International Journal of

Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Dairy Science

ISSN 1811-9743
DOI: 10.3923/ijds.2017.368.376

Research Article
Combination of Probiotic Filtrates as Antibacterial Agent Against Selected Some Pathogenic Bacteria in Milk and Cheese

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Abstract

Background and Objective: Since many years ago probiotics bacteria and its culture filtrates have been heavily used as antibacterial agent against some food borne bacteria. Dairy foods are the second leading source of viral and bacterial problems. The objective of this study was to study and evaluate the antibacterial effect of the probiotic filtrate combination against some disease borne bacteria. **Materials and Methods:** Different Milk samples were collected from different areas in Alexandria governorate during the period of 2016-2017. Bacterial isolation was achieved using two different selective medium specific for the two food borne bacterial strains; *Escherichia coli* (*E. coli*) O157:H7 and *Streptococcus pyogenes* (*S. pyogenes*) in the collected samples. Additionally, specific PCR was performed on the isolated bacteria for more confirmation and identification. The pathogenic bacterial isolates were treated with filtrate of the probiotic strains; *Lactobacillus acidophilus* (EMCC 1324), *Bifidobacterium bifidum* (EMCC 1334) and *Lactobacillus plantarum* (EMCC 1845). The antibacterial activity for the filtrates (either in separate or in combination) was recorded and analyzed by one-way ANOVA using SPSS. **Results:** The mixture of the three bacterial filtrates showed high antibacterial activity against the two examined food borne bacteria. Moreover, the MIC of the mixed filtrate was 1%. In addition, complete growth inhibition for the *E. coli* O157:H7 and *S. pyogene* was observed after 3 and 4 days post treatment, respectively. **Conclusion:** The mixed probiotic filtrates could be used in food preservation and food safety especially against food borne pathogenic bacteria.

Key words: Probiotic filtrate, pathogenic bacteria, milk and feta cheese, *E. coli* O157:H7, *S. pyogene*

Citation: Gamal Mohamed Hamad, William A. Botros and Elsayed Elsayed Hafez, 2017. Combination of probiotic filtrates as antibacterial agent against some pathogenic bacteria in milk and cheese. Int. J. Dairy Sci., 12: 368-376.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Milk is an important nutritious food for the human in different ages. It is rich with proteins, carbohydrates and a wide range of vitamins and minerals. Many of the pathogenic bacteria persisted in milk don't propagate well but remain alive[1]. Incorrect processing or storage of dairy products resulted in a transmission hazard for the consumers whom are acceptable to infect with different diseases such as; brucellosis, listeriosis, tuberculosis[2,3].

Some of the bacteria contained in milk such as *Lactobacillus* spp., *Bifidobacterium* spp. were also presented in the healthy human gastrointestinal tract, aiding in digestion and protection from other infections[4]. There are many different types of bacteria which considered as milk-borne diseases includes; *Brucella* spp., *Campylobacter jejuni*, *Bacillus cereus*, Shiga toxin-producing *E. coli* (O157:H7), *Coxiella burnetii*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium* spp., *Paratuberculosis*, *Salmonella* spp., *Yersinia enterocolitica* and certain strains of *Staphylococcus aureus* which are capable of producing highly heat-stable toxins[5].

Escherichia coli O157:H7 represents one of the most important enter pathogenic bacteria; it is the main causal of diarrhea which can be transmitted through food, water and environment[6]. *E. coli* O157:H7 was responsible on food borne disease because this kind of bacteria produces different types of potent toxins which causes wide range of human death.[7-9].

Probiotics bacteria in the dairy diet could be used in reduction and elimination of the vegetative intestinal pathogenic bacteria[10,11]. Probiotics can play an important role not only in controlling of food borne pathogenic bacteria but also can effect on the host by altering indigenous microbiota and preventing infections[12]. It was reported that the bacteria; *Bifidobacterium* spp. and *Lactobacillus* spp., could be used in control some intestinal pathogenic bacteria and stimulate the host immune system[13-15]. Moreover, *Lactobacillus* spp., showed ability in preventing the growth and toxin production by some pathogenic bacteria such as; *Campylobacter jejuni*, *Listeria monocytogenes*, *Helicobacter pylori*, *Salmonella*, *Shigella* and *Escherichia coli*[16-20]. Several studies (*in vitro* and *in vivo)* demonstrated that the antagonism of *Lactobacillus*, including *L. plantarum*, *L. acidophilus*, *L. reuteri* and *L. casei*, against a variety of pathogens[16-19]. The main objective of this study was to study and evaluate the antibacterial effect of the probiotic filtrate combination against some disease borne bacteria. This probiotic agent not only be used as biocontrol agent against food borne bacteria but also as detoxification agent against the different types of toxins produced by food pathogens.

MATERIALS AND METHODS

This study was performed at Department of Food Technology, City of Scientific Research and Technological Applications in the period of 2016-2017.

Bacterial strains, media and growth conditions: *Bifidobacterium bifidum* (DSM 20082), *Lactobacillus acidophilus* (DSM 20079) and *Lactobacillus plantarum* (DSM 20174) were individually grown in 200 mL Man, Rogosa and Sharpe (MRS) broth and incubated at 37°C for 2 days with shaking until OD at 600 nm was 0.4-0.6. After cultivation, the culture broth was centrifuged at 10,000 rpm for 10 min. The supernatant was taken to a fresh new conical tube and stored at -70°C deep freezer. Then, the culture media filtrate was lyophilized at -50°C using lyophilizer (Telstar Model 50, Spain) and the obtained powder was weighed.

Sample collection: Hundred samples of milk and milk product such as; raw milk (25), packaged Milk (50), soft cheese were collected from the market and commercial milk and milk products(cheeses) samples were directly transported to the laboratory in ice Box. It stored in the refrigerators and then analyzed within 24 h.

Isolation and Identification of pathogenic bacteria by selective media: A portion (1g or 1mL) of each sample was taken aseptically and diluted in 9 mL sterile distilled water. The diluted sample was streak inoculated on sterile nutrient agar and incubated at 37°C for 24 h. After incubation period all the colonies were inoculated on sterile selective media such mannitol salt agar (M.S.A) was used for isolation of *Staphylococcus aureus*. On the other hand, eosin methylene blue (E.M.B) was used for *Escherichia coli* isolation but xylose lysine deoxycholate agar (X. L. D) was used for *Salmonella typhi, Shigella* spp., by *Salmonella-Shigella* agar (S.S.A). Whenever, blood agar plates (B.A.P) was used for isolation of *Streptococcus pyogenes*, Sorbitol MacConkey Medium (SMAC) for *Escherichia coli* O157:H7. The obtained colonies were subjected to specific PCR for type detection[21,22].

Inhibitory effect of individual and mixed probiotic culture filtrate against pathogenic bacteria: Antimicrobial activities of each individual and mixed probiotic culture filtrate on two pathogenic bacteria (*Escherichia coli* O157:H7 and

Streptococcus pyogenes) used in this investigation was determined on Muller Hinton Agar media (M.H.A) by using agar well diffusion method. Wells of 9 mm diameter were made on the solid agar using a sterile cork borer. Approximately 200 μL mix probiotic culture filtrate was added into each wells which contains 20 μL of each pathogenic bacterium *(Escherichia coli* O157:H7 and *Streptococcus pyogenes)* (10^6 CFU mL^{-1}) (The plates were performed in triplicates). All plates were incubated at 37°C overnight. After 24 h of incubation, each probiotic culture filtrate was noted for zone of inhibition for all isolates. The diameters of the zone of inhibitions were measured by measuring scale in centimeter (cm)[23].

Determination of minimum inhibitory concentration (MIC): The mixed probiotic culture filtrate showed that high antibacterial activity against *Escherichia coli* O157:H7 and *Streptococcus pyogenes* was chosen. Their minimum inhibitory concentration (MIC) was determined using descending concentrations of the mixed probiotic culture filtrate. The MIC of mixed probiotic culture filtrate was diluted using sterile saline and was tested for their antibacterial activity against *Escherichia coli* O157:H7 and *Streptococcus pyogenes* according to Miri *et al.*[24] and Hamad *et al.*[25] with some modifications. The different prepared concentrations were tested against the bacterial strains using well diffusion assay as previously mentioned. The formed clear zones were measured and recorded and the MIC for each extract was determined.

Determination inhibitory effect of the mixed probiotic culture filtrate against *Escherichia coli* O157:H7 and *Streptococcus pyogenes* inoculated in feta soft cheese: Feta soft cheese was purchased from local supermarket, the packaging showed the presence of no artificial preservatives. Initial experiments of inoculating tryptone soya agar (TSA) plates with cheese diluted 1 in 10 with PBS and incubating at 37°C for 48 h showed no microbial contamination of either product. The procedure was based on that of Smith-Palmer *et al.*[26], 10 g of cheese was added to 90 mL of phosphate buffered saline (PBS) (Sigma-Aldrich, UK) in stomacher bags and homogenized for 2 min in a stomacher (Seward Medical, London, UK). The mixed probiotic culture filtrate of concentration 1% had antimicrobial activity against studied strains was added to the cheese mixture to achieve final concentrations of 1%. The controls contained PBS but no mixed probiotic culture filtrate. The cheese mixture was inoculated with 100 mL of cold adapted *Escherichia coli* O157:H7 and *Streptococcus pyogenes* cultures that had been prepared by growing for 24 h in 10 mL tryptone soya broth (TSB) in an orbital incubator (100 rpm). The inoculums (10^6 CFU mL^{-1}) was mixed thoroughly with the cheese mixture by gently squeezing the bags by hand and the concentration of both strains in the cheese and determined Colony Forming Units after incubation at 0, 1, 2, 3, 4, 7 and 10 days storage at 7°C using the serial dilution and spread plate technique [27]. The counts were taken on supplemented with mixed probiotic culture filtrate for BHI agar medium plates for *Escherichia coli* O157:H7 and *Streptococcus pyogenes* by surface plating the appropriate dilutions of the samples aseptically in duplicate. Prior to removing samples, the contents of the bags were mixed by gently squeezing the bags by hand. Three individual replicates of each experiment were performed in all cases.

Analysis of probiotic culture filtrate by Gas Chromatography-Mass Spectrometry: Analysis of culture filtrate was determined by GC-MS according to Abdel Rahim *et al.*[28].A Varian GC-MS (QP-2010 SHIMADZO-JAPAN) equipped with a split/split less program. The temperatures of the injector, interface and ion source were 260, 300 and 230°C, respectively. The samples were introduced into the split-injection mode (10:1). The oven temperature was maintained at 80°C for 1 min and this temperature was increased to 100°C at 5°C min^{-1} and finally to 300 °C (5 min) at the rate of 30 ºC min^{-1}. The GC-MS analysis in SIM mode was performed using an Agilent 6890N gas chromatography interfaced with an Agilent 5975B mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 cross linked capillary column (5% phenyl-95% methyl polysiloxane bonded phase; 25 m × 0.20 mm I.D., 0.11 μm film thickness). The characteristic SIM ions are showed in (Table 1) and all GC-MS-SIM analyses were performed in triplicate[29].

PCR amplification of specific gene for detection of specific strain: The isolated bacteria were subjected to DNA extraction using DNA extraction kit (Qiagen, Germany) according to the manufacture procedures. The obtained bacterial DNA was subjected to PCR amplification using strain specific primers. The primers information and primer sequences were tabulated in Table 1. The PCR amplification was performed in Thermocycler Gene Amp 6700 (Applied Bio-system, USA). The PCR reaction was carried out in total volume of 25 μL consists of; 2 μL DNA (100 ng), 2 μL of each primer (10 pm μL^{-1}), 2.5 μL 1X buffer, 2.5 μL 3.2 mM $MgCl_2$, 2.5 μL 0.6 mM dNTPs and 0.2 μL (5 units μL^{-1}) *Taq* DNA polymerase (Promega Germany). PCR conditions were: 94°C for 5 min, followed by

Table 1: Primers used in bacterial detection

Strain name	Target gene	Annealing temperature	Primer sequence 5'-'3	Amplicon size (bp)
Salmonella Typhimurium	fimA	65	CCTTTCTCCATCGTCCTGAA	85
			TGGTGTTATCTGCCCGACCA	
Staphylococcus aureus	nuclease gene	57	GCGATTGATGGTGATACGGTT	276
			CAAGCCTTGACGAACTAAAGC	
Streptococcus pyogenes	*cpa* locus	65	GGATATGAGATTGCCGAACCTATTACTTTTAAAG	600
			GGAGCCTGTTTATCTTCCATTCGAATAATATCCAC	
Shigella spp.	IpahgeneShi	60	CTTGACCGCCTTTCCGATAC	610
			CAGCCACCCTCTGAGAGTA	
E. coli-O157:H7	*hlyA* gene	45	GTAGGGAAGCGAACAGAG	361
			AAGCTCCGTGTGCCTGAA	

35 cycles of 1 min at 94°C, 75 sec at temperature depending on the type of primer (Table 1), 2 min at 72°C and 10 min of final extension at 72°C. The PCR amplifications were separated by electrophoresis on 0.8% agarose gel.

Statistical analysis: Data were expressed as mean±standard error (SE) by multiple comparisons one-way analysis of variance (ANOVA) using SPSS 16 software program (IBM is International Business Machines, an American, Armonk, New York.) at probability values <0.05 considered statistically significant.

RESULTS AND DISCUSSION

Isolation and identification of pathogenic bacteria by selective media: The results presented in Table 2 shows that 9 samples out of the collected 50 milk samples are contaminated with food borne pathogens especially *Streptococcus pyogenes* and *E. coli*-O157:H7. The *E. coli* O157:H7 was detected in samples; 7, 14, 23, 29, 36, 42 and 49 whenever, *S. pyogenes* were observed in only two samples (7 and 29). The results revealed that about 14% of the collected samples are spoiled with the two strains of pathogenic bacteria. Many scientists and researchers reported that a high incidence of *E. coli* O157:H7 were detected in different samples of milk[30-32]. It is well known that the recovery of *E. coli* from raw milk is not only regarded as an indicator of fecal contamination but more likely as an evidence of poor hygiene and sanitary practices during milking and further handling. The presence of *E. coli* itself in milk and milk products as a possible cause of food borne disease was insignificant because *E. coli* is normally a ubiquitous organism[33]. However, the occurrence of pathogenic strains of *E. coli* in milk products could be a source of hazardous for consumers. Singh *et al.*[34] detected and isolated the two pathogenic bacterial strains; *S. pyogenes* and *E. coli* O157:H7 from 100 milk samples with percentage of 13.33 and 32.14%, respectively.

Results tabulated in (Table 3) revealed that the 50 examined cheese samples include eight samples are food borne infected. The major bacterial strains detected in the eight infected samples are; *E. coli* O157:H7 and *S. pyogenes* with percentage 14%. Samples infested with *E. coli* are; 11, 16, 24, 27, 31, 39 and 49. But, *S. pyogenes* is detected only in sample 49. Abdulaal[35] succeeded in isolating *S. pyogenes* bacterium with percentage of 14% from soft cheese, while Awad[36] detected *E. coli* O157:H7 in soft cheese with 23% but Saad *et al.*[37] isolated *E. coli* with percentage of 60% from kariesh samples.

Bacterial isolation using selective medium and confirmation using specific PCR: The using of selective media specific for isolation either *E. coli* O157:H7or *S. pyogenes* was used and the results revealed that there were growing of huge number of bacterial colonies on the plates after incubation period which was 3 and 4 days, respectively. Form more confirmation the DNA of the randomly selected bacterial colonies and subjected to specific PCR for the two food borne bacteria (*E. coli* O157: H7 and *S. pyogenes*). Results in the (Fig. 1a and b) showed that amplicons with molecular sizes 361bp specific for *E.coli* O157:H7 (antigen gene) and 600 bp specific for the *S. pyogenes* (toxin regulatory protein). Nguyen *et al.*[38] used the multiplex PCR for detecting *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogens* in contaminated food, whenever, amplicons with molecular sizes; 284, 404 and ~600 bp were amplified from *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7, respectively. On the other hand, Lee *et al.*[39] used the multiplex PCR for detection of different food borne bacterial strains in the Korean food using primers of specific genes such as; antigen gene for *E.coli* O157:H7, gyrase gene for *B. cerus*, toxin regulatory gene for *V. parahaemolyticus*, the *inv A* gene of *Salmonella* spp., the *hly* gene of *L. monocytogenes* and the thermonuclease gene for *S. aureus*. It can conclude that the using specific PCR in food borne pathogens detection either specific or multiplex PCR is successful protocol, is highly accurate, not coastly and safe time.

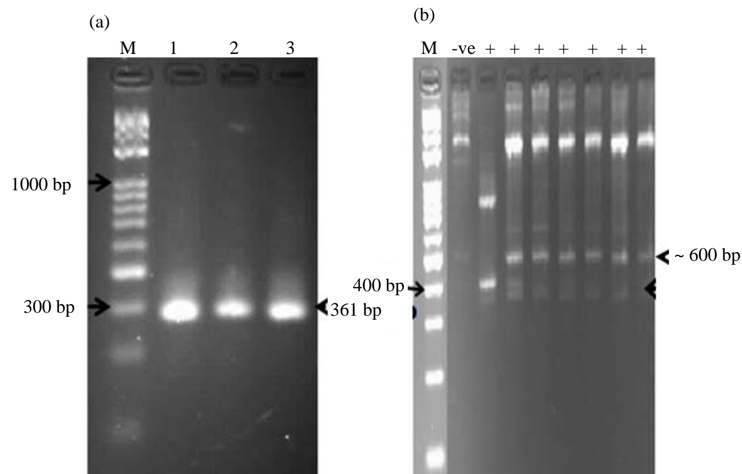

Fig. 1(a-b): (a) PCR detection of *E. coli* O157:H7 in the grown bacterial colonies on the selective medium specific for the *E. coli* O157:H7. M: 3 K DNA ladder. Lanes 1-3 randomly selected bacterial colonies which grown on the selective medium and (b) PCR detection of *S. pyogenes* in the grown bacterial colonies on the selective medium. Lanes -ve: Negative control from healthy sample. +: Amplicon with molecular size 390 bp which specific for *S. pyogenes*

Table 2: Isolation of pathogenic bacteria from different milk samples on selective media

Samples	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Shigella* spp.	*Salmonella* Typhimurium	*E. coli*-O157:H7
Milk 1-6	-	-	-	-	-
Milk 7	-	+	-	-	+
Milk 8-13	-	-	-	-	-
Milk 14	-	-	-	-	+
Milk 15-22	-	-	-	-	-
Milk 23	-	-	-	-	+
Milk 24-28	-	-	-	-	-
Milk 29	-	+	-	-	+
Milk 30-35	-	-	-	-	-
Milk 36	-	-	-	-	+
Milk 37-41	-	-	-	-	-
Milk 42	-	-	-	-	+
Milk 43-48	-	-	-	-	-
Milk 49	-	-	-	-	+
Milk 50	-	-	-	-	-

(-) = Negative (+) = Positive

The results presented in Table 4 revealed that the filtrate of *L. plantrum* shows high antibacterial activity against the two human pathogenic bacteria (*E. coli* O157:H7 and *S. pyogenes*) more than the filtrate of the other two probiotic bacterial strains. But the mixture of three filtrates of the three probiotic bacteria showed a highest antibacterial activity more than each filtrate alone. Different concentrations of the mixture were used to determine the MIC of the mixed filtrate against the two human pathogenic bacteria *E. coli* O157:H7 and *S. pyogenes* and the results tabulated in Table 5 showed that the MIC for this mix is 1%. The antibacterial activity of the mixed filtrate (1%) was examined for 10 days along against the two pathogenic bacteria and the results revealed that the mixed filtrate succeeded to make complete growth inhibition for the *E. coli* O157:H7 after 3 days of treatment but it lasts for 4 days in case of the *S. pyogenes* (Table 6). The results represented in this study are in agree with the results obtained by Arena *et al.*[40], they used different strains of *L. plantrum* bacteria as biocontrol agent against food borne pathogens such as; *Listeria monocytogenes*, *Salmonella enteritidis*, *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Whenever, Russo *et al.*[41] used the *L. plantrum* as antifungal especially against the cereals based products. Arena *et al.*[42] revealed that *L. plantrum* is a promising probiotic strain and it can be used in different applications. Uraipan and Hongpattarakere[43] postulated that *B. bifidum* bacteria have high antibacterial activity against different human pathogenic bacteria. Moreover, the same observation

Table 3: Isolation of pathogenic bacteria from different cheese samples on selective media

Samples	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Shigella* spp.	*Salmonella* Typhimurium	*E. coli* O157:H7
Cheese 1-10	-	-	-	-	-
Cheese 11	-	-	-	-	+
Cheese 12-15	-	-	-	-	-
Cheese 16	-	-	-	-	+
Cheese 17-23	-	-	-	-	-
Cheese 24	-	-	-	-	+
Cheese 25-26	-	-	-	-	-
Cheese 27	-	-	-	-	+
Cheese 28-30	-	-	-	-	-
Cheese 31	-	-	-	-	+
Cheese 32-38	-	-	-	-	-
Cheese 39	-	-	-	-	+
Cheese 40-48	-	-	-	-	-
Cheese 49	-	+	-	-	+
Cheese 50	-	-	-	-	-

(-) = Negative (+) = Positive

Table 4: Clear zones of the antibacterial activities of individual filtrate of different probiotics strains and their mix against *Escherichia coli* O157:H7 and *Streptococcus pyogenes*

	Diameter of inhibition zone (cm)	
Strains culture filtrates	*Escherichia coli* O157:H7	*Streptococcus pyogenes*
B. bifidum	2.2±0.2	1.9±0.3
L. plantarum	2.4±0.5	2.2±0.4
L. acidophilus	2±0.3	2±0.18
Mix	2.8±0.3	2.5±0.2

Values obtained as SD

Table 5: Minimum inhibitory concentrations (MIC) of antibacterial activity of the probiotic filtrate mix in concentrations of, 1, 0.5, 0.25, 0.125 and 0.0625% against *Escherichia coli* O157:H7 and *Streptococcus pyogenes*

	Diameter of inhibition zone (cm)	
Concentrations mix filtrates (%)	*Escherichia coli* O157:H7	*Streptococcus pyogenes*
1	2.7±0.2	2.5±0.7
0.5	2.3±0.13	2±0.21
0.25	2.1±0.3	1.8±0.08
0.125	1.8±0.15	1.5±0.2
0.0625	0.0±0.0	0.0±0.0

Values obtained as SD

was recorded by Hongpattarakere and Uraipan[44] and they concluded that both of *L. plantrum* and *B. bifidum* could be used as biocontrol against different human pathogens especially the food borne ones.

Based on the data represented in this study, it can conclude that the probiotic mix could be considered as a good antibacterial agent against a wide range of food borne pathogens. The obtained results in this study agree with theses obtained by Gomez *et al.*[45,46], they used lactic acid bacterial mix as biofilm to control different human pathogens (food borne) such as; *Salmonella* Typhimurium and *E. coli* O157:H7. Moreover, Benkerroum *et al.*[47] used lyophilized mixed formed from both *Lactobacillus curvatus* and *Lactococcus lactis* as biocontrol agent against *Listeria monocytogenes* in dry-fermented sausages. The previous results and those obtained in this study (Table 5) confirmed that probiotic mix as biocontrol agents against the food borne pathogenic bacteria is more effective than the individuals strains.

Data presented in Table 6 revealed that using probiotic mix as biocontrol agents against the food borne bacteria; *Escherichia coli* O157:H7 and *Streptococcus pyogenes* and this antibacterial effect lasts for long period. The antibacterial activity of the used probiotic filtrate smix succeeded to make complete control for the *Escherichia coli* O157:H7 happened after 3 days post treatment. Whenever, after 4 days post treatment complete diminish was approached for *Streptococcus pyogenes*. These results confirm that the mix is promising as antibacterial specially the human pathogenic bacteria. These results confirmed with the results obtained by Gómez *et al.*[46], they concluded the complete controlling of the food bornepathogenic bacteria by lactic acid bacteria could be achieved after days post treatment. The results obtained by this study revealed that the shelf life of the probiotic mix could be lasts for long time and their activity not diminish by the time.

GC-MS analysis of probiotic of mix probiotic culture filtrate: GC-MS analysis of probiotic culture media extract revealed presence of twenty peaks on chromatogram. The filtrates mainly comprise organic acids, alcohols, long chain fatty acids, carboxylic acids, amino acids, nitrogenous compounds and aldehydes (Table 7). The bioactive molecules are given in MS chromatogram and Table 7. GC-MS analysis revealed

Table 6: Bacterial counts (CFU mL^{-1}) of strains *Escherichia coli* O157:H7 and *Streptococcus pyogenes* in feta soft cheese samples during storage at 7°C after treatment with mixed probiotic culture filtrate

	Colony forming units (CFU mL^{-1})			
	Treatment with mix probiotic culture filtrate (1%)			
Incubation (days)	Control *Escherichia coli* O157:H7	Treatment *Escherichia coli* O157:H7	Control *Streptococcus pyogenes*	Treatment *Streptococcus pyogenes*
0	$1x10^6 \pm 0.4$	$6.8\times10^5\pm0.6$	$1\times10^6\pm0.7$	$7.9\times10^5\pm0.5$
1	$2.3\times10^6\pm0.18$	$4.2\times10^3\pm0.32$	$3.1\times10^6\pm1.2$	$3.3\times10^3\pm0.6$
2	$6.2\times10^6\pm0.2$	$1.5\times10^2\pm1.4$	$5.2\times10^6\pm0.65$	$1.8\times10^2\pm1.8$
3	$5.3\times10^7\pm0.8$	0.0 ± 0.0	$8.7\times10^6\pm0.8$	$\pm0.90.5\times10^2$
4	$8.9\times10^7\pm1.9$	0.0 ± 0.0	$2.5\times10^7\pm1.3$	0.0 ± 0.0
7	$7.25\times10^8\pm2.1$	0.0 ± 0.0	$1.16\times10^8\pm0.5$	0.0 ± 0.0
10	$6.14\times10^9\pm0.54$	0.0 ± 0.0	$7.85\times10^9\pm0.4$	0.0 ± 0.0

Values obtained as SD

Table 7: GC-MS components of combination probiotic culture filtrate

Retention time (min)	Compounds
3.264	Acetic acid
4.602	2,3-Butanediol, [R-(R*,R*)]
4.695	2,3-Butanediol, [S-(R*,R*)]
6.626	2,5-Hexanedione, 3,4-Dihydroxy-3,4-dimethyl- SS 1,6-Dideoxy-3, 6.715
	Propanoic acid, 2-Methyl-, 1-methylethyl ester SS isobutyric acid,
7.122	Glycerol i
7.627	Benzene acetaldehyde SS acetaldehyde, phenyl- S$ .alpha.-Tolualc
7.764	2,5-Dimethyl-4-hydroxy-3(2H)-furanone $S 3(2H)-Furanone, 4-hy
7.953	Butyric acid, p-fluorophenyl ester $S, 4-Fluorophenyl butyrate # SS
8.089	Pyrazine, tetramethyl- S$ BS Factor S$, Tetramethyl pyrazine SS 2,
8.793	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- $S 3,5-Dih:
8.885	2,3,5-Trimethy-1, 6-ethylpyrazine SS, 2-Ethyl-3,5,6-trimethylpyrazin
13.983	3-Pyrrolidin-2-yl-propionic acid
14.337	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- SS Hexahydropyrro
14.776	2-Decene, 3-methyl-, (Z)- SS (2Z)-3-Methyl-2-decene I SS
15.459	L-Proline, N-valeryl-, pentadecyl ester
15.563	Bromocriptine SS, ergotaman-3',6',18-trione, 2-bromo-12'-hydroxy
15.743	2-Acetylpyrrolidine SS, 1-(2-Pyrrolidinyl)ethanone # SS
16.953	Oleic Acid
17.437	Pentanamide $S, valeramide SS, n-valeramide SS, pentanimidicacic

bromocriptine bioactive component found in probiotic filtrates. Chemical structure and retention time of this bioactive molecule was presented in Table 7. The GC components involved in the probiotic mix in this study are agreed with that obtained by Kantachote *et al.*[48] and they conclude that the probiotic mix contains phenyl lactic acid (PLA), succinic acid, 1, 4-Butanediol, γ-butyrolactone, tetrahydrofuran, (N, O-bis (trimethylsilyl)-acetamide) and some biodegradable polymers. The same observation was indicated by Willke and Vorlop[49].

CONCLUSION

The probiotic bacteria filtrate is a good tool for control food borne pathogens if it is used in the right food process and the right food type. The probiotic filtrate mix effect was better than the effect obtained individual bacterium as antibacterial. Moreover, the probiotic bacteria filtrate shows no affect by time which adds a value for using these bacteria in human pathogenic bacteria. This mix could be used in food preservation and safety if both the steps and the mix contents of this study were been followed.

SIGNIFICANCE STATEMENTS

The study discovers that probiotic combination could be used as antibacterial agent against some pathogenic bacteria persisted in milk and cheese. This study will help many researchers to do further studies on discovering a new probiotic strain which could be used in biocontrol of different food borne pathogenic bacteria.

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