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Isolation of Endospore-Forming Bacteria from Milk Collected from Selected Cities of Pakistan

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ABSTRACT

Human nutritional needs are linked to milk production, processing, and consumption, as determined by the current study, which involved the microbiological analysis of milk samples from various Pakistani cities. **Objective:** To investigate the isolation of endospore-forming bacteria from milk collected from selected cities of Pakistan. **Methods:** Several milk samples were collected from Lahore, Gujranwala and Sheikhupura for microbiological evaluation and antibiotic susceptibility patterns. Isolation and characterization employing different morphological and biochemical tests was done which showed a heavy load of Bacillus endospore-forming bacterial species. **Results:** The results revealed that all the samples were contaminated with endospores-forming bacteria. Highly effective drugs in this study included Azithromycin, Rifampicin and Chloramphenicole resulted in 97%, 95.5% and 95.5% bacterial sensitivity respectively whereas Novobiocin was found to be 88.8% and Amoxilin showed 84.4% efficacy against bacterial Isolates. Metronidazole and Cefaxitin were found to be the least sensitive up to 44.4% and 17.7% respectively. Cefaxitin and Metronidazole were the most resistant medicines recorded at 83.3% and 66.6% respectively. In the case of metals, the bacterial sensitivity was found to be much lower i.e. at 1% Zn concentration, the highest recorded sensitivity was 51.1%. Bacterial Isolates were found to be highly resistant against Fe and Pb (1% metal concentration) and showed 0% and 2.2% sensitivity respectively. **Conclusions:** It was concluded that for the eradication of harmful endo-sporogenic bacteria in drinking milk, samples should be obtained employing efficient and safe standard antibacterial protocols for milk processing.

INTRODUCTION

High-quality milk and dairy products have been linked to human health and are regarded as nature's perfect food for mammals. Many active antimicrobial agents found in freshly milked milk temporarily stop the growth of contaminating microorganisms, but these agents are dependent on the temperature at which raw milk is stored. A certain amount of raw milk contamination is practically unavoidable, even in the best possible circumstances for routine milk collection. Microorganisms grow to high populations and cause milk rancidity, even if handled and stored suitably [1, 2]. The animal hide that are contaminated, the milking equipment and the animal feed, are just a few of possible sources of contamination of raw milk [3, 4]. Contaminants of milk include diverse types of microorganisms but some microorganisms produce spores in a non-vegetative form, resistant to heat, pressure and desiccation. Two main genera of endospore-forming bacteria are genus Bacillus and Clostridium. Bacillus are aerobic, facultative and anaerobic but the clostridium is strictly anaerobic. The bacteria form endospores are thick-walled structures that are released from vegetative cells up one cell lysis. Due to their dehydrated state, the endospore bacteria contain less moisture than the vegetative cell and are also more resistant to temperature, disinfectants, dehydration, and other environmental stresses as compared to the vegetative cell [5, 6].

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Commonly present members of the genus Bacillus in milk are the *B. cereus* group, *B. subtilis* group and *B. licheniformis*. Other aerobic endospore-forming bacteria isolated from milk included the genera e.g. *Paenibacillus, Oceanobaqcillus, Brevibacilolus, Lysinbacillus, Ureibacillus, Ornithinibacillus* and *Sporosarcina* [7, 8]. Clostridium species are anaerobic spore-formers that are widespread in dairy environments just like the aerobic endospore-forming bacteria. They are isolated from dairy farms from different sources such as the soil, silage, forage, hay and other raw milk and its products, silage is the major source of contamination of these clostridium species like *C. disporicum, C. tyrobutyricum* and *C. sporogenes.* Clostridium bacteria in milk convert the lactic acid into butyric acid and grow during fermentation and in cheese maturation. These are also the causative agent of the late blowing in butyric acid fermentation causing defects in chees during long fruiting time. The primary cause of late blowing is *C. tyrobutyricum* and another source of chees defects is *C. sporogenes.* Clostridium species are very important like *C. perferinges* particularly linked with food poisoning poisoning starts after heat treatment the spore-germinate and cell go through to the proliferation of vegetative cells. Some Clostridium spp. are pathogens e.g., *C. botulinum* of the food-borne botulism, Clostridium spp. baratii and butyricum are associated in a burst of food-borne, Type-E botulism. In the raw milk clostridium is low due to high RH value but during milk supply, botulinum neurotoxin is the most toxic substance that deliberately contaminates the milk. *C. botulinum* complexes and their neurotoxin types A and B are inactivated by pasteurization at high temperatures [9]. Milk processing units comprising milk loading cavities, pipelines, and filling machines also play a pivotal role in milk spoilage due to biofilms produced by various microbes [10]. Mostly the sporogenic bacteria create biofilms that are highly resistant to most of the contamination processes. Due to its resistance, it causes an additional obstacle for the dairy industry. Some strains of sporogenic bacteria can proliferate in low temperatures such as under the condition of refrigeration (4°C) foremost to the production of diverse lipolytic and proteolytic enzymes that will degrade the main constitutes of the milk, consequently affecting the sensory features [11]. Thorough investigations that have been conducted, involving the microbiological examination of multiple samples from different cities including Lahore, Gujranwala and Sheikhupura indicated that all the samples were contaminated with bacterial endospores. Numerous factors, such as management practices, sampling stage (farm vs. dairy bulk tank), farm hygiene, season, and milking practices, have been linked to the diversity and quantity of endospore-forming bacteria in raw milk. This information was not for strict legislation to save the measures from consumption of milk contaminated with bacterial endospores of a diverse nature. As it is known that bacterial endospores survive the boiling temperature of the water, these products prepared from such milk, even after boiling may pose health issues including food pasteurization to the consumers.

This study aims to examine the quality of raw milk by assessing the bacterial content resistant to antibiotics and heavy metals in three cities.

METHODS

Observational and prospective research was conducted at the Microbiological and biotechnology lab at the University of the Punjab, Lahore, Pakistan, from the period of September 2017 to 25 June 2018. Several milk samples were collected from Lahore, Gujranwala and Sheikhupura for microbiological evaluation and antibiotic susceptibility patterns. A total of 20 milk samples were collected in glass vials and transported to the laboratory immediately and maintained at 4°C, after heating at 80°C for 10 minutes 10-1, 10-2, and 10-3 dilutions were made. 100ul from 10^{-3} dilution of each sample was spread on the plates of nutrient agar and incubation was done at 37°C for 24 hours. 24 hours incubated plates containing only 2-3 bacterial colonies were Isolated. Four nutrient media (Nutrient agar media, Blood agar media, Tryptophan broth and Simmons citrate agar) were used for the separation of bacterial Isolates. After spreading of diluted sample (100ul) on the nutrient agar plates and incubation was done at 37°C for 24 hours, different types of bacterial colonies were obtained. The isolated colony was picked up by loop and inoculated in nutrient broth for biochemical testing. Also, slants were made of each bacterial strain for further work. Incubated Slants and nutrient broth bottles were kept at 37°C for 24 hours. After isolation, bacterial isolates were purified by using quadrant streaking. From the pure cultured plates, the unknown bacteria were identified by performing characterization colony morphological, and biochemical tests and different antibiotics and metals susceptibility patterns were measured. Bacteria identification succeeded through multiple biochemical analyses. The examinations analyzed motility movement of bacteria in combination with cellular observation using Gram staining and endospore staining and bacterial enzyme evaluations through conductance of oxidase and catalase testing. The tests for indole production and citrate utility were conducted to examine further metabolic traits. The disc diffusion method on nutrient agar medium detected the sensitivity of antibiotics to bacteria. A test included novobiocin (5 µg), metronidazole (5 µg), rifampicin (5 µg), azithromycin (15 µg), chloramphenicol (30 µg),

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streptomycin (10 µg), cefoxitin (30 µg), amoxicillin (25 µg) and ticarcillin-clavulanic acid (85 µg) used at their stated concentrations. The investigators placed their antibiotic discs gently onto prepared surfaces of nutrient agar where bacterial cultures rested. Plates were placed in an incubator at 37°C for 18-24 h to which the radial zones of inhibitions measured. Cu, Pb, Zn, Cr and Fe were used to test the susceptibility of bacterial Isolates i.e. sensitivity or resistance to these metals. For this purpose, two concentrations of each metal were formed one was 1% and the second was 0.5%. Filter paper discs were poured with 6ul solution of each metal in two concentrations. Sterilization was performed by autoclaving the disc placed in a vial containing plastic caps. Nutrient agar plates were formed and each bacterial Isolate was spread on the agar plates by a sterilized glass spreader under aseptic conditions. These metal discs were placed on the agar plates with sterilized forceps and incubated at 37°C for 24 hours. The CFU data corresponding to the cities of Sheikhupora, Lahore and Gujranwala were statistically analyzed using the Kruskal-Wallis Test as a non-parametric, alternative to one-way ANOVA. Results showed significance at $p<0.05$.

RESULTS

Bacterial growth was observed after 24 hours of incubation, two to three types of colonies appeared one type of colony is small just like puncta form, transparent, convex, shiny and smooth. The other type of colony was opaque white in medium size with a rough texture and flat and curled edges. The third type of colony on the nutrient agar was filamentous, large, flat, opaque grey and with rough texture. Further morphological details of bacterial colonies are given in table 1.

Table 1: Colony Morphology and Configuration Analysis of the Procured Bacterial Isolates from Different Milk Samples

Sr. No.	No. of obs.	Morphology of Bacterial Colonies					
		Name of Strain	Texture	Elevation	Margin	Appearance	Size
1	S1	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
2	S2	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
3	S3	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Small
4	S4	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
5	S5	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Small
6	L6	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
7	L7	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
8	L8	A	Rough	Raised	Curled	Opaque white	Moderate
		A	Smooth	Convex	Entire	Transparent	Punctiform
9	L9	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
10	L10	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
11	G11	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Small
		Fg	Rough	Flat	Filamentous	Gray	Large
12	G12	A	Rough	Raised	Curled	Opaque white	Large
		A	Rough	Convex	Entire	Transparent	Punctiform
13	G13	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
14	G14	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
15	G15	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
16	G16	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
		B	Rough	Flat	Filamentous	Gray	Large
17	G17	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
		B	Rough	Flat	Filamentous	Gray	Large
18	G18	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
19	G19	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
20	G20	A	Rough	Raised	Curled	Opaque white	Large
		A	Smooth	Convex	Entire	Transparent	Punctiform
		B	Rough	Flat	Filamentous	Gray	Large

All of the purified bacterial strains (n=45) were identified based on cultural characteristics, microscopic morphology with Gram's reaction and biochemical profiles. All the bacterial isolates were endospore-forming gram-positive bacilli, oxidase-positive and negative, indole-positive, citrate-positive and motile are presented in table 2.

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Table 2: Microscopic and Biochemical Characterization of Bacterial Isolates Procured from Different Milk Samples

Sr. No.	Samples from Different Cities	Strains Derived from Each Sample	Morphological Characteristics			Biochemical Tests			
			Gram Staining	Endospore Staining	Motility	Catalase	Oxidase	Citrate	Indole Production Test
1	S1	A	+	+	+	+	+	+	+
		a	-	+	+	+	+	+	+
2	S2	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
3	S3	A	+	+	+	-	+	+	+
4	S4	a	+	+	+	-	+	-	+
		A	+	+	+	+	+	+	-
		a	+	+	+	+	+	+	+
5	S5	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
6	L6	A	+	+	+	+	+	-	+
		a	+	+	+	-	+	-	+
7	L7	A	+	+	+	-	+	-	+
		a	+	+	+	+	+	-	-
8	L8	A	+	+	+	+	+	-	+
		a	+	+	+	-	+	-	-
9	L9	A	+	+	+	+	+	+	+
		B	+	+	+	+	+	-	+
10	L10	A	+	+	+	+	+	-	+
		a	+	+	+	+	-	-	+
11	G11	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
		Fg	+	+	+	+	+	+	+
12	G12	A	+	+	+	+	-	-	+
		a	+	+	+	+	+	+	+
13	G13	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	-
14	G14	A	+	+	+	+	+	+	+
		a	-	+	+	+	-	+	+
15	G15	A	+	+	+	+	-	+	+
		a	+	+	+	+	+	+	+
16	G16	A	+	+	+	+	+	-	+
		a	+	+	+	+	+	-	+
		B	+	+	+	-	+	-	-
17	G17	A	+	+	+	+	+	-	+
		a	+	+	+	+	+	+	+
		B	+	+	+	-	-	-	+
18	G18	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
		B	+	+	+	+	+	+	+
19	G19	A	+	+	+	+	+	+	+
		a	+	+	+	+	+	+	+
20	G20	A	-	+	+	+	+	+	+
		a	+	+	+	-	+	+	+
		B	+	+	+	+	+	+	+

The inhibition zone produced by antibiotics was measured in mm from the 24 hours post-incubated plates, a clear large zone of inhibition was produced when bacterial Isolates showed sensitivity against antibiotic discs, whereas resistant bacteria don't produce a zone of inhibition. The results were recorded and interpreted in(Table 3)

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Table 3: Against 45 Bacterial Isolates from Milk Samples Diameter of Zone of Inhibition in Mm of Different Antibiotics

Sr. No.	Milk Samples from Different Cities	Bacterial Isolates from Each Sample	Inhibition Cell Wall			Inhibition Protein Synthesis			Inhibition Nucleic Acid Synthesis		
			Inhibition FOX Zone in Mm	Inhibition TIM Zone in Mm	Inhibition AML Zone in Mm	Inhibition S Zone in Mm	Inhibition AZM Zone in Mm	Inhibition C Zone in Mm	Inhibition RD Zone in Mm	Inhibition NV Zone in Mm	Inhibition MTZ Zone in Mm
1	S1	A	7	18	16	22	28	30	20	16	7
		a	R	14	18	R	16	26	20	16	R
2	S2	A	R	R	R	20	26	22	8	R	R
		a	R	12	14	22	28	26	16	16	R
3	S3	A	8	14	14	8	24	28	20	18	14
		a	R	14	14	R	20	25	18	16	R
4	S4	A	R	14	18	R	20	30	18	20	R
		a	R	14	12	16	28	28	20	14	R
5	S5	A	R	14	18	16	14	28	18	22	R
		a	R	14	18	16	14	28	22	20	R
6	L6	A	R	R	R	8	10	R	R	18	R
		a	R	R	R	8	R	R	R	R	R
7	L7	A	R	8	9	19	14	18	11	12	R
		a	R	8	10	6.2	14	18	15	14	10
8	L8	A	9	8	9	7	13	17	11	15	6.2
		a	7	8	R	R	9	22	10	12	10
9	L9	A	R	9	10	R	15	22	12	14	7
		B	7	7	12	R	15	19	9	8	8
10	L10	A	R	9	10	21	15	18	14	13	7
		a	R	8	9	R	14	15	11	13	R
11	G11	A	R	13	11	8	17	25	14	16	7
		a	8	9	9	15	14	21	11	13	9
		Fg	R	8	9	R	19	21	13	12	R
12	G12	A	R	9	10	19	17	16	12	10	R
		a	R	8	9	26	14	19	13	13	6.2
13	S13	A	R	6.2	9	R	13	14	11	10	R
		a	6.2	9	10	7	18	21	16	14	7
14	S14	A	R	R	R	16	24	11	8	11	R
		a	R	8	9	R	17	20	16	9	R
15	S15	A	R	R	9	R	17	22	16	10	R
		a	8	10	9	18	14	21	16	16	9
16	L16	A	R	7	9	17	16	21	13	16	R
		a	R	7	10	16	11	20	16	11	7
		B	R	R	R	12	20	25	9	R	9
17	L17	A	R	R	9	17	10	24	11	16	10
		a	R	8	9	R	15	22	12	12	6.2
		B	R	12	13	21	16	25	22	18	6.2
18	L18	A	R	12	14	7	18	22	15	14	6.2
		a	R	R	10	22	24	22	21	15	R
		B	R	6.2	7	18	18	22	15	16	R
19	L19	A	R	R	7	R	18	18	10	14	R
		a	R	R	8	18	35	34	14	R	R
20	L20	A	R	R	7	R	14	11	16	11	R
		a	R	8	9	R	13	16	10	10	R
		B	R	R	8	R	10	14	8	12	R

R=Resistance, S=Sensitivity, FOX=Cefaxitin, AML=Amoxillin, TIM=Clavulanic acid, AZM=Azithromycin, C=Chloramphenicole, S=Streptomycin, NV=Novobiocin, MTZ=Metronidazole, RD=Refampicin

Antibiotic susceptibility patterns of the bacterial Isolates (n=45) derived from milk samples (n=20) of Lahore, Gujranwala and Sheikhupura against different antibiotics. The results were recorded and interpreted in Figure 1.

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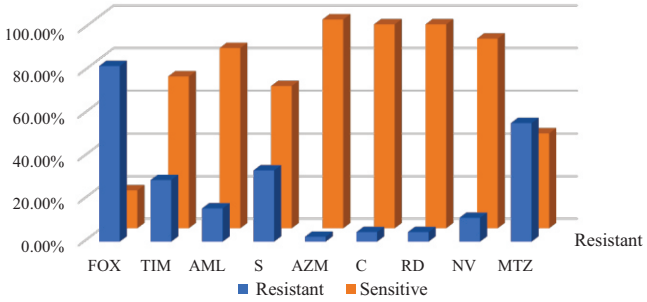

Figure 1: Antibiotic Susceptibility Patterns

R=Resistance, S=Sensitivity, FOX=Cefaxitin, AML=Amoxicillin, TIM=Clavulanic acid, AZM=Azithromycin, C=Chloramphenicole, S=Streptomycin, NV=Novobiocin, MTZ=Metronidazole, RD=Refampicin

The antibiotic susceptibility analysis of bacteria strains received interpretation in figure 2.

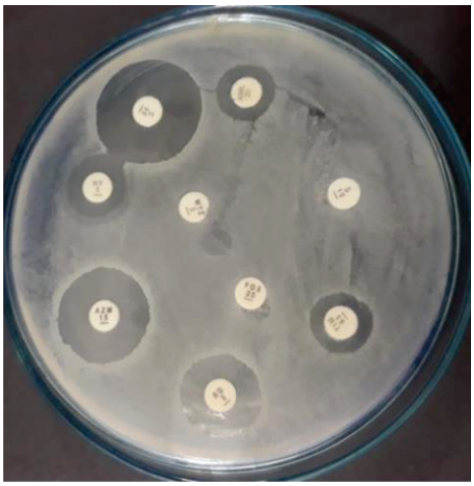

Figure 2: Bacterial Strain analysis

The zone of inhibition produced by the heavy metals Cu, Pb, Zn, Cr and Fe was measured in mm from the 24-hour incubated plates, the sensitive bacterial Isolates showed a large zone of inhibition.Resistant bacteria did not produce a zone of inhibition.The results were recorded and interpreted in table 4.

Table 4: Diameter of Zone of Inhibition in Mm of Different Metals Against 45 Bacterial Isolates from Milk Samples

Sr. No.	Samples from different cities	Strains derived from each sample	Copper (Cu)		Ferric (Fe)		Chromium (Cr)		Zink (Zn)		Lead (Pb)	
			Zone of inhibition at Cu1%	Zone of inhibition at Cu0.5%	Zone of inhibition at Fe1%	Zone of inhibition at Fe0.5%	Zone of inhibition at Cr1%	Zone of inhibition at Cr0.5%	Zone of inhibition at Zn1%	Zone of inhibition at Zn0.5%	Zone of inhibition at Pb1%	Zone of inhibition at Pb0.5%
1	S1	A	20	R	R	R	26	22	30	30	R	R
		a	25	20	R	R	28	22	32	26	R	R
2	S2	A	R	R	R	R	40	36	38	36	R	R
		a	28	20	R	R	R	R	22	18	R	R
3	S3	A	R	R	R	R	R	R	28	26	R	R
		a	22	18	R	R	16	18	18	16	R	R
4	S4	A	R	R	R	R	R	R	18	R	R	R
		a	24	R	R	R	R	R	26	R	R	R
5	S5	A	24	16	R	R	R	R	R	R	R	R
		a	22	18	R	R	R	R	24	22	R	R
6	L6	A	R	R	R	R	46	40	48	46	38	40
		a	R	R	R	R	R	R	R	R	R	R
7	L7	A	24	R	R	R	R	R	28	26	R	R
		a	R	R	R	R	28	22	20	18	R	R
8	L8	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
9	L9	A	R	R	R	R	R	R	28	26	R	R
		B	R	R	R	R	36	34	R	R	R	R
10	L10	A	R	R	R	R	28	22	30	26	R	R
		a	R	R	R	R	36	28	R	R	R	R
11	G11	A	R	R	R	R	28	26	R	R	R	R
		a	26	14	R	R	R	R	R	R	R	R
		Fg	26	20	R	R	28	24	30	26	R	R
12	G12	A	28	R	R	R	20	18	28	R	R	R
		a	R	R	R	R	28	26	R	R	R	R
13	G13	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	18	16	R	R
14	G14	A	R	R	R	R	R	R	R	R	R	R
		a	24	R	R	R	R	R	R	R	R	R

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15	G15	A	R	R	R	R	26	30	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
16	G16	A	R	R	R	R	30	30	28	26	R	R
		a	R	R	R	R	R	R	18	16	R	R
		B	R	R	R	R	20	R	R	24	R	R
17	G17	A	R	R	R	R	28	26	R	R	R	R
		a	R	R	R	R	32	28	R	R	R	R
		B	R	R	R	R	R	R	18	16	R	R
18	G18	A	R	R	R	R	R	R	28	16	R	R
		a	R	R	R	R	R	R	R	24	R	R
		B	R	R	R	R	R	R	R	R	R	R
19	G19	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
20	G20	A	R	R	R	R	R	R	R	R	R	R
		a	R	R	R	R	R	R	R	R	R	R
		B	R	R	R	R	28	26	26	22	R	R

R=Resistance, S=Sensitivity, Cu1%, Cu0.5%, Fe1%, Fe0.5%, Cr1%, Cr0.5%, Zn1%, Zn0.5%, Pb1%, Pb0.5%

Heavy metals susceptibility pattern of the bacterial strains (n=45) isolated from milk samples (n=20) of Lahore, Gujranwala and Sheikhupura in contradiction of diverse Metals. R=Resistance, S= Sensitivity, Cu1%, Cu0.5%, Fe1%, Fe0.5%, Cr1%, Cr0.5%, Zn1%, Zn0.5%, Pb1%, Pb0.5%. The results were recorded and interpreted in Figure 3.

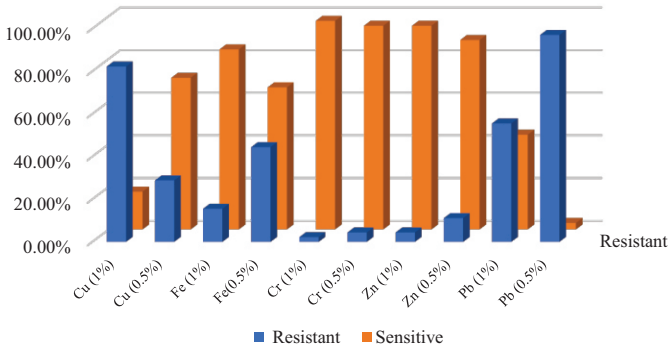

Figure 3: Heavy Metals Susceptibility Pattern of the Bacterial Strains (n=45) Isolated from Milk Samples (n=20)

Heavy Metals Susceptibility analysis of the Bacterial Strains was recorded in Figure 4.

Figure 4: Heavy Metal Susceptibility Analysis of the Bacterial Strains

Following the significance Post Hoc Test was applied, test results are shown in which shows that milk samples from Gujranwala and Sheikhupora were significantly different from each other while the comparison of Sheikhupora, Lahore and Gujranwala-Lahore showed to be non-significant-respectively at $p<0.05$. The results were recorded and interpreted in Figure 5.

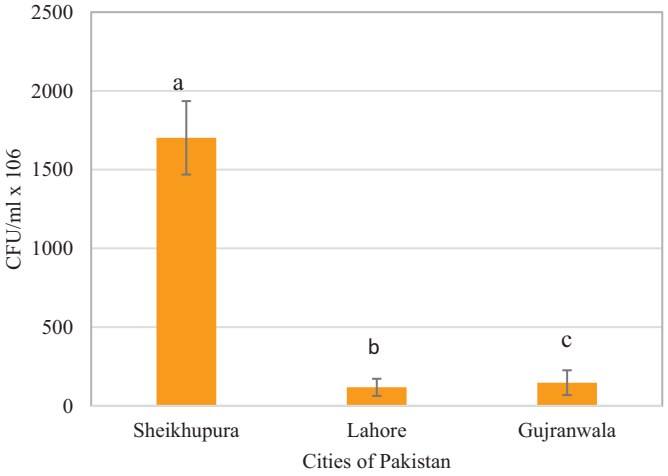

Figure 5: CFU\ml median values of Gujranwala, Lahore and Sheikhupora Letters Showing Significance at $p<0.05$

CFU\ml count for coliform in fresh milk samples collected from Gujranwala, Lahore and Sheikhupora. The results are shown in Table 5.

Table 5: CFU\ml Count for Coliform in Raw Milk Samples

Sr. No	Samples from Different Cities	CFU\ml
1	S1	$2.11x10^9$
2	S2	$2.41x10^9$
3	S3	$1.37x10^9$

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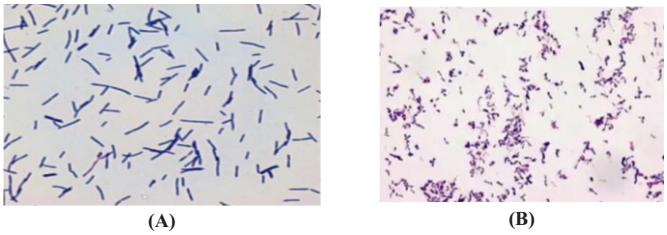
4	S4	$1.27x10^{9}$
5	S5	$1.35x10^{9}$
6	L6	$2.3x10^{8}$
7	L7	$2.7x10^{8}$
8	L8	$2.98x10^{7}$
9	L9	$2.95x10^{7}$
10	L10	$2.88x10^{7}$
11	G11	$7.2x10^{6}$
12	G12	$6.0x10^{6}$
13	G13	$3.1x10^{7}$
14	G14	$4.6x10^{6}$
15	G15	$3.9x10^{6}$
16	G16	$7.4x10^{8}$
17	G17	$3.3x10^{8}$
18	G18	$3.1x10^{6}$
19	G19	$3.4x10^{8}$
20	G20	$4.8x10^{6}$

To check the number of endospore-forming bacteria in the milk samples endospore staining was done at forty-eight hours' culture broths employing the Schaeffer Fulton method. The slide was observed under a bright field light microscope at 40x objective lens magnification [12]. The endospore-forming bacteria were motile when microscopically checked, at twenty-four hours of incubation. Gram's reaction, shape, size and arrangement of bacteria and released spores were also observed microscopically in Figure 6.



(A) (B)

Figure 6: Gram-positive Bacteria Are in A and Endospore-Forming Bacteria in B

DISCUSSION

In this study, twenty milk samples were collected from three different cities of Pakistan Lahore, Gujranwala and Sheikhupura from which forty-five bacterial isolates were obtained. Following isolation characterization of these Isolated bacteria was done based on their different morphological, and biochemical profiles and antibiotic and metal susceptibilities and their results were recorded. Samples were collected in glass vials under aseptic conditions and transported to the lab by maintaining temperature of 4°C. Heat treatment (80°C for 10 minutes) was applied to kill the non-sporogenic bacteria. Some samples were collected from dairy farms directly whereas other samples were collected from the shops. Interestingly the samples from dairy farms contained more bacteria than the samples from the shops. This low bacterial count in different samples obtained from milk shops was probably due to the addition of chemicals that on one hand maintain the milk for a long shelf-life. On the other hand, highly dangerous to human health. But in dairy farms, these chemicals were not added so a high number of bacteria were observed. When the heat treatment was applied, the majority of no spore-former bacteria died but as the sporogenic bacteria survived formed vegetative cells showed resistance to this high temperature, these spore-forming bacteria caused the milk spoilage even after the pasteurization process [14]. It has been inferred from the research that milk samples collected from Sheikhupura contain a higher number of spore-forming bacteria than Lahore. Morphology of the different isolated bacteria was presented which are separated from different samples. Three types of colonies were observed in Lahore and Sheikhupura's milk samples while two types of colonies were present in Gujranwala's samples. The colony morphology was noted by observing the texture, elevation, margin and size of the bacterial colonies. In Table. 2 the biochemical test results of the indole test, citrate utilization test, catalase test and oxidase test were described [15, 16]. In the oxidase test different bacteria have variable abilities to produce cytochrome oxidase enzyme by addition of the following test reagent p-amino dimethyl, aniline oxalate, the development of pink then dark purple coloration on the surface of colonies showed production of cytochrome oxidase whereas no colour change on colonies specified the absence of oxidase activity [17]. Table 2 shows the results of the catalase test that converts the hydrogen peroxide into oxygen and water. In the positive test bubbles were formed which indicated the presence of Bacillus spp. and negative results may show the presence of Clostridium spp according to [18]. An indole synthesis assay was done to determine the ability of the bacterial isolates to convert the amino acid tryptophan into indole, which revealed the presence of the tryptophanase enzyme. For this purpose, Kovac's reagent was added to 4 ml of tryptophan broth culture and a ring was observed for a positive indole test [18]. To check the ability of bacterial strains to utilize sodium citrate as a sole source of carbon and inorganic ammonium salts as a nitrogen source, a citrate utilization test was performed. Each bacterial Isolate was inoculated on Simon's citrate agar and incubated at 37°C for 18-24 hours. The appearance of a blue colour indicated a positive citrate test [12]. The bacterial susceptibility to various antibiotics was determined by the Kirby-Bauer disc diffusion technique (9) using commercially available antibiotic discs on the nutrient agar plates. Variable zone of inhibition produced by bacterial Isolates susceptible to different antibiotics: Novobiocin (5 µg), Metronidazole (5 µg), Rifampicin (5 µg), Azithromycin (15 µg), Chloramphenicol (30 µg),

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Streptomycin (10 µg), Cefoxitin (30 µg), Amoxil (25 µg), Ticarcillin Clavulanic acid (85 µg) resulted in that were measured, Clear large zones of inhibition were produced when bacterial Isolates were found to be sensitive against antibiotic discs, whereas resistant bacteria didn't produce a zone of inhibition. Highly effective drugs included Azithromycin, Rifampicin and Chloramphenicole showed 97%, 95.5% and 95.5% sensitive results respectively, Novobiocin was found to be 88.8% and Amoxilin showed 84.4% sensitivity, results in (Table 3). Metronidazole and Cefaxitin were found to be the least sensitive 44.4% with and 17.7% sensitivity. Further, Cu, Pb, Zn, Cr and Fe were used to test the susceptibility of bacterial Isolates i.e. sensitivity or resistance to these metals. Zone of inhibition produced by the heavy metals Cu, Pb, Zn, Cr and Fe were measured in mm scale. The sensitive bacterial Isolates showed a large zone of inhibition whereas, resistant bacteria didn't produce a zone of inhibition. The results were recorded and interpreted in table 4. But in the case of metals the bacterial sensitivity was found to be much lower Zn (1%) had the highest recorded that was 51.1% while others had low sensitivity i.e. <50%. Fe and Pb were found to be highly resistant and showed 0% and 2.2% sensitivity respectively as shown in Table 4. The resistance of metals was probably due to the presence of heavy metals in the water and the silage of animals which were drunk. The research infers that the population of the endospore-forming bacteria did not decrease till the end of milk processing and even after heat treatment (10 minutes at 80°C). Therefore, the endospore-bacterial population causes additional contamination during milk processing [19, 20]. Further studies on the identification of endospore-forming bacterial Isolates may unveil the human health risk associated with the consumption of such contaminated milk and milk products. It is suggested that such milk be consumed after endospore processing recommended for such products by international standards.

CONCLUSIONS

It was concluded that the population of the endospore-forming bacteria did not decrease till the end of milk processing and even after heat treatment (10 minutes at 80°C). As it is known that bacterial endospores survive the boiling temperature of the water, these products prepared from such milk, even after boiling may pose health issues to the consumers. Therefore, the endospore-bacterial population causes additional contamination during milk processing. Further studies on the identification of endospore-forming bacterial Isolates may unveil the human health risks associated with the consumption of such contaminated milk and milk products. It is suggested that such milk be consumed after endospore processing is recommended for such products by international standards.

Authors Contribution

Conceptualization: JIQ
Methodology: SJ[1], SJ[2], AH[2]
Formal analysis: MAR, SA
Writing review and editing: AH[1]

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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