Journal of the Association of Arab Universities for Basic and Applied Sciences (2015) 18, 7–11

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Journal of the Association of Arab Universities for Basic and Applied Sciences
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حيوية بكتيريا *بفيدوبكتيريوم بفيدم* (ب.ب) في الزبادي المحضر من حليب البقر والابل والمتبل بالقرفة والثوم

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الملخص:

تم دراسة تأثير المستخلص المائي للثوم والقرفة في الزبادي على بكتيريا *بفيدوبكتيريوم بفيدم* **(ب.ب)** طيلة ٢١ يوم من التخزين المبرد ومن خلال محاكاة الهضم المعدي– المعوي. وتم تحضير نوعين من الزبادي المحضر من حليب البقر او الابل في وجود المستخلص المائي للثوم او القرفة. وقد وجد أن عدد الخلايا الحيوية من ب. ب في اليوم الاول للزبادي المحضر من حليب البقر و مستخلص الثوم او القرفة كانت أعلى معنويا (8.1 ×10^9 وحدة– مكونة مستعمرة/مل (ومم/مل) و6.6 ×10^9 ومم/مل على التوالي) من الزبادي العادي (1.9 ×10^9 ومم/مل). بالمقابل فإن عدد الخلايا الحيوية من *بكتيريا* ب. ب في الزبادي الطازج المحضر من حليب الابل كانت 1.99 ×10^9 ومم/مل بينما أدى وجود مستخلص الثوم او القرفة في الزبادي الى زيادة معنوية في اعداد البكتريا الى 19.61 ×10^9 ومم/مل و 25.55×10^9 ومم/مل على التوالي . وكذلك فإن عدد الخلايا الحيوية من ب.ب في كل انواع الزبادي الحاوي على مستخلص الاعشاب والمحضر من حليب البقر او الابل انخفضت معنويا خلال التخزين المبرد في الثلاجة . كان عدد الخلايا الحيوية من ب.ب تقريبا 1.3 ×10^9 ومم/مل في نوعي زبادي الاعشاب الطازج المحضر من حليب البقر بعد ساعة من الهضم في المعدة .أما الهضم المعوي لمدة ساعة ادى الى زيادة اعداد الخلايا الحيوية من ب.ب في كل أنواع الزبادي الطازج ولكن ليس في أنواع الزبادي التي عمرها 7 أيام (الزبادي العادي والزبادي الحاوي على خلاصة الثوم) . ولكن فإن اطالة الهضم المعوي لساعة اخرى أدى إلى خفض معنوي في اعداد الخلايا الحيوية من ب.ب في كل أنواع الزبادي الطازج والمخزن. بالمقابل فإن الزبادي الطازج المحضر من حليب الابل اظهر اعدادا من الخلايا الحيوية من ب.ب أقل او يساوي 1×10^9 ومم/مل بعد الهضم في جهاز محاكاة الهضم المعدي– المعوي. هذا وقد أظهر زبادي الثوم المحضر من حليب الابل والمخزن في الثلاجة لمدة ٧ ايام خلايا حيوية اقل لاعداد البكتيريا بعد الهضم في المعدة مقارنة بالزبادي العادي وزبادي القرفة. كذلك فإن اعداد الخلايا الحيوية من ب.ب انخفضت معنويا في كل انواع الزبادي المحضر من حليب الابل بعد ساعتين من الهضم في الامعاء. في الختام فأن المستخلص المائي للثوم والقرفة قد حسن نمو ب.ب. في كلا النوعين من الزبادي اثناء التخزين في الثلاجة ولكن ليس اثناء الهضم.

Journal of the Association of Arab Universities for Basic and Applied Sciences (2015) **18**, 7–11

University of Bahrain

Journal of the Association of Arab Universities for Basic and Applied Sciences

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ORIGINAL ARTICLE

Survival of *Bifidobacterium bifidum* in cow- and camel-milk yogurts enriched with *Cinnamomum verum* and *Allium sativum*

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Received 5 September 2013; revised 19 December 2013; accepted 23 February 2014
Available online 17 March 2014

KEYWORDS

Allium sativum;
Cinnamomum verum;
B. bifidum;
Simulated gastrointestinal digestion;
Yogurt

Abstract The effects of *Allium sativum* and *Cinnamomum verum* water extracts on the survival of *Bifidobacterium bifidum* during 21 days of refrigerated storage and after simulated gastrointestinal digestion (SGD) were investigated. Two types of yogurt (cow- and camel-milk yogurts) were prepared in the presence of *A. sativum* or *C. verum*. The viable cell counts (VCC) of *B. bifidum* in fresh *A. sativum*- or *C. verum*-cow milk yogurt (1 day) were higher (8.1×10^9 cfu/ml and 6.6×10^9 cfu/ml, respectively; $p < 0.05$) than plain-yogurt (1.9×10^9 cfu/ml). In contrast, *B. bifidum* VCC in fresh plain-camel milk yogurt was 1.99×109 cfu/ml whereas the presence of *A. sativum* or *C. verum* in yogurt increased ($p < 0.05$) VCC to 19.61×109 cfu/ml and 25.55×109 cfu/ml, respectively. The VCC of *B. bifidum* in both herbal-yogurts decreased ($p < 0.05$) during refrigerated storage for both types of yogurt. The VCC of *B. bifidum* was $\sim 1.3 \times 10^9$ cfu/ml in all fresh cow milk yogurts after 1 h gastric digestion. Intestinal digestion (1 h) increased VCC of *B. bifidum* in all fresh yogurts but not in 7 day old yogurts (plain- and *A. sativum*-yogurts). However, prolonged digestion to another 1 h in intestine reduced ($p < 0.05$) VCC of *B. bifidum* in all fresh and storage yogurts. In contrast, all fresh camel milk yogurts showed VCC of *B. bifidum* $\leqslant 1 \times 10^9$ cfu/ml after SGD. Seven day old *A. sativum* – camel milk yogurt showed the lowest survival of *B. bifidum* after gastric digestion compared to plain- and *C. verum*-yogurt. The VCC reduced ($p < 0.05$) in all camel milk-yogurts after 2 h intestinal digestion.

1. Introduction

Nowadays, there has been a worldwide increasing interest about the survival of probiotic bacteria in yogurt. Probiotics are live microorganisms that provide health benefits on the host when administered in sufficient amounts (Wang et al., 2012). Yogurts containing probiotics are claimed to provide

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Peer review under responsibility of University of Bahrain.

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http://dx.doi.org/10.1016/j.jaubas.2014.02.006

several health benefits such as improve lactose utilisation (De Vrese et al., 2001), prevent cancer (Rafter, 2003), maintain intestinal microflora balance (Mainville et al., 2005) and reduce serum cholesterol level (Baroutkoub et al., 2010). Moreover, yogurt containing *Bifidobacterium bifidum* Bb-12 improved immunoglobulin A (IgA) production in the intestine that enhances local immunity against gastrointestinal infection (Kabeerdoss et al., 2011). It also has inhibitory effects on commonly known food borne pathogens (Goderska and Czarnecki, 2007) and ability to control intestinal infections by producing inhibitory/antimicrobial substances such as organic acids, hydrogen peroxide, deconjugated bile acids, antibiotics and bacteriocins (Schiffrin and Blum, 2001).

Viable numbers of probiotics in the final product suggested being at least 10^6–10^7 cfu/g to be accepted as the therapeutic minimum (Madureira et al., 2011). Several studies have investigated the survival ability of probiotic cultures during refrigerated storage (Donkor et al., 2007; Ramchandran and Shah, 2010).

The ability of probiotic bacteria to survive through the gastrointestinal tract varies according to species and even strain-dependent (Wattiaux and Howard, 2000). In addition, functional properties of this probiotic can be affected by the food matrix used in delivery (Lahtinen et al., 2007; Ranadheera et al., 2012) because the buffering capacity of food would help to enhance the viability of probiotics during gastric transit (Kailasapathy and Chin, 2000Kailasapathy and Chin, 2000; Mainville et al., 2005). Ranadheera et al. (2012) reported that the addition of certain ingredients such as cocoa powder and stabilizers guar gum and dextrose in the ice cream enhanced the viability of probiotics by providing some protection. Other study showed that the presence of *Allium sativum* or *Cinnamomum verum* in yogurt enhanced the growth of lactic acids' bacteria (Shori and Baba, 2012). The objective of this work is to evaluate the viability of *B. bifidum* in *C. verum*- or *A. sativum*-yogurt during 21 days of refrigerated storage and the survival of these bacteria after simulated gastrointestinal digestion.

2. Materials and methods

2.1. Plant water extraction

Commercially available dried *A. sativum* or *C. verum* powder was mixed with sterile dH_2O in the ratio of 1:10 in a 250 ml bottle. The final concentration of both herbal extracts was 0.1 g/ml. The mixture was left for 12 h (Shori and Baba, 2011a) in a water bath at 70 °C (Julabo, Model Sw-21c) followed by centrifugation (1000 rpm, 15 min at 4 °C). The supernatant was removed and used as herbal water extract in the making of herbal-yogurt.

2.2. Preparation of starter culture and bio-yogurt

Commercially available direct vat set (DVS) starter culture powder used in yogurt preparation consisting of a mixture of *Lactobacillus acidophilus* LA-5, *Bifidobacterium* Bb-12, *Lactobacillus casei* LC-01, *Streptococcus thermophilus* Th-4 and *Lactobacillus delbrueckii ssp. bulgaricus* (Chris-Hansen, Denmark) was in the ratio of 4:4:1:1:1. The preparation of starter culture from cow or camel milk was carried out using the method described by Shori and Baba (2011b). Two groups of bio-yogurt made from cow and camel milk and three types of yogurt (plain-, *A. sativum*- and *C. verum*-yogurts) were prepared in each group as described by Shori and Baba (2011b).

2.3. In vitro gastrointestinal model

2.3.1. Preparation of gastric and duodenum juices

The gastric and duodenum solutions were freshly prepared according to the protocols described by Huang and Adams (2004). To simulate the *in vivo* saliva, 100 ml of a sterile electrolyte solution (6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l $CaCl_2$, 1.2 g/l $NaHCO_3$) was added to lysozyme (10 mg) to obtain a final concentration of 100 ppm. To simulate the stomach environment (gastric juice), the electrolyte solution was added to 0.3% pepsin and the pH was adjusted to 3 by adding 5 M HCl. To simulate the intestinal digestion (duodenum juice), the electrolyte solution (6.4 g/l $NaHCO_3$, 0.239 g/l KCl, 1.28 g/l NaCl) containing 0.3% bile salts and 0.1% pancreatin (v/w concentrations) was adjusted to pH 7.2 by using 5 M NaOH.

2.3.2. Simulation of gastrointestinal digestion (SGD)

Yogurt samples were mixed with the artificial saliva solution in the ratio of 1:1 followed by incubation at 37 °C for 5 min. Samples were then mixed with artificial gastric fluid solution in the ratio of 3:5 prior to a second incubation at 37 °C for 1 h. After 1 h, 30 ml of samples from the "stomach digestion" was taken out for analysis. The remaining solutions from "stomach digestion" were then mixed with artificial duodenal secretion in the ratio of 1:4 followed by a third incubation at 37 °C for 2 h. Samples (30 ml) were taken out for analysis after every hour interval of "intestinal digestion". All samples were manually agitated and stirred intermittently during the incubation time in order to ensure adequate enzymatic digestion to mimic gastrointestinal movement.

2.4. Viable cell counts (VCC) of B. bifidum

Cultures of *B. bifidum* were enumerated using MRS-LP agar. The formulation of MRS-LP was prepared according to Vinderola et al. (2000) where 0.2% (w/v) of lithium chloride (solid–powder) and 0.3% (w/v) of sodium propionate (solid–powder) were added to the MRS media (62 g/930 L dH_2O, 45 °C). Yogurt samples (1 ml) were mixed with 9 ml of 0.15% sterile buffered peptone water (20 g/L dH_2O). The mixture was thoroughly stirred and serial decimal dilutions were prepared by using buffered peptone water. One millilitre of diluted yogurt with buffered peptone water was mixed with 15 ml of autoclaved melted MRS–LP media using the pour plate method. The probiotic cultures were anaerobically incubated (GasPak System-OXOID) at 37 °C for 72 h. The viable *B. bifidum* counts were calculated (Sivakumar and Kalaiarasu, 2010) as follow:

$$\mathrm{CFU}*/\mathrm{ml} = \frac{\text{Number of colonies formed} \times \text{dilution factor of sample}}{\text{1 ml of sample}}$$

*CFU: colony forming unit.

2.5. Statistical analysis

The experiment was carried out using three different batches of yogurt ($n = 3$). Data were expressed as mean ± SE. The statistical analysis was performed using one way analysis of variance (ANOVA, SPSS 14.0), followed by Duncan's post hoc test for mean comparison. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. VCC of *B. bifidum* in yogurt during storage (4 °C)

Both fresh *A. sativum*- and *C. verum*-yogurts made from cow milk showed higher VCC of *B. bifidum* (8.10×10^9 cfu/ml and 6.59×10^9 cfu/ml, respectively) than plain-yogurt (1.89×10^9 cfu/ml; Fig. 1). Refrigerated storage of plain-yogurt increased *B. bifidum* VCC to the highest counts (2.68×10^9 cfu/ml) on day 7 followed by significant decrease to 0.26×10^9 cfu/ml by day 21 of storage. The VCC of *B. bifidum* in *A. sativum*- and *C. verum*-yogurts decreased during refrigerated storage but they were still higher than plain-yogurt even on day 21 of storage (0.48×10^9 cfu/ml and 1.01×10^9 cfu/ml, respectively; Fig. 1).

The VCC of *B. bifidum* in fresh plain-yogurt made from camel milk was 1.99×10^9 cfu/ml (Fig. 2). The presence of *A. sativum* or *C. verum* in yogurt increased the VCC to 19.61×10^9 cfu/ml and 25.55×10^9 cfu/ml, respectively. Refrigerated storage of yogurt up to 7 days increased the VCC of *B. bifidum* in plain-yogurt (6.05×10^9 cfu/ml) followed by reduction to 0.75×10^9 cfu/ml on day 21 of storage (Fig. 2). The VCC of *B. bifidum* in both *A. sativum*- and *C. verum*-yogurts decreased to 1.41×10^9 cfu/ml and 1.11×10^9 cfu/ml for *A. sativum*- and *C. verum*-yogurts, respectively on day 21 of storage.

3.2. VCC of B. bifidum after SGD

The VCC of *B. bifidum* in cow milk both in the presence and absence of *A. sativum* or *C. verum* water extract were less than

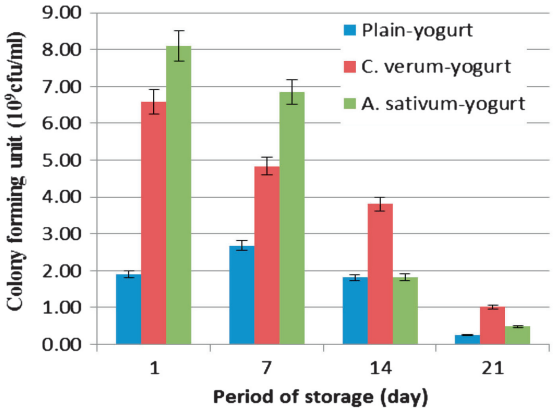

Figure 1 Changes in viable cell counts of *B. bifidum* ($\times 10^9$ cfu/ml) in cow milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4 °C). Error bars represent a pooled standard error of the mean ($n = 3$). The level of significance was preset at $p = 0.05$ compared to plain-yogurt at the same storage period.

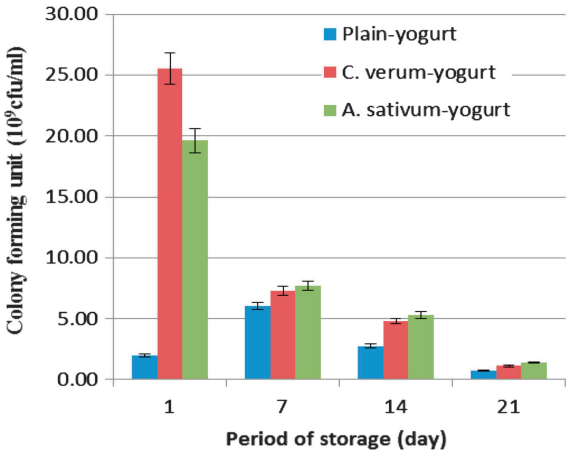

Figure 2 Changes in viable cell counts of *B. bifidum* ($\times 10^9$ cfu/ml) in camel milk-yogurt in the presence and absence of *A. sativum* or *C. verum* water extract during 21 days of refrigerated storage (4 °C). Error bars represent a pooled standard error of the mean ($n = 3$). The level of significance was preset at $p = 0.05$ compared to plain-yogurt at the same storage period.

1×10^9 cfu/ml during 3 h SGD (Fig. 3). Similar VCC was shown in all fresh yogurts ($\sim 1.3 \times 10^9$ cfu/ml, 1 day) after 1 h gastric digestion. Intestinal digestion (1 h) increased the VCC of *B. bifidum* to the highest value in plain-yogurt (33.4×10^9 cfu/ml) followed by *C. verum*- (30.4×10^9 cfu/ml) and *A. sativum*- (6.6×10^9 cfu/ml) yogurts. Prolonged digestion for another 1 h reduced the VCC of *B. bifidum* to 2.4×10^9 cfu/ml for both plain- and *C. verum*-yogurts and to 0.8×10^9 cfu/ml for *A. sativum*-yogurts (Fig. 3). The VCC of *B. bifidum* in refrigerated storage (7 days) plain-yogurt was 49.9×10^9 cfu/ml after 1 h gastric digestion. The VCC was not affected by either the presence of *A. sativum* or *C. verum* in yogurt (0.6×10^9 cfu/ml and 12.4×10^9 cfu/ml, respectively). Intestinal digestion (1 h) decreased VCC of *B. bifidum* in plain-

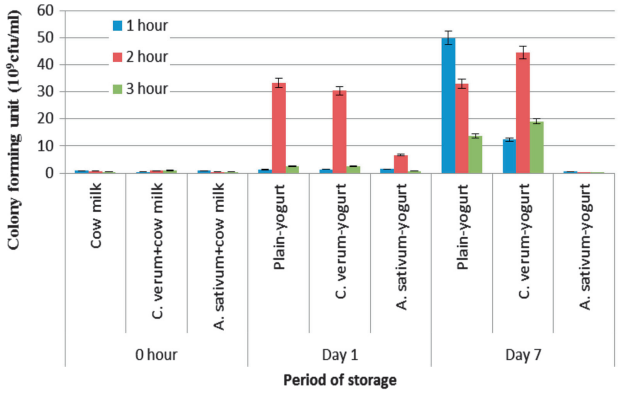

Figure 3 VCC of *B. bifidum* ($\times 10^9$ cfu/ml) in cow milk at initial time of fermentation (0 h) and after fermentation (1 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 h (1st hour represents gastric digestion, 2nd and 3rd hours represent 1 and 2 h in intestinal digestion, respectively). Error bars present a pooled standard error of the mean ($n = 3$). The level of significance was preset at $p = 0.05$ compared to plain-yogurt at the same storage period.

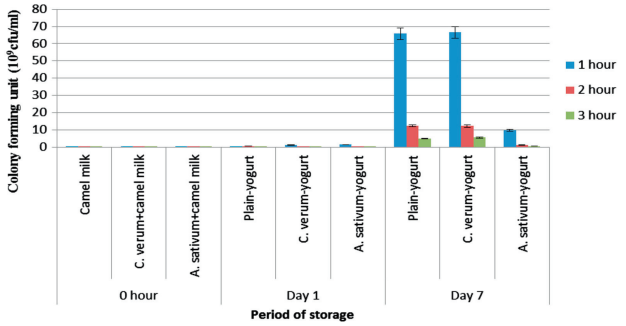

Figure 4 VCC of *B. bifidum* ($\times 10^9$ cfu/ml) in camel milk at initial time of fermentation (0 h) and after fermentation (1 day) and during refrigerated storage (7 days) under simulated gastrointestinal condition for 3 h (1st hour represents gastric digestion, 2nd and 3rd hours represent 1 and 2 h in intestinal digestion, respectively). Error bars present a pooled standard error of the mean ($n = 3$). The level of significance was preset at $p = 0.05$ compared to plain-yogurt at the same storage period.

and *A. sativum*-yogurts (32.9×10^9 cfu/ml and 0.2×10^9 cfu/ml, respectively) but not in *C. verum*-yogurt (44.5×10^9 cfu/ml; $p < 0.05$). A further one hour digestion in intestinal section decreased VCC to the lowest value in *A. sativum*-yogurt (0.04×10^9 cfu/ml) followed by plain- (13.69×10^9 cfu/ml) and *C. verum*- (19.04×10^9 cfu/ml) yogurts (Fig. 3).

The VCC of *B. bifidum* in all camel milk treatments at initial time of fermentation (0 h) and after fermentation (1 day) were $\leqslant 1 \times 10^9$ cfu/ml (Fig. 4). Refrigerated storage (7 days) of plain- and *C. verum*-yogurts showed similar VCC of *B. bifidum* after 1 h gastric digestion (66.0×10^9 cfu/ml) whereas *A. sativum*-yogurt had only 9.7×10^9 cfu/ml VCC of *B. bifidum*. Intestinal digestion for 2 h reduced VCC to 4.85×10^9 cfu/ml, 0.50×10^9 cfu/ml and 5.55×10^9 cfu/ml for plain-, *A. sativum*- and *C. verum*-yogurts, respectively.

4. Discussion

The survival of probiotic microflora in yogurt is governed by physicochemical factors such as yogurt acidity, dissolved oxygen, species interaction and storage conditions (Rybka and Kailasapathy, 1995). The present study showed that the addition of *A. sativum* or *C. verum* water extract in both cow- and camel-milk yogurts increases ($p < 0.05$) the VCC of *B. bifidum* compared to the respective plain-yogurts during 21 days of storage. This could be related to the essential growth factors present in *A. sativum* or *C. verum* such as vitamins, minerals, amino acids and polyphenolics (Abdullah et al., 1988; Al-Numair et al., 2007). The presence of higher free amino groups in herbal-yogurt made from camel- than cow-milk (Shori and Baba, 2011a,b) may explain the higher VCC of *B. bifidum* in the former than in the latter. Furthermore, the higher buffering capacity in camel milk than cow milk (Ramet, 2001) may help to stabilise the pH in yogurt (Shori and Baba, 2011a,b) thus allowing more *B. bifidum* growth prior to the development of inhibitory acidic environment. The present study showed significant reduction in *B. bifidum* VCC of *A. sativum*- and *C. verum*-yogurts made from either cow or camel milk during refrigerated storage. This observation was in agreement with Vinderola et al. (2000) whereby the reduction of VCC of *B. bifidum* was shown dependent on the milk type. Thus, the faster reduction ($p < 0.05$) of these bacteria in herbal- camel milk- than cow milk-yogurts after 7 days of storage would suggest that specific milk composition in the former may be responsible for the reduction. In particular, the anti-microbial compounds are present in higher concentrations in camel milk than other mammalian milk (El-Agamy et al., 1992).*In vitro* limited tolerance of probiotic strains to gastric acid has been demonstrated elsewhere (Mishra and Prasad, 2005; Madureira et al., 2011). In the present study, the effect of *A. sativum* or *C. verum* on the survival of *B. bifidum* was dependent on milk type and type of herbal extract used. During intestinal digestion, *B. bifidum* in *C. verum*-cow milk yogurt showed the ability to grow in such condition and/or recover from sub lethally-injured cells. This may suggest possible interaction between phenolic compounds and cow milk proteins that could provide considerable protection for *B. bifidum* against exposure to intestinal juice. This possibility shown also from Ranadheera et al. (2012) suggested that the addition of ingredients i.e., cocoa powder and stabilizers (guar gum and dextrose) in goat milk ice cream has provided protection towards probiotic survival during simulated gastrointestinal digestion. Camel milk was reported to have a higher antimicrobial lactoperoxidase system (Anonymous, 2003) which may be caused by further inhibitory effects on *B. bifidum* growth. Prolonged exposure to intestinal digestion (2 h) showed substantial reduction of *B. bifidum* VCC in both types of yogurt. This is in agreement with other studies of (Saxelin et al., 2010; Vinderola et al., 2011; Ranadheera et al., 2012) who related that to the antimicrobial nature of bile salt that arises mainly from its detergent property.

5. Conclusion

The growth of *B. bifidum* improved in the presence of *A. sativum* or *C. verum* water extract in both cow- and camel-milk yogurts during fermentation and they continued to survive even during refrigerated storage. *A. sativum*- and *C. verum*-yogurts made from either cow or camel milk provided higher viable *B. bifidum* over 2 weeks of storage. Therefore, these yogurts may be considered as probiotic yogurt with promising therapeutic properties upon daily consumption.

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