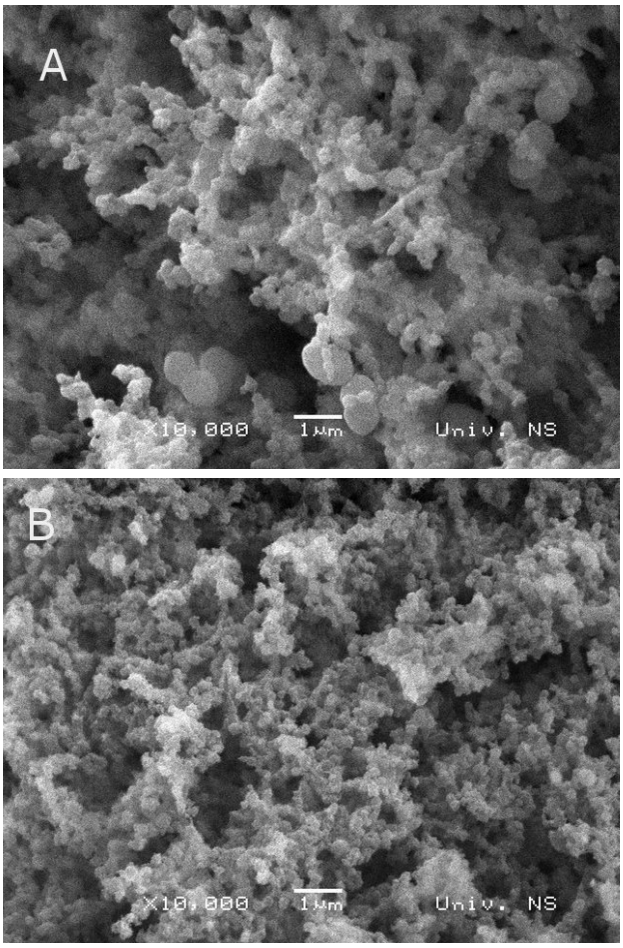


Fig. 3: Viability of lactic acid bacteria over 21 days of storage. Different small letters denote significantly different results (P<0.05, Holm-Sidak test). WPC: Whey protein concentrate additions, and H: Acacia honey additions



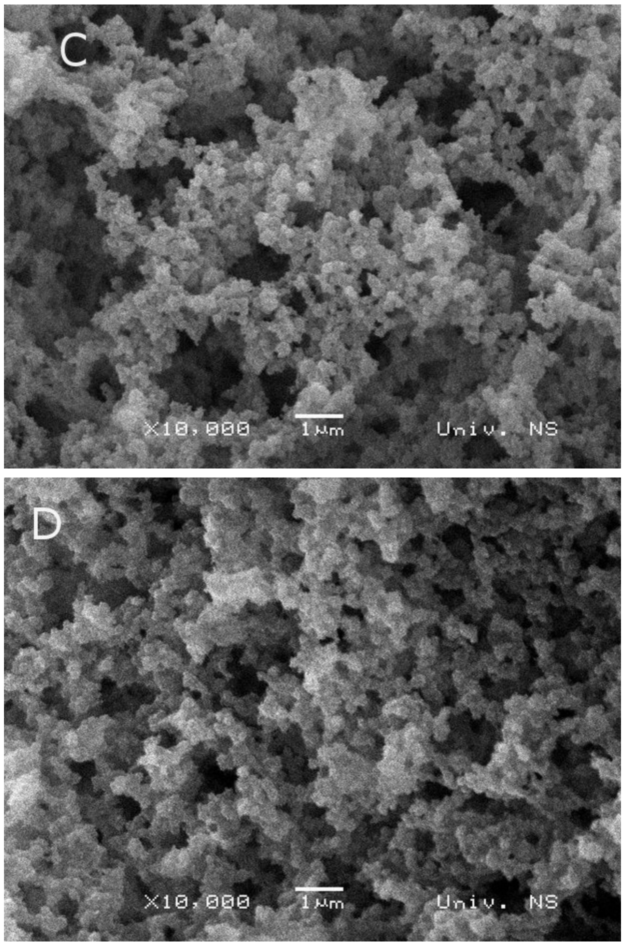


Fig. 4: Microstructure (SEM) of yoghurt produced without any addition (control) (**A**), and with the addition of 1% WPC (**B**), 1% WPC + 2% acacia honey (H) (**C**) and 1% WPC + 4% honey (**D**)

Adding WPC increased the diameter of particles (Aziznia *et al*., 2008), while adding honey did not influence the microstructure of the produced yoghurts.

Discussion

As shown in Fig. 1, WPC and honey additions decreased fermentation time compared to the control yoghurt. It has been previously reported that the addition of WPC to milk followed by heat treatment induced a decrease of fermentation time (Antunes *et al*., 2005; Milanovic *et al*., 2009). Several authors have showed that adding honey to milk decreased fermentation time (Riazi and Ziar, 2008; Stijepic *et al*., 2009; Slačanac *et al*., 2011), while others reported no accelerated fermentation (Sert *et al*., 2011). Further acidification kinetics show that acacia honey enhances lactic acid production during fermentation (Table 1), which is probably due to the assimilation of glucose from honey and its conversion to lactic acid by LAB activity (Varga, 2006; Riazi and Ziar, 2008).

During the storage period, WPC and honey enriched yoghurts had the lowest pH, while the control sample had the highest (Fig. 2A). These results agree with those obtained for the yoghurt produced with LAB and 5% (w/v) unifloral or polyfloral honey over 28 days of storage (Riazi and Ziar, 2008). Opposite results (Sert *et al*., 2011) were found when sunflower honey was added to skim milk at 2, 4, or 6% (w/v) levels.

As Fig. 2B shows, the control sample had higher lactic acid content than the 1% WPC sample on the first day of storage (P<0.05), confirming the findings of Chick *et al*. (2001). Similar to the results of Dave and Shah (1998), Antunes *et al*. (2005) and Sady *et al*. (2007), adding WPC to milk did not enhance lactic acid production during the first week of storage. Honey proportion did not significantly influence the production of lactic acid in yoghurt samples over storage either. Changes of lactic acid content were found to be mostly related to the storage period than honey or WPC addition. Generally speaking, acacia honey or WPC addition did not support the overproduction of lactic acid, which can positively influence yoghurt stability during the 21 day storage period. The lower acidification of WPC + honey supplemented products can increase the shelf life of low fat yoghurts, which is often limited by excessive acidification during storage (Akalin *et al*., 2007).

Total solid contents of the yoghurt samples significantly increased with WPC and WPC + honey addition (P<0.05). As Table 2 shows, ash content also increased, but not significantly (P>0.05). The highest total solid contents were found in samples produced by adding 1% WPC + 4% H.

As seen in Fig. 3, the addition of 1% WPC did not improve the viability of LAB over storage. This result was similar to those reported by Antunes *et al*. (2005). Significantly higher LAB counts in samples containing honey indicate the metabolism of honey sugars by the LAB during fermentation and storage periods. Shamala *et al*. (2000) and Slačanac *et al*. (2011) have reported protective effects of honey on the viability of lactobacilli or streptococci. In fact, honey concentration and type are considered as important factors which stimulate or inhibit LAB growth and viability. The 5% honey level was not inhibitory to *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Chick *et al*., 2001). The protective effect on LAB cell viability over 28 storage days has also been observed at the 10% honey level (Riazi and Ziar, 2008). However, no change in LAB viability over 42 storage days was observed in yoghurt enriched with 1, 3 or 5% honey (Varga, 2006). Furthermore, an increase in the viability of *L. bulgaricus* in yoghurt produced by adding 4 or 6% sunflower was reported to increase *S. thermophilus* by adding 2 or 4% honey, respectively (Sert *et al*., 2011).

As Fig. 4 shows, the microstructure of produced yoghurts consists of casein chains with a typical microstructure for yoghurt (Tamime *et al*., 2007). The extensive rearrangements of protein particles and honey sugars during fermentation could lead to the formation of smaller pores, a dense protein matrix and a compact structure. However, adding honey does not significantly influence the microstructure of yoghurts, as WPC and acacia honey enriched probiotic yoghurt do (Stijepic *et al*., 2011).

The results obtained from this study show that formulations of WPC + honey accelerated fermentation and had stimulatory effects on LAB growth over a 21 day storage period, and did not significantly affect the viability of LAB bacteria (P>0.05). Moreover, adding honey to WPC did not support the overproduction of lactic acid, which can positively influence yoghurt stability during the 21 day storage period.

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