American Journal of Biomedical Research, 2020, Vol. 8, No. 1, 1-6
Available online at http://pubs.sciepub.com/ajbr/8/1/1
Published by Science and Education Publishing
DOI:10.12691/ajbr-8-1-1

Putative Amplification of 16S rRNA Markers of *Escherichiacoli* and *Salmonellaspp* Isolates From Raw Milk Sample of Cattle

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Received January 11, 2020; Revised February 20, 2020; Accepted March 10, 2020

Abstract Milk derived from milk of dairy cows harbour a variety of microorganisms and can be sources of food borne pathogens. The presence of specific amplicons of stx, stx2, in timin genes, InvA, and stn genes are responsible for the virulence of the organisms. The aim of this study was to amplify the 16S rRNA marker genes of E.coli and Salmonella spp.A total number of ten raw milk samples were collected from Fulani dairy farm at Ago-Aare, Oke-Ogun area, Oyo State, Nigeria. Both total viable bacteria counts (TBC) and total coliform counts (TCC) analysis were performed to determine the microbial load of the raw milk samples. The antimicrobial susceptibility testing for both E.coli and Salmonella were carried out using the disk diffusion method. A more specific identification of the bacterial 16S rRNA genes was performed by PCR and Sanger sequencing technique by using specific primers which are specific for the amplification of E.coli and Salmonella genes. Total viable bacteria count of all the samples ranged from 1.23 x105 cfu/ml (R7) to 2.60 x105 cfu/ml (R1). Total coliform count ranged from 1.05 x105 cfu/ml (R7) to 1.93 x105 cfu/ml (R9). Both counts were significant at (P >0.05). The raw milk samples had 80% *E.coli*, 70% *Salmonella*, 20% *Shigella spp*, 60% *Klebsiella spp,* while 50% level of contamination was observed with *Staphylococcus aureus,* 20% Proteus spp and 20% Pseudomonas spp. Isolates were catalase (+), glucose and maltose (+) and oxidase (-). Antibiotic susceptibility test showed that Salmonella spp and E.coli were resistant to most commonly used antibiotics and most susceptible to Ofloxacin and ceftriaxone. Molecular characterization of both E.coli and Salmonella spp indicated the presence of specific amplicons of stx1, stx2, intimin genes, InvA and stn genes respectively. The presence of these genes could justify the degree of virulence in the organisms that harboured the genes. Conversely the need for continuous molecular surveillance and genetic monitoring of raw milk and dairy products in order to minimize epidemics with Salmonella and E.coli.

Keywords: *16S rRNA, diary, susceptibility-test, virulence, amplicons, marker genes*

Cite This Article: Adeoti O.M, Fehintola O.I, Adeoye K.A, Olaoye O.J., Olufemi S.O, Adedokun E.O, and Adeshina D.A, "Putative Amplification of 16S rRNA Markers of *Escherichiacoli* and *Salmonellaspp* isolates from Raw Milk Sample of Cattle." *American Journal of Biomedical Research*, vol.8, no. 1 (2020): 1-6. doi: 10.12691/ajbr-8-1-1.

1. Introduction

Milk and milk products constitute important nutritional components for human diet and plays a prominent role in human nutrition [1]. Good quality milk meets the nutritional needs of the body better than any single food as it contains essential food constituents such as fat, proteins, carbohydrates, minerals, vitamins. As a result of the presence of these nutritional components, milk is an excellent culture medium for many microorganisms, especially bacterial pathogens [2]. Cow's milk has rejuvenator, health protecting, and health promoting properties and hence can be referred to as one of the best vitalizers [3]. Most of the changes which take place in the flavorand appearance of milk, after it is drawn from the udder are the results of the activities of microbes. Milk as it is secreted by the gland of the mammals is free of microorganisms.

However, microorganisms associated with the teat move up the teat canal and into the interior of the udder. This causes even aseptically drawn milk to contain microorganisms, mostly bacteria. Bacteria in aseptically drawn milk are usually limited in number and include mostly, *Micrococci*, *Lactococci*, *Staphylococci*, *Streptococci* and *Bacillus*. Milk is nutritious food for human beings, also serves as a good medium for the growth of many

microorganisms, especially *Escherichiacoli*, *Salmonellatyphii*, *Pseudomonasaeruginosa* and *Staphylococcusaureus*. Raw or processed milk is a well-known good medium that supports the growth of several microbes with resultant spoilage of the product i.e., infections or intoxications in consumers [4].

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2. Materials and Methods

2.1. Sample Collection

Ten raw milk samples were collected from ten different cows at a local Fulani dairy farm settlement at Ago-Aare Oke-Ogun area Oyo State. All the samples were aseptically collected in sterile wide-mouthed sterile bottles, labelledas (R1to 10)and were transported as quickly as possible in ice box to the Laboratory of Microbiology Department, Faculty of Science of the Oke-Ogun Polytechnic Saki, Oyo State for the conventional identification and isolation of possible bacteria present in the Raw Milk samples.

2.2. Preparation of Culture Media

Different media were used for the isolation of different microorganisms from the samples and they include: Nutrient Agar (NA), Mac Conkey Agar, Blood Agar, and Chocolate Agar. Some media were also employed to identify and characterize the bacterial isolates which included Eosin Methylene Blue (EMB), Salmonella Shigella Agar and MRS agar. Each medium was prepared using the manufacturer’s instructions.

2.3. pH Determination

The pH of the samples was determined using a pH meter (pancellentmeters). The pH meter was calibrated, with two standard solutions (pH 4.0 and 7.0) 30ml of each sample was transferred into a clean beaker and the probe of the pH meter was rinsed with distilled water and dipped into the milk samples. The probe was rinsed at intervals with distilled water after each sample. The pH value of each sample was displayed on the meter and subsequently recorded.

2.4. Total Bacterial Count

A modification of the methods reported by [5] was performed. Ten-fold serial dilutions of the samples were made as follows: 1mL of each raw milk sample was withdrawn aseptically using a sterile pipette and transferred into a test tube containing 9 mL of distilled water. After shaking, 1 mL of the first diluted sample (10^{-1}) was aseptically withdrawn and transferred into another 9mL of sterile distilled water contained in a test tube and shaken again, this represents 10^{-2}. The dilution was done up to 10^{-10}. Subsequently 1 mL each from dilutions 10^{-3} and 10^{-5} was aseptically taken and plated on nutrient agar (NA) using the pour plate method. Each of the plates was incubated at 37°C for 24 hours.

2.5. Isolation of *Salmonella* Bacteria

The 10^{-3} and 10^{-7} dilutions of the samples were inoculated in Salmonella-Shigella Agar (SSA) and incubated at 37°C for 24 hours. Cultural characteristics and colonial morphology of the isolates were interpreted using standard microbiological procedures.

2.6. Isolation of *E.coli* Bacteria

After the serial dilution of the raw milk, 1 mL each from dilutions 10^{-3} and 10^{-7} were aseptically taken and plated on Mac Conkey agar using pour plate method. After the 24 hours incubation, plates that showed growth of organisms and colour change indicating presence of enteric bacteria with distinct colonies on the agar were sub-cultured by inoculating distinct colonies on Eosin Methylene Blue agar (EMB) plates and incubated at 37°C for 24 hours. Typical colonies indicating a shining metallic greenish colour is suspected to be *Escherichia coli* before further characterization was performed.

2.7. Gram Staining Techniques

A sterile wire loop was used to pick a bacterial colony and a smear of the bacterial isolates were made on a slide with a drop of distilled water. These were air-dried and then heat fixed. The slides were then flooded with crystal violet (primary stain) for 60 seconds, the stain was drained off, washed and flooded with Lugol’s Iodine (mordant) for 60 seconds. It was then flooded with 95 % alcohol for 15 seconds after which it was then rinsed with distilled water and finally counterstained with safranin for 30 seconds. The slides were then gently washed with distilled water and air dried. The slide was observed under oil immersion lens (× 100). Gram positive cells stained purple while Gram negative ones stained pink or red.

2.8. Identification of Isolates by Biochemical Tests

The pathogenic bacteria isolates were confirmed and characterized by indole, catalase, oxidase, citrate, and sugar fermentation test according to Bergey’s manual of determinative bacteriology.

2.9. Antimicrobial Susceptibility

Antimicrobial susceptibility testing of the bacteria isolates was performed by the Kirby Bauer disc diffusion

method using Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI). The following used antibiotics were tested: Augmentin (30µg), Erythromycin (5 µg), Of loxacin (5 µg.), Ceftazidime (30 µg.), Gentamicin (10 µg.), Cloxacillin (5 µg.), Ceftriaxone (30 µg.), Cefuroxime (30 µg.).

Briefly, a bacterial suspension with equivalent turbidity to 0.5 McFarland standards (1.5 × 108 CFU/mL) was prepared in sterile Phosphate Buffered Saline (PBS) (137 mMNaCl, 10 mM phosphate, 2.7 mMKCl, pH 7.4). A sterile swab stick was dipped into the bacterial suspension and then inoculated uniformly on the surface of the agar to make bacteria lawn on the surface of the Mueller Hinton agar. Afterward, antibiotic disks were placed on the plates and incubated at 35°C for 24 hrs. Inhibition zones on agar plates were measured, and the results were recorded in accordance with interpretive criteria provided by Clinical and Laboratory Standards Institute (CLSI).

2.10. Molecular Analysis

Bacteria DNA Extraction (Cell Lysis)

To extract the DNA of the test organisms, 300 µl of cell Lysis solution was pipetted into a clean and sterile 2ml Eppendorf tubes as previously described by [4], one or two clean colonies of the bacteria samples was scraped using the tip of the pipette and was transferred into the tube and was then Pipetted up and down to lyse the cells. 2µl of lysozyme was then added into the Eppendorf tube and then incubated at 55°C for 60 minutes.

Protein Precipitation

100 µl of protein precipitation solution was added to the cell lysate and was Vortex rigorously for 20 secs to mix well and was then centrifuged at maximum speed for 2 min. The resulting precipitated proteins form a tight dark pellet which was incubated on ice for 5mins and centrifuged again.

DNA Precipitation

300 µl of 99% Isopropanol solution was pipetted into a clean 2 ml Eppendorf tube and the supernatant from the protein precipitation was mixed with it by inverting gently for 1 minutes. The mixture was centrifuged at maximum speed for 1 min. DNA became visible as a small white pellet. The supernatant was discarded, and the tube was drained briefly on clean absorbent paper. 500 µlof Washing Buffer was added and the tube was inverted several times to wash the DNA pellet. The DNA pellet was centrifuged at maximum speed for 1 min and the ethanol was carefully discarded and dried at room temperature for about 10 to 15 minutes[4].

2.11. PCR Protocol

The PCR was performed to amplify the 16S rRNA as determined by [6] using specific type primers 27F (5'AGAGTTTGATCMTGGCTCAG3') and 511R (5'GCGGCTGCTGGCACRKATGT3'). The 25µl PCR mix contains 5µl of 5x PCR mix by Jena Bioscience (Jena Germany), 1µl each of the primers, 13µl of PCR grade water and 5µl of the template (bacteria DNA). The PCR program included one denaturing step at 95°C for 3 min, 25 cycles of 95°C for 40 sec, 55°C for 40 sec and 72°C for 40 sec followed by final extension at 72°C for 7 min. Gel electrophoresis was used to check the existence of a single band in an expected size for each PCR product. A 2% agarose gel was used and this was passed across an electric field with 100 volts.

2.12. Statistical Analysis

Data were subjected to descriptive statistics and one-way ANOVA using SPSS 2016 and differences in mean was separated using Duncan's multiple Range Test (DMRT) of the same software.

3. Results

The total viable bacteria count is one of the most common tests applied to indicate the microbiological quality of food. In this study, the total viable bacteria count obtained in the raw milk samples ranges from 1.22 - 2.60 Log CFU / g. The sample raw milk 1(R1) had the highest total viable bacteria count while the sample raw milk 7 (R7) had the least total viable bacteria counts. The presence of coliforms bacteria in food and food products is undesirable, because it is an indication of poor hygiene or inadequate processing of food products and post-process contamination of foods. The mean coliform count as obtained in this present study ranged from 1.05 - 1.93 Log CFU/g. Raw milk 9 (R9) had the highest coliform count, while raw milk 7 (R7) had the least coliform counts. There are significant differences ($p > 0.5$) in the different chemical parameters monitored. pH was significantly different across the different milk samples analyzed. Values ranged from 6.30 - 7.30. Raw milk sample 9 (R9) had the highest pH range and Raw milk sample 1 (R1) had the least pH range. The mean pH was 6.62 close to a neutral pH. The result of the pH screening agreed with the result of [7] collected a total of 20 raw milk samples and reported the mean pH of all the milk samples to be 6.80. The colonial characteristics of all the bacteria isolate in this work was from macroscopic and microscopic colonies of each of the isolate on different selective and differential media. *Escherichiacoli* appeared as bright pink colonies on MacConkey agar with a moist glistening growth, on Eosin Methylene Blue agar, the colonies of *Escherichiacoli* appeared as a deep purple growth with a distinctive metallic green sheen. When viewed microscopically, it was viewed as a cluster of Gram-negative, rod shaped bacteria, and motile. *Salmonella* appeared as a colorless, transparent, smooth and raised colony on MacConkey agar, its inability to ferment lactose present in the media is responsible for this characteristic growth attributable of *Salmonella* on MacConkey agar. When viewed microscopically, *Salmonella* also appeared as a cluster of gram-negative, rod shaped motile bacteria. Hence the result is consistent with the previous findings of [8].To further characterize the bacteria isolates in this study, the bacteria isolates were subjected to series of biochemical tests. *Escherichiacoli* was observed to be Indole positive (+), Catalase positive (+), Oxidase negative (-), Citrate (-), Lactose positive (+), Glucose (+), Maltose (+), and Sucrose (+). *Salmonellaspp* reacted negatively to Indole test (-), it was Catalase positive (+), Oxidase negative (-), Citrate negative (-), Lactose negative (-), Glucose positive

(+), Maltose positive (+) and Sucrose negative (-).There was a striking resemblance between the biochemical data results reported in this study and the one obtained by [9,10] and with other works which reported the presence of *Staphylococcusspp*, *Klebsiellaspp*, *Pseudomonasspp*, *Proteusspp*, *Enterobacterspp*, *Escherichiacoli* and *Salmonellaspp* in raw milk samples [11].

Table 1. Mean pHand Mean microbial population from raw milk samples

Codes	pH	TBC (CFU/ml) x10^5	TCC (CFU/ml) x10^5
R1	6.30^d	2.60^a	1.79^b
R2	6.56^{cd}	2.23^c	1.92^a
R3	6.50^{cd}	2.37^{bc}	1.52^e
R4	6.53^{cd}	1.80^d	1.84^b
R5	6.33^{cd}	1.56^e	1.32^f
R6	6.73^c	2.42^{abc}	1.71^c
R7	6.57^{cd}	1.22^f	1.05^g
R8	7.00^b	2.30^{bc}	1.64^d
R9	7.30^a	2.20^c	1.93^a
R10	6.40^{cd}	2.50^{ab}	1.57^e
SEM	0.06	0.08	0.04

SEM = Standard error of mean. Superscript (a, b, c, d, e and f) means with similar
Super-script across the same row are not significant. R1 - |Raw milk sample 1, R2 - Raw milk sample 2, R3- Raw milk sample 3, R4 - Raw milk sample 4, R5 - Raw milk sample 5, R6 - Raw milk sample 6,R7 - Raw milk sample 7,R8 - Raw milk sample 8,R9- Raw milk sample 9,R10- Raw milk sample 10

Table 2. Bacteria isolates that were present and isolated from the raw milk samples

Samples	Isolates
R1	Klebsiella species, Escherichia coli, Staphylococcus aureus, Salmonella species
R2	Enterobacter species, Staphylococcus aureus, Escherichia coli, Salmonella species
R3	Pseudomonas aeruginosa, Klebsiella species, Proteus species, Escherichia coli
R4	Staphylococcus aureus, Salmonella species, Escherichia coli, Enterobacter species
R5	Enterobacter species, Shigella species, Salmonella species, Escherichia coli
R6	Klebsiella species, Salmonella species, Enterobacter species, Proteus species
R7	Escherichia coli, Staphylococcus aureus, Klebsiella species, Enterobacter species
R8	Enterobacter species, salmonella species, Klebsiella species, Shigella species
R9	Escherichia coli, Salmonella species, Klebsiella species, Staphylococcus aureus
R10	Pseudomonas species, Shigella species, Proteus species, Escherichia coli

R1 - |Raw milk sample 1, R2 - Raw milk sample 2, R3- Raw milk sample 3, R4 - Raw milk sample 4, R5 - Raw milk sample 5, R6 - Raw milk sample 6,R7 - Raw milk sample 7,R8 - Raw milk sample 8, R9- Raw milk sample 9,R10- Raw milk sample 10.

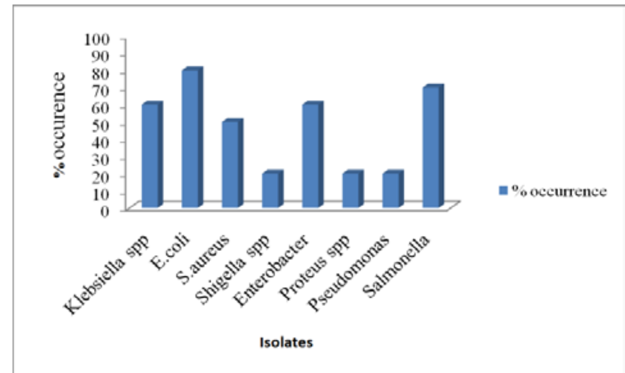

Figure 1. Percentage occurrence of the isolate in raw milk samples.

5. Discussion

The bacteria isolated from the raw milk were subjected to antibiotic sensitivity testing using disc diffusion method. Eight antibiotics: Augmenting, Erythromycin, Ofloxacin, Ceftazidime, Gentamicin, Cloxacillin, Ceftriaxone, and Cefuroxime were all tested against*Salmonella spp.* There was resistance noticedaugmentin, cefuroxime, erythromycin and Cloxacillin. However, the *Salmonella* isolates was susceptible to ceftriaxone and Ofloxacin, and was found to be intermediate to both gentamicin and ceftazidime.

Escherichiacoli were resistance to Augmentin, Erythromycin, Cloxacillin and Cefuroxime. The bacteria isolate was however susceptible to Ofloxacin, ceftazidime, and ceftriaxone, but intermediate to gentamicin.

An increase in antimicrobial resistance of *Salmonellaspp*, which may be linked to over-use of antibiotics in animals and humans is a major public health concern owing to the high mortality rates associated with *Salmonellosis.*

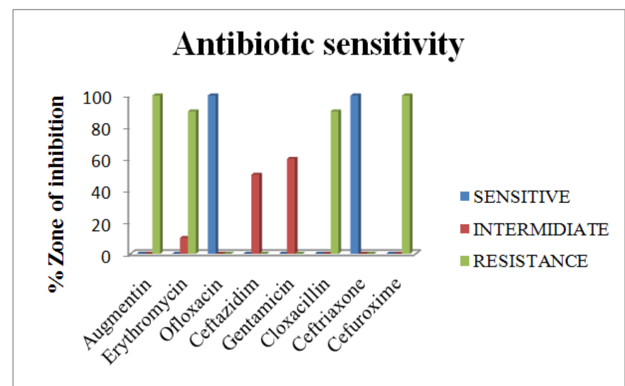

Figure 2. Antibiotic resistance patterns of all the isolatesagainst commonly used antibiotics

Table 3. Antibiotics sensitivity testing of the bacteria isolates from raw milk

Test antibiotic	Antibiotic (:g/disc	SA	SS	KP	ES	PS	EC	P.S
		Zone of inhibition in diameter (mm)						
Augmentin	30 µg	-R	-R	-R	-R	-R	-R	-R
Erythromycin	5µg	5 (R)	-R	- R	-R	5(R)	-R	12(I)
Ofloxacin	5µg	30(S)	27(S)	28 (S)	19(S)	29(S)	30(S)	20 (S)
Ceftazidime	30 µg	14(I)	13(I)	12(I)	-R	13(I)	-R	14(I)
Gentamicin	10µg	5(R)	12(I)	12(I)	12(I)	12(I)	12(I)	13(I)
Cloxacillin	5µg	(R)	-R	-R	4(R)	12(I)	-R	6(R)
Ceftriaxone	30 µg	28(S)	32(S)	17(S)	20(S)	20(S)	28(S)	26(S)
Cefuroxime	30 µg	-R	-R	R	R	-R	-R	-R

SA=*Staphylococcusaureus*; SS=*Salmonella spp;* KP = *Klebsiella spp;* ES= *Enterobacter spp*.EC= *Escherichia coli*; P.S= *Pseudomonas spp;* PS; =Proteus spp. S = (Sensitive> 14) R = (Resistant 0 -11)I = (Intermediate ranged from 12-14).

In this study, by optimizing the concentration of each probe in the assay, it was possible to achieve robust sensitivity in the multiplex assay and positively identified *E.coli* and *Salmonella* strains from raw milk samples using *E.coli* and *Salmonella* specific primers.

Nowadays, whole genome sequencing have been explored for a more efficient and more comprehensive approach for bacteria detection, the application of bioinformatics tools have made it really possible to determine the nucleotides sequences of most bacteria and also to deduce the protein structure of bacteria isolates as well as to determine the phylogeny of bacteria isolates through alignment by using the Basic Local Alignment Search Tools (BLAST). Although whole genome sequencing potential with bacteria characterization and surveillance is apparent, bacteria detection and characterization will likely continue to rely on a combination of culture and non-culture methods, the latter including real-time PCR. Multiplex real-time PCR targeting *invA*, *stx1* and *stx2* genes for detection of *E.coli* and *Salmonella* has become a routine test for preliminary screening in clinical laboratories as the Center for Disease Control and Prevention recommended. Although whole genome sequencing is not yet a routine testing, future prediction would include this technology as a means to track the mobility of pathogenic microbes. The multiplex real-time PCR developed in this study have been demonstrated to be reliable, efficient and sensitive assay and may serve as a useful tool and method for the detection of *E.coli* and *Salmonella* in epidemiological surveillance programs as well as in food analytical laboratories.

Molecular characterization of the *E.coli* isolates for virulent genes indicated the presence of *shigatoxin* 1 (s*tx* 1), *shigatoxin* 2 *(stx* 2) and *intimin* (*eaeA)* genes. *E.coli* can cause Hemolytic Uremic Syndrome (HUS) mainly by secretion of Shiga toxins encoded by the genes *stx* 1 and/or *stx* 2 and their variants. The toxin is effective against small blood vessels, such as found in the digestive tract, the kidney, and lungs, but not against large vessels such as the arteries or major veins. A specific target for the toxin appears to be the vascular endothelium of the glomerulus. This is the filtering structure that is a key to the function of the kidney. Destroying these structures leads to kidney failure and the development of the often deadly and frequently debilitating hemolytic uremic syndrome. Food poisoning with Shiga toxin often also has effects on the lungs and the nervous system. It has been proposed as reported that *stx* 2 has a lower receptor binding density which enables it to stay longer in circulation and to reach the kidneys more easily than *stx* 1. Shiga toxins can be released by antibiotics that may cause bacteria lysis and liberate the free Shiga toxins in the intestinal tract or enhance the expression of shiga toxin genes. The existence of *invA* gene mostly in all *Salmonella* serovars and its absence from the other bacteria rather than *Salmonella* proved it as a genetic marker for the identification of *Salmonella*. The *invA* gene usually codes for protein in the inner bacteria membrane that is responsible for invasion of intestinal cells of the host. The invA gene contains unique sequences specific to the genus *Salmonella* and has been proved as a specific PCR target with important diagnostic applications

Likewise, the presence of *stn* gene, a virulence gene with an enterotoxic activity could be a key factor in acute gastroenteritis and diarrhea and could contribute to *Salmonella* virulence.

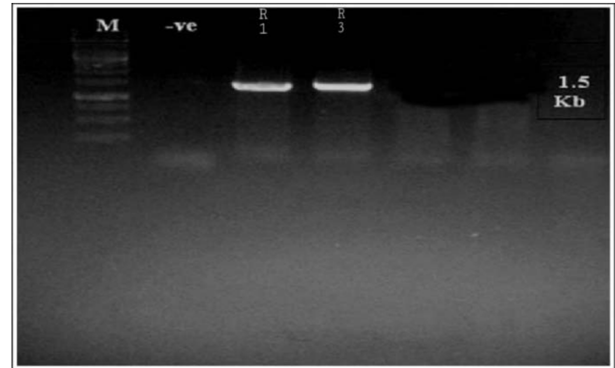

Plate 1. Gel electrophoresis purification of polymerase chain reaction amplicons of representatives bacteria isolates from raw milk samples (R1 and R3) using *E.coli* and *Salmonella* species type specific primers.

6. Conclusion

The microbial community within raw milk is complex. The dominant, and subdominant, microorganisms present in raw milk can have a variety of influences on the flavor, taste and texture of raw milk-derived products. A number of these microorganisms also have the potential to contribute to health through the production of antimicrobials or possessing other probiotic-associated traits. Through modern genomics-based analysis, it has been established that many of these microorganisms have become adapted to milk niches from various sources, including plant and gut environments, through genomic evolution and gene gain or loss. Despite the beneficial impact of many milk-associated microorganisms from flavor, technological or health-related perspective, it is clear that there can be significant risks associated with the consumption of raw milk and raw milk-derived products or, more specifically, of the pathogens that can be found therein. While many of these microorganisms gain entry to the milk from equipment or personnel, zoonotic pathogens can also be introduced into milk from unhealthy animals. Because of this risk, pasteurization or other treatments are employed to remove disease-causing microorganisms. In the food industry, the negative impact of removing lactic acid bacteria and other bacteria on subsequent food fermentations has been addressed for some time through their re-introduction in the form of starter and adjunct cultures. Similarly, once established definitively, it may be possible to restore the benefits associated with the consumption of raw milk and specific microorganisms therein, through the re-introduction of these microorganisms after processing. Thus, the microbial composition of raw milk is likely to continue to be the focus of much attention into the future.

The multiplex real-time PCR developed in this study has been demonstrated to be reliable, efficient, and sensitive assay and may serve as a useful tool and method for the detection and identification of *E.coli* and *Salmonella* in epidemiological surveillance programs as well as in food analytical laboratories. The multiplex real-time PCR assay was successfully tested in this study

for the detection of *E.coli and Salmonella* from raw milk samples. It is worth noting that on the other hand, in the development of PCR assay, great efforts were made toward getting high sensitivity and specificity by optimizing the amplification conditions and by minimizing the interferences among probes, primers, and target genes to reduce the false negative rate in detection. Also, in the use of the PCR assay, precautions are needed in interpretation of the positive results because some certain strains of free phages might harbor *stx* genes or even some *Shigella* strains may acquire *stx* genes.

Recommendation

The bacteriological study of raw milk has drawn attention to poor microbiological quality of milk and milk products consumed and purchased by people. In order to safeguard the public health various suggestions are recommended.

I Based on the results obtained from this study, it is recommended that strict hygienic measures should be applied during production, processing and distribution of milk and its products to avoid contamination by bacteria pathogens.

II It is recommended that both consumers and suppliers of dairy products to adequately store dairy products at suitable temperatures in order to control the levels of bacteria and to retard the rate of milk spoilage.

III It is recommended that various tests such as the phosphatase and methylene blue reduction tests should be routinely performed on each batch of milk processed by dairy plants as this will ensure safe milk for human consumption

IV It is recommended that people should desist from the consumption of raw milk and its varied products as such products harbors a lot of pathogenic bacteria that can leads to health related problems.

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