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Effects of Fermentation and Storage on Bioactive Activities in Milks and Yoghurts

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Abstract

Fermentation of milk enhances its nutritional value through improved bioavailability of nutrients and production of bioactive substances which have biological functions. The goals of this research were to study the effect of fermentation and storage on antioxidant and antimicrobial activities in buffalo, goat and cow milks and yoghurts. Samples of buffalo, goat, cow milks and their yoghurts during their fermentation and storage were determined for proximate analysis and bioactive activities including antioxidant activities of DPPH, ABTS and reducing power assays, and antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *Escherichia coli*. Results showed that buffalo, cow and goat yoghurts had antioxidant activities in all assays and their activities significantly increased during their fermentation. Pasteurization did not affect the antioxidant activities. The activities of all yoghurts remained unchanged after a storage time of 21 days at 4°C. For the antimicrobial activities, only yoghurts from buffalo, cow and goat milks had the activities, while all milks did not show any activities. However, buffalo yoghurt could inhibit only Gram positive strains (*S. aureus* and *B. cereus*), while goat and cow yoghurt inhibited all tested strains. A chemical responsible for antimicrobial activities in yoghurts was lactic acid formed by lactic acid bacteria. However, bioactive peptides produced by protein digestion during milk fermentation by lactic acid bacteria could not be ruled out for antioxidant and antimicrobial activities present. The antimicrobial activities of all yoghurts remained constant during their storage. It is concluded that all yoghurts would retain milk nutrition and bioactive functions during their storage in a refrigerator and may be served as a functional food with benefits from those activities for consumers.

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: Antioxidant activity; Antimicrobial activity; Milk; Yoghurt; Fermentation; Storage

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Nomenclature

LAB	Lactic acid bacteria
DPPH	2,2-Diphenyl-1-picrylhydrazil
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

1. Introduction

Bioactive peptides are short chains of amino acids that are produced during gastrointestinal digestion or food processing. The peptides have showed a wide range of biological activities such as anti-hypertension, anti-oxidant, anti-microbial, anti-angiotensin converting enzyme (anti-ACE) and anti-carcinogenic activities[1]. More recently, a great interest has been focused on peptides that can lower the blood pressure in hypertensive patients, since hypertension is a disease that is increasing at high rates, especially in developed countries[2]. Sources of bioactive peptides are found to be milk and milk products. Fermentation of milk enhances its nutritional value through improved bioavailability of nutrients and production of substances which have a biological function[3,4]. Fermented dairy products, in addition to providing both energy and nutrients, are the excellent sources of bioactive peptides. They provide numerous peptides with bioactive properties and form lactic acid and flavor compounds during fermentation and storage[5]. A large number of oligopeptides are generated by casein degradation by extracellular proteases from microbial cells. Consequently, amino acids and small peptides are generated by further breakdown by intracellular peptidases[6,7]. The proteolytic activities result in the release of bioactive peptides from specific amino acid sequences within the parent proteins and they can provide physiological benefits[8,9,10]. The size of bioactive peptides may vary from 2 to 20 amino acid residues with their activities depending on their amino acid sequence and composition[3].

Lactic acid bacteria (LAB) are commonly used to ferment milk into yoghurt and other fermented milk products. The types of LAB usually used in the dairy industries are thermophilic and mesophilic strains of *Streptococcus*, *Lactococcus*, and *Lactobacillus* species[6]. During fermentation of milk, the cell wall associated proteinase of LAB hydrolyses caseins into large peptides which are taken up into their cells, then broken down by intracellular peptidases resulting in a range of bioactive peptides showing, for example, hypertensive or angiotensin-I-converting enzyme (ACE)-inhibitory activity[6].

Cow milk and its products have been studied for bioactive peptide activities. Based on FAO Statistics (2009), cow milk is the most important milk among the different types of milk produced and its production has been growing at the rate of about 6.9% annually. The percentage proportions of milk production are as follow: cow milk (73.4%), goat milk (12.7%), buffalo milk (8.9%) and sheep milk (5.0%). Among the countries, spectacular annual growth rates for milk production have been recorded in Thailand (24.1%) and Indonesia (13.4%). Consumers drink goat or buffalo milk less than cow milk because of a problem in taste. However, in recent years, the trend of goat and buffalo milk consumption is increasing due to consumers' awareness of their health and nutrition. Some people are also sufferering from cow milk allergy and digestive problems. In addition, goat and buffalo milk products are available commercially worldwide. Unfortunately, no studies have been done to investigate bioactive activities in such products. The goals of this work were to determine bioactive activities of cow, buffalo and goat milk and their products and to study the effect of fermentation and storage on bioactive activities of the milk products.

2. Methods

2.1 Samples and Sample Preparation

Raw and processed milk including their yoghurts made from cow, goat and buffalo were collected from local farms. In addition, their yoghurt samples during fermentation and during storage for 21 days at 4°C were collected. Samples were prepared by centrifugation at 6,000 g, 4°C for 15 minutes. The supernatant (water soluble peptide extract) was kept and determined for bioactive activities.

2.2 Proximate Analysis

All milk samples were analyzed for proximate composition: ash, moisture, protein and lipid, including pH and lactose content[11].

2.3 Bioactive Activity Analysis

2.3.1 Antioxidant Activity

a. DPPH assay (2,2-Diphenyl-1-picrylhydrazil)[12]

To a 500 µL sample in the micro plate was added 500 µL of 100 µM DPPH, and incubated at 37°C for 30 minutes in a dark room. After then, the absorbance at 517 nm was measured using a micro plate reader. Negative control was distilled water and positive control was 10 mM ascorbic acid.

% Inhibition (%I) = 100 x [(Ac–Acb) – (As–Asb)] / (Ac–Acb)

Where Ac is absorbance of control; Acb is absorbance of control blank; As is absorbance of sample; Asb is absorbance of sample blank.

b. Reducing power assay[13].

To 60 µL of sample was added 400 µL of phosphate buffer (pH 6.6, 0.2 M). Then was added 400 µL of $K_3Fe(CN)_6$ (1% w/v). After incubation at 50°C for 20 minutes, 400 µL of TCA (10% w/v) was added. The mixture was centrifuged at 3000 g for 10 minutes. 100 µL of 0.01% $FeCl_3$ was added to 500 µL of the supernatant immediately (in the dark). Absorbance was measured at 700 nm.

% Inhibition (%I) = 100 x [(As–Asb) – (Ac–Acb)] / (Ac–Acb)

c. ABTS assay (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)[14]

ABTS 7 mM was mixed with $K_2S_2O_8$ (ABTS:$K_2S_2O_8$ = 1:20) 140 mM and the mixture was incubated in the dark room at room temperature for 16 hs. Then it was diluted with 5 mM PBS (pH 7.4 to obtain the absorbance of 0.7 ± 0.2 at 734 nm); 1000 µL ABTS was added to 10 µL of sample. Finally, the blue colour of ABTS developed at 30°C in 1-6 minutes after mixing was measured at 734 nm.

% Inhibition (%I) = 100 x [(Ac–Acb) – (As–Asb)] / (Ac–Acb)

2.3.2 Antimicrobial Activity

All milk samples were analyzed for antimicrobial activity using an agar well diffusion method[15]. Muller-Hinton agar was poured into a Petri dish and allowed to set for 30 minutes at room temperature. It was swab inoculated with 0.1% of an overnight culture of the pathogen strain in NB and TSB *(*Gram positive bacteria: *B. cereus* and *S. aureus*; Gram negative bacteria: *S. typhimurium* and *E. coli*). Then holes (diameter of 7 mm) were punched in the agar and filled with 80 µl of samples. Plates were incubated overnight at 37°C for growth of the indicator strain pathogen. Samples were measured as the diameter of the inhibition zone (in cm). Positive controls tested were 1% lactic acid and 2% tetracycline. Negative control was distilled water.

2.4 Statistical Analysis

The experiment was done in triplicate. Results were reported as mean values ± standard deviations. One way ANOVA and Duncan tests were employed to determine the significant differences between treatments ($p< 0.05$).

3. Results and discussion

3.1 Proximate analysis

3.1.1 Effect of pasteurization

The effect of pasteurization on proximate and chemical composition of milks is shown in Table 1.

Table 1 Chemical compositions of raw and pasteurized cow, goat and buffalo milks

Milks*		Moisture (%)	Lactose (%)	Protein (%)	Fat (%)	Ash (%)	pH
Cow	R	87.7 ± 0.6^{aB}	5.2 ± 0.6^{aAB}	3.7 ± 0.6^{aA}	3.4 ± 0.6^{aB}	0.67 ± 0.01^{aA}	6.56 ± 0.02^{aA}
	P	86.9 ± 0.6^{a}	5.2 ± 0.6^{a}	2.4 ± 0.6^{a}	2.9 ± 0.2^{a}	0.67 ± 0.01^{a}	6.56 ± 0.02^{a}
Goat	R	89.1 ± 0.6^{aA}	4.7 ± 0.6^{aB}	4.0 ± 0.6^{aA}	3.3 ± 0.6^{aB}	0.67 ± 0.01^{aA}	6.45 ± 0.06^{aB}
	P	88.0 ± 0.6^{a}	4.6 ± 0.6^{a}	2.8 ± 0.6^{a}	3.3 ± 0.6^{a}	0.68 ± 0.00^{a}	6.42 ± 0.06^{a}
Buffalo	R	83.15 ± 0.04^{aC}	6.1 ± 0.6^{aA}	4.0 ± 0.6^{aA}	7.3 ± 0.6^{aA}	0.67 ± 0.01^{aA}	6.52 ± 0.03^{aAB}
	P	82.7 ± 0.6^{a}	6.0 ± 0.6^{a}	3.0 ± 0.6^{a}	6.4 ± 0.6^{a}	0.68 ± 0.01^{a}	6.50 ± 0.03^{a}

Values in the same analysis with different letters (a,b,c,....) were significantly different among raw and pasteurized milks with 3 replicates. Values in the same analysis with different letters (A,B,C,....) were significantly different in different types of raw milks with 3 replicates by Duncan's multiple range test ($p<0.05$). *R = Raw milk; P = Pasteurized milk

Table 1 shows pasteurization has no effect ($p<0.05$) significantly different for proximate and chemical analysis. P asteurization is heating process that not intended to kill all micro-organisms in the food. Instead, it aims to reduce t he number of viable pathogens so they are unlikely to cause disease (assuming the pasteurized product is stored as i ndicated and is consumed before its expiration date. For home pasteurization, which is to heat milk at 63°C (145°) f or 30 minutes based on FDA standard.

3.1.2 Effect of fermentation

Fermentation has a strong effect, resulting in a decrease of fat and pH value in yoghurts. After fermentation when milk became yoghurt, the fat content of each yoghurt was decreased (Tables 1 and 2). The results are in line with other observations[16,17].

Table 2 Chemical compositions of yoghurts produced from cow, goat and buffalo milks

Yoghurt*		Moisture(%)	Lactose (%)	Protein (%)	Fat (%)	Ash (%)	pH
Cow	Y1	83.1 ± 0.6^{aA}	4.4 ± 0.6^{aAB}	4.3 ± 0.6^{aA}	2.31 ± 0.02^{aB}	0.67 ± 0.01^{aA}	4.39 ± 0.01^{aA}
	S1	82.2 ± 0.6^{a}	4.3 ± 0.6^{a}	4.2 ± 0.6^{a}	2.1 ± 0.6^{a}	0.68 ± 0.01^{a}	4.39 ± 0.01^{a}
	Y2	83.1 ± 0.6^{aA}	4.4 ± 0.6^{aAB}	4.3 ± 0.6^{aA}	2.72 ± 0.01^{aB}	0.67 ± 0.01^{aA}	4.39 ± 0.01^{aA}
	S2	82.2 ± 0.6^{a}	4.2 ± 0.6^{a}	4.2 ± 0.6^{a}	2.0 ± 0.6^{a}	0.67 ± 0.01^{a}	4.39 ± 0.01^{a}
Goat	Y2	82.3 ± 0.6^{aB}	3.7 ± 0.6^{aB}	4.7 ± 0.6^{aA}	2.3 ± 0.6^{aB}	0.67 ± 0.01^{aA}	4.39 ± 0.02^{aA}
	S2	81.4 ± 0.6^{a}	3.6 ± 0.6^{a}	4.6 ± 0.6^{a}	1.8 ± 0.6^{a}	0.68 ± 0.01^{a}	4.39 ± 0.01^{a}
Buffalo	Y2	82.04 ± 0.02^{aB}	5.2 ± 0.6^{aA}	4.7 ± 0.6^{aA}	5.51 ± 0.01^{aA}	0.67 ± 0.01^{aA}	4.41 ± 0.03^{aA}
	S2	82.02 ± 0.01^{a}	5.1 ± 0.6^{a}	4.6 ± 0.6^{a}	5.2 ± 0.6^{a}	0.68 ± 0.01^{a}	4.39 ± 0.01^{a}

Values in the same analysis with different letters (a,b,c,....) were significantly different among fresh and stored yoghurts with 3 replicates. Values in the same analysis with different letters (A,B,C,....) were significantly different in different types of yoghurts with 3 replicates by Duncan's multiple range test ($p<0.05$). * Y1 = Yoghurt with monoculture (*B. bifidum*); Y2 = Yoghurt with 2 cultures (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*); S1 = Stored yoghurt with monoculture (*B. bifidum*); S2 = Stored yoghurt with 2 cultures (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*).

Table 2 shows that fermentation affected pH values. Their pH decreased during milk fermentation. The acidity increased (pH decreased) as a function of fermentation time as lactic acid is produced by lactic acid bacteria in yoghurts. Acid production depends upon the growth of the organisms and their abilities to ferment some available carbohydrates in milks. Yoghurt in two cultures, *S. thermophilus* and *L. bulgaricus* readily utilize lactose, which is the major fermentable sugar in milk, and they produce substantial amounts of acid in milk. After 4 h of fermentation, the pH obtained was around 4.40. The decreasing of protein content in yoghurt depends on the proteolytic activity of lactic acid bacteria, which hydrolyses protein (caseins) into peptides and amino acids[18].

3.1.3 Effect of storage

Storage at refrigerator temperature had no significant effect ($p<0.05$) on the proximate and chemical analyses (Table 2). Milk composition is dependent on the type of breeds, storage, feeding systems, milking frequency, milking method, seasonal changes and lactation period. In general, buffalo milk had higher total protein, lactose and ash contents than goat milk. However, the major differences between buffalo and goat milks are related to the different proportions of the different kinds of caseins (αs1-casein, αs2-casein, κ-casein, etc.) and the different structure and size of fat globules and protein micelles[19]. In addition, goat milk has less αs1-casein, which is present in variable proportions depending on the individual goat breed. For fat content, goat milk contains a higher proportion of smaller fat globules than buffalo milk[19].

3.2 Antioxidant activity

Fig. 1 shows antioxidant activities determined by three assays in cow, buffalo and goat milk and their products. Antioxidant activity was present in raw milk, because they have antioxidant compounds like aromatic amino acid residues (tyrosine, phenylalanine, tryptophan) and free sulfhydryl groups. In fact, the antioxidant activity of milk could also be due to the contribution of natural antioxidants, such as α-tocopherol, carotenoids, conjugated linoleic acid, casein and lactoferrin ocurring in whey[20].

3.2.1 Effect of pasteurization

Pasteurization of milks had no significant effect on antioxidant activities measured by all assays (DPPH, reducing power, ABTS activity) (Fig. 1), though there was a tendency for antioxidant activity to be a little higher in a pasteurized milks. Therefore, heat treatment of milk may be associated with a small increase in its antioxidant activity. Heat treatment would increase antioxidant activity because of protein unfolding and exposure of thiol groups that can act as hydrogen donor. Under severe heating, pro-oxidant molecules may be consumed in the Maillard reaction pathway, generating melanoidin with strong antioxidant activity[21].

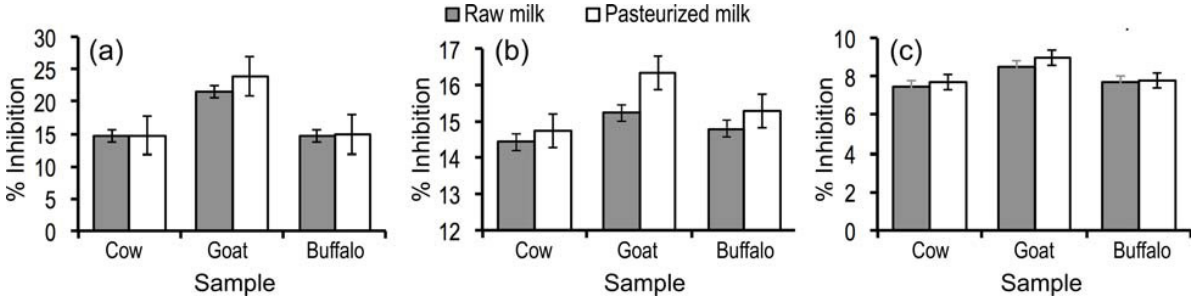

Fig. 1. Changes in antioxidant activity of milk in (a) DPPH assay, (b) reducing power activity, (c) ABTS during pasteurization.

Methods of antioxidant activity determination also influenced the measurements as shown in Fig. 1. The DPPH method was the most sensitive method giving the highest antioxidant activities among the three methods. That would indicate milk samples had more hydrogen donating ability of antioxidant to radicals or the sample contained peptides acting as electron donors and could react with free radicals to form more stable products. The ABTS assay gave the lowest antioxidant activities among the three methods probably because milk had less antioxidant compounds to reduce $ABTS^{+}$. In general, different results of antioxidant assays were observed probably because relative differences in the ability of antioxidant compounds in the milk extract to quench aqueous peroxyl radicals and to reduce $ABTS^{+}$ (2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid)), the DPPH free radical and ferric iron in the in vitro systems.

3.2.2 Effect of fermentation

Fermentation of all types of milks to become yoghurts increased antioxidant activities determined by all assays as shown in Fig. 2.

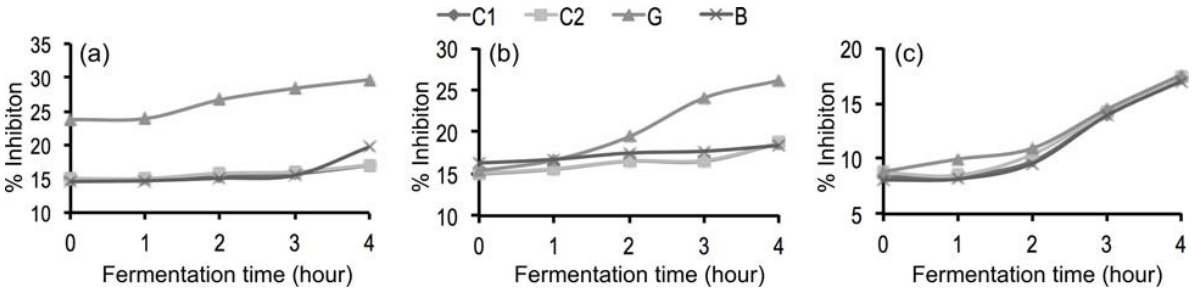

Fig. 2. Antioxidant activity of yoghurt in (a) DPPH assay, (b) reducing power activity, (c) ABTS assays during fermentation *C1 = Cow yoghurt with monoculture; C2 = cow yoghurt with 2 cultures; G = Goat yoghurt; B = buffalo yoghurt

Yoghurts of all milks at 4-hour fermentation had the strongest activity. Increased antioxidant activities in yoghurts may result from bioactive (antioxidative) peptides released from protein digestion by bacterial fermentation. A number of bioactive peptides have been identified in milk proteins, such as casein and whey proteins, where they are presented in an encrypted form, stored as propeptides or mature C-terminal peptides only released upon proteolysis. Peptides generated in milk digestion may act as electron donors reacting with free radicals to form more stable products[22]. In addition, lactic acid bacteria produce metabolic compounds acting as scavengers or degraded products of milk proteins acting as hydroxyl radicals[23]. The fermented milk also contained reductones formed during fermentation, which could react with free radicals to stabilize and terminate radical chain reactions[24].

The bacterial strains used did not have any effects on antioxidant activities since the same proteolytic system may be found in both monoculture and two cultures used to produce yoghurts. Yoghurt had more oxidative stability than milk because microorganism action could yield antioxidant peptides acting as electron donors. They reacted with free radicals and reduced radical scavenging activity[25].

Among the three yoghurts, goat yoghurt showed the strongest antioxidant activities by all assays. That could mean goat yoghurt had more hydrogen donating ability of antioxidant to radicals or contained peptides acting as electron donors reacting with free radicals to form more stable products (from DPPH assay). Goat yoghurt would also contain compounds with high reducing powers.

Goat yoghurt may have more antioxidant activity than others because goat milk has higher β-casein (51%) than cow milk (34%) and also had higher αs-2 casein (21%) than others [26]. Studies have shown that caseins, especially β- and αs-2 casein, and also whey protein have good antioxidant properties, presumably based on their ability to bind transition metals and scavenge free radicals[26].

3.2.3 Effect of storage

Storage had no effects on antioxidant activities in yoghurts determined by all assays (p<0.05) as shown in Fig. 3.

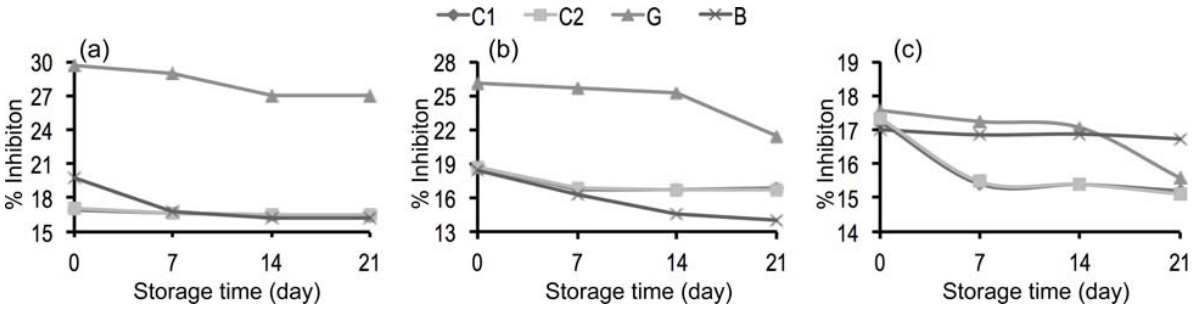

Fig. 3. Antioxidant activity of yoghurt in (a) DPPH assay (b) reducing power activity (c) ABTS during storage *C1 = Cow yoghurt with monoculture;C2 = cow yoghurt with 2 cultures; G=Goat yoghurt; B=buffalo yoghurt

Fig. 3 showed that storage had no effects on antioxidant activities in yoghurts determined by all assays (p<0.05). During the storage, antioxidant activity tended to be stable or a little decreased. Therefore, consumers could get full benefits from these activities from yoghurt after 3-weeks storage (21st days) in a refrigerator or before an the expired date of the product.

3.3 Antimicrobial Activity

3.3.1 Effect of pasteurization

Raw and pasteurized milks of all animals did not show any antimicrobial activities against all tested bacteria as seen in Table 3.

Table 3 Antimicrobial activities of raw and pasteurized cow, goat, and buffalo milks

Milk*		Inhibition zone** (cm)			
		S. aureus	*B. cereus*	*S. typhimurium*	*E. coli*
Cow	R	-	-	-	-
	P	-	-	-	-
Goat	R	-	-	-	-
	P	-	-	-	-
Buffalo	R	-	-	-	-
	P	-	-	-	-
Positive control		++++ (2.0)	++++ (2.0)	++++ (2.0)	++++(2.0)
1% Lactic acid		++(1.0)	++(1.0)	++ (1.0)	++ (1.0)

Positive control = 2% Tetracycline, ** – no inhibition; + weak inhibition; ++ moderate inhibition; +++ strong inhibition; ++++ very strong inhibition; *R= Raw milk; P= Pasteurized milk

Raw and pasteurized milks of all animals did not show any antimicrobial activities against all tested bacteria as seen in Table 3. It was probably because only a little small amount of antimicrobial compounds were found in raw milk and or antimicrobial peptides were still not activated or not excreted before fermentation.

3.3.2 Effect of fermentation

Antimicrobial activities were found in all yoghurts after 3 h fermentation (Table 4).

Table 4 Antimicrobial activities of cow, goat and buffalo yoghurts

Yoghurt		Inhibition zone (cm)*			
		S. aureus	*B. cereus*	*S. typhimurium*	*E. coli*
Cow	Y1 (0 h)	-	-	-	-
	Y1 (1 h)	-	-	-	-
	Y1 (2 h)	-	-	-	-
	Y1 (3 h)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	Y1 (4 h)	++ (1.0)	++ (1.0)	++ (1.0)	++ (1.0)
	S1 (7 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	S1 (14 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	S1 (21 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	Y2 (0 h)	-	-	-	-
	Y2 (1 h)	-	-	-	-
	Y2 (2 h)	-	-	-	-
	Y2 (3 h)	+ (0.9)	+ (0.8)	+ (0.8)	+ (0.8)
	Y2 (4 h)	++ (1.1)	++ (1.0)	++ (1.0)	++ (1.0)
	S2 (7 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	S2 (14 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	S2 (21 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
Goat	Y2 (0 h)	-	-	-	-
	Y2 (1 h)	-	-	-	-
	Y2 (2 h)	-	-	-	-
	Y2 (3 h)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
	Y2 (4 h)	+++ (1.2)	+++ (1.2)	+++ (1.2)	+++ (1.2)
	S2 (7 d)	+++ (1.2)	+++ (1.2)	+++ (1.2)	+++ (1.2)
	S2 (14 d)	+++ (1.2)	+++ (1.2)	++ (1.0)	++ (1.0)
	S2 (21 d)	+ (0.8)	+ (0.8)	+ (0.8)	+ (0.8)
Buffalo	Y2 (0 h)	-	-	-	-
	Y2 (1 h)	-	-	-	-
	Y2 (2 h)	-	-	-	-
	Y2 (3 h)	+ (0.8)	+ (0.8)	-	-
	Y2 (4 h)	+++ (1.2)	+++ (1.2)	-	-
	S2 (7 d)	+++ (1.2)	+++ (1.2)	-	-
	S2 (14 d)	+++ (1.2)	+ (0.8)	-	-
	S2 (21 d)	+ (0.8)	+ (0.8)	-	-
Positive		++++(2.0)	++++(2.0)	++++ (2.0)	++++(2.0)
Lactic acid 1%		++(1.0)	++(1.0)	++(1.0)	++(1.0)

Positive control = 2% tetracycline; * – no inhibition; + weak inhibition; ++ moderate inhibition; +++ strong inhibition; ++++ very strong inhibition; Y1 = Yoghurt with monoculture (*B. bifidum*) ; Y2 = Yoghurt with 2 cultures (*S. thermophilus* and *L. delbrueckii* subsp.*bulgaricus*); S1 = Stored yoghurt with monoculture (*B. bifidum*); S2 = Stored yoghurt with 2 cultures (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*); Y (0, 1, 2, 3, 4 h) = fermentation at 0, 1, 2, 3 and 4 hours; S (7, 14, 21 d) = storage for 7, 14 and 21 days in a refrigerator.

Antimicrobial activities may be caused by the actions of lactic acid bacteria (LAB) during milk fermentation. Antimicrobial activities of LAB are largely due to their production of organic acids (*e.g.* lactate, acetate, citrate and butyrate), ethanol, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and bacteriocins[27].

Effect of lactic acid on antimicrobial activity of yoghurts appeared as an inhibition zone of diameter around 1 cm in all of Gram positive and negative bacteria (Table 4). This means that LAB can effect antimicrobial activity by their organic acid production. Lactic acid and other organic acids produced by LAB during fermentation resulted in antimicrobial activity found in yoghurts.

Cow yoghurt with starter culture of *B. bifidum* (Y1) and two starter cultures of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Y2) gave the same inhibition zones against four tested strains at 3 and 4 hours fermentation (Table 4). It seems that they could provide the same of acids and/or other compounds exhibiting antimicrobial activity.

3.3.3 Effect of storage

Storage of yoghurts in refrigerator temperature during 21 days did not have any effects on their antimicrobial activities, especially in goat and buffalo yoghurst (Table 4). It is indicated that a low temperature could keep their antimicrobial activities in the products and the product still could have this benefit to consumers before its expired date.

3.3.4 Effect of different kinds of milks

It should be noted that yoghurt from different types of milks affected Gram positive and Gram negative bacteria differently (Table 4). Yoghurt from cow and goat milks inhibited all tested strains, while that from buffalo milk inhibited only Gram positive bacteria (*S. aureus* and *B. cereus*). In addition, yoghurt from goat milk showed the strongest activities against all tested strains compared to the other yoghurts possibly since goat milk has a specific composition resulting in the increased antimicrobial compound production during fermentation. Lysozyme is found in the milk of goats (0.23 mg/L) at a higher level than cows (0.16 mg/L)[28]. Buffalo yoghurt inhibited Gram positive bacteria only (Table 4). It may be because buffalo milk has more α-lactoglobulin. Buffalo milk yoghurt showed stronger antimicrobial activity against Gram-positive bacteria than cow yoghurt. In Table 4, the results showed buffalo yoghurt after storage for 7 days still had a bigger inhibition zone (1.2 cm) than that of cow yoghurt (around 0.8 cm).

4. Conclusions

Cow, buffalo and goat yoghurts had strong antioxidant activities in all assays and their activities significantly increased during their fermentation. The activities of both products remained unchanged during the storage time of 21 days at 4°C. For the antimicrobial activities, buffalo yoghurt could inhibit only Gram positive strains (*S. aureus* and *B. cereus*), while goat yoghurt inhibited all tested strains. The antimicrobial activities of both products were still the same during storage time. It is concluded that the antioxidant and antimicrobial activities could be found in cow, buffalo and goat yoghurt. All products may have benefit from those activities for consumers. These activities increased as a result of the bacterial fermentation of cow, buffalo and goat milk to become its yoghurt products and there were no changes during the storage time of 21 days. The fermentation leads to protein digestion in milk to produce short chains of amino acids that may act as bioactive peptides and lactic acid production is possibly responsible for those activities.

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