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Research Paper

Utilization of bioactive peptides derived from camel milk proteins as biopreservatives in Kareish cheese

Mervet M. El-Bayoumi

Breeding of Animal and Poultry Department, Desert Research Center, Cairo, Egypt.

E-mail: merfat13373@gmail.com

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Abstract. Kareish cheese is one of the popular white soft cheese produced in Egypt. The manufacture of Kareish cheese is characterized by a long preparation and making period. Meanwhile, Kareish cheese has a convenient content of nutrients for microbial growth. It has also higher water content; therefore its shelf life is short due to fast microbial deterioration caused by microbial activity, which results in production of many undesirable biohazards. The antimicrobial peptides derived from milk proteins have been found to be active against broad range of pathogenic organisms. These bioactive peptides have the potential to be used as natural preservatives. The aim of this study was focused on studying the antibacterial activity of bioactive peptides derived from camel milk fermented whey protein and casein solution by some probiotics against some pathogens as *Enterobacteria* spp. and *Staphylococcus* sp. in Kareish cheese. Meanwhile, antibacterial activity of fermented whey and casein solution against pure strains of *Escherichia coli* (ACCT8739) and *Staphylococcus aureus* (ATCC6538) was also studied. Camel milk samples of whey and casein solution (2%) were heat treated at 65°C/30 min, cooled to 42°C and then divided into four portions, which were inoculated with *Bifidobacterium bifidium* (ATCC15708), *L. acidophilus* (ATCC4356), *L. helveticus* (ATCC15009) and *Lactobacillus delbrueckii* ssp. bulgaricus (ATCC7995) and incubated at 42°C for 24 hours. After fermentation, samples were centrifuged at 15000 ×g for 15 min at 4°C .The resulted supernatants were then sterilized using Millipore Membrane Filter, 0.45 µm and kept at 4°C for treatments as preservation solutions. Kareish cheese samples were immersed in all sterilized supernatants of fermented whey and casein solutions, at 4°C and kept for 72 h under static conditions. Samples were taken at zero time, 12, 24, 36, 48, 60 and 72 h for microbiological analyses. Results revealed that all supernatants of fermented whey and casein solution of camel milk have the ability to inhibit the growth of examined pathogenic bacteria in Kareish cheese stored at 4^{o}C for 72 h. However, fermented rennet whey has higher antibacterial activities against both *Enterobacteria* spp. and *Staphylococcus* sp. than fermented casein solution. The maximum antibacterial activity was found in the Kareish cheese treated with supernatant of fermented whey by all probiotics after 48 and 72 h. Fermentation of rennet whey or casein solution by *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. bulgaricus had a remarkable higher antibacterial activity against both pathogens than fermentation by *Bifidobacterium bifidium* or *Lactobacillus acidophilus.* Among all probiotics, the highest antibacterial activity was found in Kareish cheese preserved in the supernatants produced from fermented whey and casein by *Lactobacillus delbrueckii* ssp. bulgaricus (ATCC7995). Results of this study may provide knowledge to utilize a new method to preserve and enhance the quality of Kareish cheese.

Keywords: Bio-preservation, antibacterial activity, probiotics, pathogenic bacteria, Kareish cheese- whey and casein, camel milk proteins.

INTRODUCTION

Foods are important for nutritional needs and improving the health of consumers. The probiotic bacteria have a vital role in both fermentation and preservation of milk and milk products. Some probiotics as lactic acid bacteria and bifidobacteria were used to improve quality and safety of foods due to their antagonistic activity against some pathogenic microorganisms (Saarela *et al*., 2002). Milk proteins can be regarded not only for its nutritive value but also as a possible resource to increase the natural defense of the organism against invading pathogens. Many dairy- starter cultures used in yoghurt and cheese making have characterized by formation of bioactive peptides from milk proteins during fermentation of dairy products (Gomez-Ruiz *et al*., 2002; Fuglsang *et al*., 2003; Matar *et al*., 2003; Gobbetti *et al*., 2004; FitzGeraid and Murray, 2006; Donkor *et al*., 2007; Gobbetti *et al*., 2007). The antimicrobial activity of bioactive peptides derived from milk proteins has many different mechanisms for inhibition of many strains of microorganisms. These mechanisms include production of inhibitory compounds, competition for binding sites, immunostimulation and nutrient competition. From these inhibition activities, the production of organic acids, e.g., lactic acid, results in decreased pH. So, organic acid liposoluble is able to break down the cell membrane and enter to the cytoplasm of pathogens (Haller *et al*., 2001).

Additionally, some probiotic strains are able to produce bioactive compounds such as fatty acids, formic acid ,bacteroicins, ethanol and hydrogen peroxide, that have antimicrobial activity (De Vuyst, 2007). Some probiotics or their antimicrobial contents were used in foods for inhibition of borne pathogens, e.g., *Listeria monocytogenes* and Staphylococcus genera (De Vuyst and Leroy, 2004; Singh and Prakash, 2009). Probiotics were also proposed as an additional bio preservative for inhibition of Listeria growth in fermented foods (Moreno *et al*., 2006). Bioactive peptides produced from milk proteins have been confirmed to have a broad range of different health-related activities such as antimicrobial, antihypertensive, antioxidant, growth stimulation, mineral binding activities (Clare and Swaisgood, 2000; FitzGeraid and Meisel, 2003; Kilara and Panyam, 2003; FitzGeraid *et al*., 2004; Korhonen and Pihlanto, 2003; Pihlanto and Korhonen, 2003; Yamamoto *et al*., 2003; Meisel, 2005; Silva and Malcata, 2005; Gauthier *et al.*, 2006; Korhonen and Pihlanto , 2006; Lopez-Fandino *et al*., 2006; Pihlanto, 2006; Korhonen and Pihlanto, 2007; Lopez-Exposito and Recio, 2008). Camel milk differs from cow milk in its protein content, composition and structure, so functional properties and bioactive properties are different from cow milk. Camel milk is characterized by higher contents of protective proteins, such as immunoglobulins, lysozyme and lactoferrin (El-Agamy and Nawar, 2000; El-Agamy, 2009) and differ in caseins, alpha-lactalbumin, beta-lactoglobulin, serum albumin, proteose-peptone fractions and other minor peptides (El-Agamy, 2016). Kareish cheese is one of the most popular white cheese produced in Egypt from skimmed milk. It is a fresh, soft, low salt, lactic acid and low fat type cheese. Kareish cheese contains all skimmed milk constituents therefore it has a higher nutrient content and due to its higher water content and long storage duration, it is considered a good medium for microbial growth which results in fast deterioration (Robinson,1990; Ray, 1996). Thus the main objective of this study was focused on founding a new method for increasing the shelf life of Kareish cheese by using the supernatants of fermented rennet whey and casein solution of camel milk, which inoculated by some probiotic bacterial strains, and could be used as biopreservatives.

MATERIALS AND METHODS

Milk samples

Camel (*Camelus dromedarius*) milk samples were obtained from Maryoot Research Station at Al-Amaryria, Alexandria, Egypt.

Bacterial strains

All bacterial strains include**:** *Bifidobacterium bifidium* (ATCC15708), *Lactobacillus acidophilus* (ATCC4356), *Lactobacillus helveticus* (ATCC15009) and *Lactobacillus delbrueckii* ssp bulgaricus (ATCC7995) as well as *Escherichia coli* (ACCT8739) and *Staphylococcus aureus* (ATCC6538) were obtained from MIRCEN Center, Faculty of Agriculture, Ain Shams University, Egypt.

Kareish cheese

Traditionally-made Kareish cheese samples were collected from Al-Nasria local market, Al-Amaryria, Alexandria, Egypt.

Culture media

MacConkey and Mannitol salt agar media for enumeration and counting *Enterobacteria* spp. and *Staphylococcus* sp., respectively were obtained from Biolife Company, Italy.

Casein preparation

Camel milk acid casein was prepared according to Warner (1944) by slow acidification at 25°C with 0.1 N HCl. pH was monitored during preparation using pH meter, Model HI 8424; HANNA instrument, Porto, Portugal). After preparation, casein solution (2%, w/v) was prepared using 0.10 M sodium phosphate buffer, pH 7.0.

Preparation of rennet whey

Raw camel milk skimmed by centrifugation at 2000 x*g* for 20 min after that renneted by using calf rennet (locally prepared liquid rennet, from the Dairy Pilot Plant, Faculty

Table 1. CFU/ml of *Enterobacteria* spp. in Kareish cheese treated with supernatant produced from fermented rennet whey of camel milk inoculated with different probiotic bacteria.

Probiotic strain supernatant	Initial pH	pH after fermentation	Time (h)						
			0	12	24	36	48	60	72
Bifidobacterium bifidum (ATCC15708)	7.11	4.1	$3.0×10^8$	$2.0×10^{10}$	$8.0×10^9$	$6.0×10^8$	$3.0×10^5$	-	-
L. acidophilus (ATCC4356)	7.11	4.2	$7.0×10^6$	$4.0×10^{10}$	$6.0×10^9$	$2.0×10^4$	-	-	-
L. helveticus (ATCC15009)	7.11	4.5	$2.8×10^6$	$3.0×10^9$	$3.0×10^7$	-	-	-	-
L. bulgaricus (ATCC7995)	7.11	4	$2.0×10^6$	$1.5×10^8$	$2.0×10^6$	-	-	-	-

of Agriculture, Alexandria University, Egypt). Clear rennet was obtained by centrifugation at 15000x*g* for 20 min.

Fermentation with probiotic bacteria

Samples of whey and casein solutions were heat treated at 65°C/30 min, cooled to 42°C and then divided into four portions, which were inoculated with *Bifidobacterium bifidium* (ATCC15708), *L. acidophilus* (ATCC4356), *L. helveticus* (ATCC15009) and *Lactobacillus delbrueckii* ssp. bulgaricus (ATCC7995) and incubated at 42°C for 24 h. After fermentation, samples were centrifuged at 15000 x*g* for 15 min at 4°C.The resulted supernatants were then sterilized by filtration using Millipore Membrane Filter, 0.45 µm pore size and kept at 4°C for treatments as preservation solutions.

Kareish cheese treatment

Kareish cheese samples were immersed in the different sterilized supernatants of fermented whey and casein solutions, at 4°C and kept for 72 h under static conditions. Samples were taken at zero time, 12, 24, 36, 48, 60 and 72 h for microbiological analyses.

Microbial growth and enumeration of microorganisms

Kareish cheese samples (10 g) were taken at zero time, 12, 24, 36, 48, 60 and 72 h and mixed with 90 ml of 0.1% sterilized peptone water and then homogenized for 5 min with lab Blender (MX32). Homogenized samples were serially diluted in peptone solution and plated for bacterial enumeration according to pour plate method. 1ml of the serial diluted samples were inoculated into molten MacConkey media and Mannitol salt agar media for *Enterobacteria* spp. and *Staphylococcus* sp. count. Plates were incubated at 37°C for 48 h. Then CFUs of the microbes were counted on plates. The experiments were performed in quadruplicates and then the average of the four parallel measurements of the count in CFU/ ml were reported.

Antimicrobial activity measurement against pathogenic microorganisms

Antibacterial activity of fermented whey and casein solution against *Escherichia coli* (ACCT8739) and *Staphylococcus aureus* (ATCC6538) was determined using inhibition zone assay according to (Collins *et al*., 1995). The experiments were performed in quadruplicates and the average of the four parallel and measurements of inhibition zone in cm was reported.

RESULTS AND DISCUSSION

Effect of milk proteins fermentation on bacterial activity

Kareish cheese samples were initially tested for the presence of *Enterobacteria* spp. and *Staphylococcus* sp. to verify the initial microbiological quality of the product. Tables 1, 2, 3 and 4 show CFU/ml of *Enterobacteria* spp. and *Staphylococcus* sp. counts in Kareish cheese samples immersed in sterilized supernatant of fermented rennet whey (FRW) and fermented casein solution (FCS) by *Bifidobacterium bifidium* (ATCC15708), *Lactobacillus acidophilus* (ATCC4356), *Lactobacillus helveticus* (ATTCC15009), and *Lactobacillus delbrueckii* ssp. bulgaricus (ATCC7995) at 4°C for 0, 12, 24, 36, 48, 60 and 72 h under static condition. Generally, it was noticed that, the CFU/ml of *Enterobacteria* spp. was gradually decreased with storage time progress. The antibacterial activities of four supernatants were different. After, 36 h of storage, CFU/ml slightly decreased in *B. bifidium* supernatant (BBS), while it dramatically decreased with *Lactobacillus acidophilus* (LAS). On the other hand, no colonies were found in both treatments of *Lactobacillus helveticus* supernatant (LHS) and *Lactobacillus delbrueckii* ssp. bulgaricus (LBS) supernatant. That means LHS and LBS treatments were more effective against *Enterobacteria* spp. activity after 36hr than BBS and LAS treatments (Table 1). The same behavior of LHS and LBS treatments was noticed against *Staphylococcus* sp. (Table 2).

Although the pH values of the four treatments after fermentation were different to some extended among 4.0

Table 2. CFU/ ml of *Staphylococcus* sp. in Kareish cheese treated with supernatant produced from fermented rennet whey of camel milk inoculated with different probiotic bacteria.

Probiotic strain supernatant	Initial pH	pH after fermentation	Time (h)						
			0	12	24	36	48	60	72
Bifidobacterium bifidum (ATCC15708)	7.11	4.1	5.0×10^9	5.0×10^{10}	6.0×10^{11}	3.0×10^7	-	-	-
L. acidophilus (ATCC4356)	7.11	4.2	5.0×10^8	2.0×10^9	4.0×10^7	3.1×10^7	3.1×10^5	-	-
L. helveticus (ATCC15009)	7.11	4.5	7.2×10^7	7.0×10^8	3.0×10^7	-	-	-	-
L. bulgaricus (ATCC7995)	7.11	4	4.0×10^7	1.0×10^{10}	1.9×10^6	-	-	-	-

Table 3. CFU/ml of *Enterobacteria* spp. in Kareish cheese treated with supernatant produced from fermented casein solution (2%) of camel milk inoculated with different probiotic bacteria.

Probiotic strain supernatant	Initial pH	pH after fermentation	Time (h)						
			0	12	24	36	48	60	72
Bifidobacterium bifidum (ATCC15708)	7	6.2	9.1×10^8	6.2×10^{10}	4.7×10^8	8.0×10^7	2.6×10^7	6.0×10^5	5.0×10^4
L. acidophilus (ATCC4356)	7	6.3	3.4×10^9	6.7×10^8	9.0×10^7	4.0×10^6	4.0×10^5	2.0×10^5	1.0×10^3
L. helveticus (ATCC15009)	7	6.4	6.5×10^8	8.0×10^{10}	5.0×10^9	7.2×10^6	5.0×10^6	3.0×10^6	7.0×10^4
L. bulgaricus (ATCC7995)	7	6	2.1×10^7	4.0×10^9	5.0×10^8	6.1×10^6	7.3×10^4	2.6×10^4	2.0×10^2

Table 4. CFU/ ml of *Staphylococcus* sp. in Kareish cheese treated with supernatant produced from fermented casein solution (2%) of camel milk inoculated with different probiotic bacteria.

Probiotic strain supernatant	Initial pH	pH after fermentation	Time (h)						
			0	12	24	36	48	60	72
Bifidobacterium bifidum (ATCC15708)	7	6.2	3.0×10^8	2.0×10^4	4.0×10^6	6.0×10^4	5.0×10^3	5.0×10^2	-
L. acidophilus (ATCC4356)	7	6.3	3.6×10^8	5.5×10^{12}	7.0×10^8	2.0×10^6	5.3×10^4	4.0×10^4	1.0×10^3
L. helveticus (ATCC15009)	7	6.4	7.2×10^7	8.0×10^9	3.0×10^9	4.0×10^8	5.0×10^7	5.0×10^5	2.0×10^5
L. bulgaricus (ATCC7995)	7	6	8.2×10^8	7.0×10^9	5.0×10^9	6.0×10^8	7.0×10^7	7.0×10^5	2.5×10^2

to 4.5, it was noticed that no bacterial colonies of either *Enterobacteria* spp. or Staphylococcus sp. were detected in all cheese samples of four supernatants treatments after 60 h (Tables 1 and 2).

The antibacterial activities of fermented casein solution with the four different probiotic against *Enterobacteria* spp. or *Staphylococcus* sp. were shown in Tables 3 and 4. It was noticed that the CFU/ml of both *Enterobacteria* spp. and *Staphylococcus* sp. were decreased by storage time progress. No remarked differences were found among the four treatments in antibacterial activity after 72 h, except for that of BBS against *Staphylococcus* sp. Since no colonies of *Staphylococcus* sp. were detected after 72 h in cheese samples treated with BBS.

From these results it is clear that fermented camel milk rennet whey has higher antibacterial activities against both *Enterobacteria* spp. and *Staphylococcus* sp. than fermented camel milk casein solution.

The higher antibacterial activity of fermented whey supernatant (FWS) than fermented casein supernatant (FCS) against *Enterobacteria* spp. and *Staphylococcus* sp. may be due to higher acidic pH of FWS than FCS (Tables 1, 2, 3 and 4). This decrease in pH may have a significant influence on antibacterial activity of whey components. Weschennfelder *et al*. (2009) reported that the maximum antibacterial activity in Kefir whey found at pH 5.8 against *E. coli*. Santos *et al*. (2013) reported also that the antibacterial activity in Kefir with a pH 6.05 against different pathogens was due to presentence of substances with antibacterial activity from Kefir grains resulted from fermentation process as bacteroicins.

Weschenfelder *et al*. (2018) reported a similar result, where they found the antibacterial activity of whey was most effective and higher against *E. coli*. The higher inhibitory effects of fermented camel rennet whey than fermented casein might be also due to protective proteins, i.e., immune proteins such as lysozyme, lactoperoxidase and lactoferrin, which are present in high concentration in camel milk whey (El-Agamy, 2016). Meanwhile, this could also be due to the reduction in the pH of the fermented whey which resulted from lactic acid a metabolite of lactic acid bacteria which creates an

Table 5. Zone of inhibition assay of supernatants produced from fermented whey camel milk inoculated with different probiotic bacteria against *E. coli* (ACCT8739) and *S. aureus* (ATCC6538).

Probiotic strain supernatant	Zone of inhibition * (cm)	
	***E. coli* (ACCT8739)**	***S. aureus* (ATCC6538)**
Bifidobacterium bifidum (ATCC15708)	1.0	1.2
L. acidophilus (ATCC4356)	0.4	1.0
L. helveticus (ATCC15009)	1.0	0.5
L. bulgaricus (ATCC7995)	2.0	1.0

*The experiments were performed in quadruplicates and then the average of the four parallel measurements of the inhibition zone in cm were reported

Table 6. Zone of inhibition assay of supernatant produced from fermented casein solution (2%) of camel milk inoculated with different probiotic bacteria against *E. coli* (ACCT8739) and *S. aureus* (ATCC6538).

Probiotic strain supernatant	Zone of inhibition * (cm)	
	***E. coli* (ACCT8739)**	***S. aureus* (ATCC6538)**
Bifidobacterium bifidum (ATCC15708)	2.6	2.8
L. acidophilus (ATCC4356)	1.0	1.0
L. helveticus (ATCC15009)	2.5	1.0
L. bulgaricus (ATCC7995)	2.0	1.0

*The experiments were performed in quadruplicates and then the average of the four parallel measurements of the inhibition zone in cm were reported.

environment that is not conducive for the growth of other microorganisms. Other factors may be also responsible for inhibition of *E. coli* and *S. aureus* might be due to the presence of lactobacillus species, which have the ability to produce antimicrobial substances such as bacteroicins. Gilliland and Speck (1977) and Warny *et al*. (1999) reported that lactobacillus species exhibit growth inhibitory effects on various Gram positive and Gram negative bacteria through production of bacteroicins and organic acid such as lactic and acetic acids .This substance inhibit growth of pathogenic bacteria (Adebolu and Ademulegun, 2006). From the results of the present study it is clear that among all probiotics, the highest antibacterial activity against *Enterobacteria* spp. and Staphylococcus sp was found in Kareish cheese preserved in the supernatants produced from fermented whey and casein by *Lactobacillus delbrueckii* ssp. bulgaricus (ATCC7995) comparing with the other strains. Tebyanian *et al*. (2017) found that the *L. fermentum* and *L .bulgaricus* had a significant inhibition against *E. coli*, *S. aureus*, *Shigella dysenteriae* and *Salmonella paratyphi* A and it might be used as bio proactive agent. *L. bulgaricus* have the highest inhibitory effect on the growth of the *E. coli* 0157:H7 whereas *L. casei*, *L. acidophulis* and *L. helveticus* showed a similar inhibitory effect on the growth of *E. coli* 0157:H7 (Ali *et al*., 2014). During fermentation, lactic acid bacteria degrade casein and whey proteins to grow in milk, given the proteolytic nature of lactic acid bacteria such as *L. lactis* (Pritchard and Coolbear, 1993; Kunji *et al*., 1998; Minervini *et al*., 2003). *L. helveticus* is used as a microbial catalyst for generation of bioactive peptides (Nakamura *et al*., 1995; Tsakalidou *et al*., 1999).

In order to confirm the antibacterial activity of fermented rennet whey and casein solutions of camel milk against pure strains of *Escherichia coli* (ACCT8739) and *Staphylococcus aureus* (ATCC6538), inhibition zone assay was performed using the four different supernatants (Tables 5 and 6). From results shown in Table 5, it was noticed that, whey BBS and LAS treatments have more inhibition effect against *S. aureus* (ATCC6538) than *E. coli* (ACCT8739). On the contrary, whey LHS and LBS treatments have more inhibition effect against *E. coli* (ACCT8739) than *S. aureus* (ATCC6538). Concerning the antibacterial activity of camel milk fermented casein solutions, results (Table 6) showed that LHS and LBS treatments have more inhibition effect against *E. coli* (ACCT8739) than *S. aureus* (ATCC6538). However, there were no differences in inhibition of *E. coli* (ACCT8739) and *S. aureus* (ATCC6538) by LAS treatment. But BBS treatment had a slight inhibition effect on *S. aureus* (ATCC6538) than *E. coli* (ACCT8739). From these results, it can be concluded that fermented rennet whey or casein solutions by *Lactobacillus helveticus* and *L. delbrueckii* ssp. bulgaricus had a remarkable higher antibacterial activity against both pathogens than those solution fermented by *B. bifidium* or *Lactobacillus acidophilus*. Mohanty *et al*. (2014) found that *E. coli* MTCC82 and *S. aureus* MTCC96 were inhibited with bioactive peptides derived from milk, while, Galia *et al*. (2009) found that

Streptococcus thermophiles was able to produce antimicrobial peptides from casein during proteolytic activity. Miclo *et al*. (2012) found that *Lactobacillus delbrueckii* subsp. lactis CRL581, hydrolyzed beta and alpha-s-casein and antimicrobial peptides were produced. Guzel–Seydim *et al*. (2011) reported that the bacteria in Kefir grains were able to release bioactive peptides during fermentation that display inhibitory activity.

CONCLUSIONS

All supernatants, resulted from fermented whey and casein of camel milk, have the ability to inhibit the growth of *Enterobacteria* spp. and *Staphylococcus* sp. in Kareish cheese. The highest antimicrobial activity was found in the supernatant produced from fermented whey or casein solution by *Lactobacillus delbrueckii* ssp. bulgaricus (ATCC7995). Therefore, results of this study may provide knowledge to utilize a new method to preserve and enhance the quality of Kareish cheese.

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