Detection and Antimicrobial Profile of *Bacillus cereus* in Milk from Lactating Farm Animals

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Abstract: The occurrence of *Bacillus cereus* in milk from does and ewes was not well documented in Ethiopia in spite of the presence of some reports on the prevalence of the bacteria in the milk from bovine. Similarly, the antimicrobial susceptibility profile of isolates from milk was not well documented. To fill this gap, a cross-sectional study was carried out from November 2015 to March 2016 to estimate the prevalence of *B. cereus* in milk of farm animals and to assess the *in-vitro* antimicrobial susceptibility profile of the isolates using commercially available antimicrobial discs. For this purpose, milk samples were collected from 146 lactating animals (91 cows, 24 ewes, 31 does) and examined using standard microbiological procedures. The result of the study revealed that overall prevalence of *B. cereus* in milk samples from the dairy animals was 29.5%. The prevalence for each species were 30.8%, 29% and 25% in the milk of cows, does and ewes, respectively. The study further indicated the significant association ($p < 0.05$) of *B. cereus* with cow's milk sample which was positive for California Mastitis Test (CMT). Based on *B. cereus* count, it was found that 48.8% of the milk samples harbored bacterial load above the tolerable limit (>10^5CFU/ml) for human consumption. The disc diffusion test further indicated that *B. cereus* isolates showed high resistance to specific antimicrobials such as penicillin (100%), ampicillin (100%), cefoxitin (94.7%) and amoxicillin (89.5%), while 3 (15.8%) of the tested isolates (n=19) exhibited multidrug resistance to five antimicrobials (kanamycin, ampicillin, penicillin, amoxicillin and cefoxitin). From the study it was concluded that higher proportion of milk samples from lactating cows, sheep and goats had high *B. cereus* load. The resistance of *B. cereus* to a wide range of antibacterial agents suggested the need to consider effective measures on environmental hygiene and monitor the use of antimicrobials in the treatments of mastitis in dairy animals so that animal health and welfare will be further improved and public health hazards minimized.

Keywords: *Antimicrobial susceptibility, Bacillus cereus, Bacterial load, Subclinical mastitis*

Introduction

In Ethiopia, milk production is crucial, because milk and milk products paly important role through feeding. In addition, milk is a source of cash, which helps families to get other foodstuffs and social services. Especially, in times of crop failure, livestock and their products are a "near-cash" capital item. It was estimated that the total cow milk production for the rural sedentary areas of the country during the period from 2016 to 2017 was about 3.32 billion liters, which makes milk constituting a significant proportion of the value of all livestock food products in Ethiopia (CSA, 2018).

In animals, *Bacillus cereus* has been known to cause an acute hemorrhagic mastitis, inadequate milk production, and loss of weight in cattle. *Bacillus cereus* is a spore former and may remain dormant in the mammary gland for long periods, unaffected by the presence of antibiotics (Constable *et al.*, 2017). In sheep, *B. cereus* was identified as a bacterium causing sporadic abortion and also confirmed as an abortifacient experimentally (Misty *et al.*, 2017). Moreover, *B. cereus* can cause mild to severe (gangrenous) mastitis in goats (David *et al.*, 2002).

Bacillus cereus is one of the food-borne pathogens that is often present in milk and dairy products and considered as an emerging opportunistic pathogen of humans (Larsen and Jorgensen, 1997; Samapundo *et al.*, 2011). Especially, the accelerated emergence of antibiotic resistance among the *Bacillus species* is a threat to the management of infectious diseases (Farrar and Reboli, 2006). Treatments with broad spectrum antibiotics are often effective. However, drugs to be used should be determined by culture and sensitivity (Larsen and Jorgensen, 1997). This is particularly important where most of *B. cereus* isolates are reported to show multi drug resistance patterns (Abraha *et al.*, 2017).

In Ethiopia, some researchers (Alemneh, 2012; Shibabaw, 2014; Yared, 2014) conducted research and reported the prevalence of *B. cereus* in milk of dairy cattle managed under smallholder dairy farms. However, there is no report on *B. cereus* from milk of lactating ewes and does. Abraha *et al.* (2017) reported the occurrence of *B. cereus* in 40% of raw cow milk sold at different market places, in the eastern part of the country. Majority of these samples had a bacterial load above the recommended level for human consumption.

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However, the studies didn't report the possible sources for milk contamination, especially the role of animal's udder as a source of milk contamination with *B. cereus* is not specified. Thus, it was considered that there is limited information on the prevalence of *B. cereus* in milk of lactating animals in the country in general and in the study districts in particular. Moreover, to the best of the researchers' knowledge, there is limited information on the antimicrobial susceptibility profile of *B. cereus* isolated from milk samples of animals in the country. Thus, the aim of the present study was: to estimate the occurrence of *B. cereus* in lactating cows, ewes and does in Haramaya and nearby towns; to assess the association of *B. cereus* occurrence with subclinical mastitis; and to determine the antimicrobial susceptibility patterns of the isolates.

Materials and Methods

Description of the Study Area

The study was conducted at Haramaya district (Haramaya town, Aweday town and Haramaya University farms). Haramaya district is located at an altitude of about 2000 m above sea level. The area receives 790 mm average annual rainfall with a bimodal pattern of short rain season ranging from February to May and long rain season ranging from June to September. The rainfall reaches peak in mid-August and April. The mean maximum and minimum temperatures are 24 ^{O}C and 9 ^{O}C, respectively (HADB, 2014).

Study Animals

The animals used for sampling were apparently healthy lactating dairy cows, ewes and does. Animals that were treated with antimicrobials in the past 4 weeks prior to sample collection were not included in the study. In the small household farms, all cows were local breeds kept under traditional barns, which are characterized by having wood side walls, muddy floor and corrugated iron roof. The houses were often contaminated with dung and urine and there was no separate milking parlor. The cows in Haramaya University were all crossbred (Holstein Friesian x Zebu) and managed under intensive husbandry practice in stall barn made of concrete floor. In both farming systems, animals were hand milked twice a day (in the morning and afternoon) by the farm attendants and/animal owners and the usual feed was hay and mixture of cereal grains byproducts. However, in Haramaya University, lactating cows were milked under separate milking parlor and were supplied with silage. The ewes and does were local breeds sheltered under loose housing system and spending much of the day time grazing and browsing in the farms/household vicinities. They were not regularly milked for commercial purpose and milking was done occasionally.

Study Design and Sampling Techniques

A cross-sectional study was conducted from November 2015 to March 2016 to determine the occurrence of *B. cereus* in milk samples collected from the study animals. The farms were selected purposively based on the willingness of owners. Due to a limited number of target animals all lactating animals in the selected herds/flocks were sampled. Accordingly, all lactating cows (n=32) and does (n=31) of the University were included in the study. The rest 59 lactating cows and 24 ewes were found from Haramaya and Aweday towns. Accordingly, 146 animals (91 cows, 24 ewes, 31 does) were included in the study. The sampling units were lactating animals.

Milk Sample Collection for Microbiological Analysis

Pooled milk sample from each animal was collected into a sterile bottle directly from the teat of animals (cow, ewe and doe). Before sampling, a 70% ethanol-soaked cotton ball was used to clean the teats. Udders and teats with paste of dirty materials were washed using tap water before the application of antiseptic. At each sampling time, hands of the researchers were disinfected using 70% ethanol and samples were then taken after proper evaporation of the alcohol from the surface of teats and hands. After disposing the first two to three streams of milk, the representative milk samples (about 10 ml) were collected from teats into a sterile universal bottle. The universal bottles were clearly labeled and transported in a box containing an icepack to Haramaya University, College of Veterinary Medicine, Veterinary Microbiology Laboratory. Samples were subjected to bacteriologic culture up on arrival.

Laboratory Analysis of Milk Samples

California mastitis test (CMT): Milk samples taken from cow's teat were screened for subclinical mastitis using CMT test according to Quinn *et al.* (1999) to assess the relationship between subclinical mastitis and *B. cereus* prevalence. Then CMT positive samples from the same animal were mixed and subjected to bacterial cultures. Likewise, all CMT negative milk samples from the same animal were pooled and cultured for the isolation of *B. cereus*.

***Bacillus cereus* isolation and identification:** *Bacillus cereus* selective agar base (CM0167, Oxoid) was the media used to isolate *B. cereus* from the milk samples. The medium was prepared based on egg yolk and mannitol supplementation. The medium was made selective by the addition of Polymyxin B Supplement (SR99) in such a way that results in a final concentration of 100 IU of polymyxin B per ml of the medium. Milk sample in each bottle was thoroughly mixed using a vortex mixer and a loopful was streaked onto the solidified media according to Quinn *et al.* (1999). After 18-24 hours of incubation at 37°C in aerobic conditions, the growth of *B. cereus* was determined based on the colonial morphology, precipitation of hydrolyzed lecithin around colonies and the failure of *B. cereus* to utilize mannitol sugar

according to Mossel *et al.* (1967) and Fricker *et al.* (2008). Generally, colonies appearing as crenate, about 5mm in diameter and having a distinctive turquoise to peacock blue color surrounded by egg yolk precipitation of the same color were presumptively considered as *B. cereus*. The isolates were confirmed by microscopic examination of Gram's reagent-stained smear and biochemical tests. On Gram's staining, *B. cereus* appear as gram-positive (purple colored), rod shaped cells with short to long chains and lightly stained non-swollen spore. The growth of other organisms (contaminants) was ruled out by their colonial appearance and bacterial characteristics on Gram's staining. *Bacillus cereus* isolates were identified using different biochemical tests such as, catalase test, oxidase test and oxidation-fermentation test as described by Quinn *et al.* (1999).

Enumeration of *Bacillus cereus*: Serial dilutions from 10^{-1} to 10^{-6} were prepared and 0.1 ml of the contents were surface inoculated on *B. cereus* selective agar base (CM0167, Oxoid). The experiment was done in duplicate. Plates were incubated for 18-72 hrs at 37°C and observe for colonies with characteristics of *B. cereus*. The number of CFU/ml of samples was calculated using the standard equation of Nicoletta and Royston (2008) as described below:

$$N = \frac{\sum C}{(n_1 + 0.1x\, n_2) \times d}$$

Where: N = total viable colony counts; $\sum C$ = sum of colonies counted from all plates; n_1 = number of plates counted at first dilution; n_2 =number of plates at second dilution; C= number of colonies counted; d = dilution factor from which the first counts obtained (least counted dilution).

The bacterial count was interpreted as those having above and below recommended level as described by International Dairy Federation (IDF, 2016). Accordingly, those having $>10^5$CFU/ml were recorded as above legal limit.

Antimicrobial Susceptibility Test of Isolates

Antimicrobial susceptibility test was performed following the standard agar disk diffusion method according to Quinn *et al.* (1999) and CLSI (2012) using ten commercially available antimicrobial disks described in Table 1. Due to the limited antimicrobial discs, only 19 *B. cereus* isolates were randomly selected from different samples. Briefly, each isolated bacterial colony from pure fresh culture was transferred into a test tube having 5 ml Tryptone Soya Broth (TSB) (Oxoid, England) and incubated at 37°C for 6 hrs. The test broth was adjusted to McFarland 0.5 turbidity to obtain desired bacterial population. Mueller-Hinton agar (Bacton Dickinson and Company, Cockeysville USA) plates were prepared according to the manufacturer guidelines. A sterile cotton swab was immersed into the inoculum suspension and rotated against the side of the tube above the level of the broth to remove excess fluid and then streaked in three directions uniformly on the surface of Mueller-Hinton agar plates. After the plates get dried, antibiotic disks were placed on the inoculated plates using sterile forceps. The antibiotic disks were gently pressed onto the agar to ensure firm contact with the agar surface, and incubated at 35°C for 18-24 hrs. Following this, the diameter of inhibition zone formed around each disk was measured using a transparent ruler after placing of the Petri dishes on a black background with reflected light. As there was no specific manual for interpretation of *B. cereus* zone of inhibition, the result was classified as susceptible, moderately susceptible and resistant according to the standardized table supplied by Quinn *et al.* (1999) and CLSI (2012) for *Staphylococcus* and related organisms (Table 1).

Table 1. Guideline for antimicrobial discs used for susceptibility test of *B. cereus* with their respective concentrations according to CLSI (2012)

Antimicrobial agents	Symbols	Disc potency (µg)	Zone diameter, nearest whole mm		
			Resistance (≤)	Moderate	Susceptible (≥)
Amoxicillin	AML	20	13	14-17	18
Ampicillin	AMP	10	28	-	29
Cefoxitin	FOX	30	21	-	22
Erythromycin	E	15	13	14-22	23
Kanamycin	K	30	13	14-17	18
Tetracycline	TE	30	14	15-18	19
Vancomycin	VA	30	9	10-11	12
Doxycycline	D	30	12	13-15	16
Enrofloxacin	ENR	5	15	16-20	21
Penicillin	P	10 unit	28	-	29

Data Management and Analysis

The total prevalence was calculated by dividing the number of milk samples positive for *B. cereus* to the total number of milk sample tested. Risk factor analysis was done using SPSS Version 20 software program. Chi-square test was performed to assess the association of different variables, such as species of animals, study location and CMT result with the occurrence and count of *B. cereus*. A p-value of less than 0.05 ($p<0.05$) was considered as statistically significant association.

Results

Prevalence of Bacillus cereus in Milk Samples

The overall prevalence of *B. cereus* in milk of lactating animals (cows, ewes and does) was 29.5% (95% CI=22.2-37.6) (Table 2). Analyses were done to look into the association between the occurrence of *B. cereus* with species of animals and districts. The statistical analysis showed that, species and site were not associated ($p > 0.05$) with the occurrence of *B. cereus*.

Table 2. Prevalence of *Bacillus cereus* and its association with different factors.

Risk factor	Category	Number of tested samples	Number of *Bacillus cereus*:		X^2	*p*-value
			Positive (%)	Negative		
Species	Bovine	91	28 (30.8)	63	0.3076	0.857
	Ovine	24	6 (25)	18		
	Caprine	31	9 (29)	22		
	Total	146	43 (29.5)	103		
Study site*	Aweday	30	10 (33.3)	20	0.2447	0.970
	Haramaya	29	8 (27.6)	21		
	HU	32	10 (31.3)	22		
	Total	91	28 (30.8)	63		
Breed	Cross bred	32	10 (31.3)	22	0.005	0.942
	Local	59	18 (30.5)	41		

** Means only cows are considered for comparison; HU= Haramaya University.*

Association of CMT test Result with the Occurrence of Bacillus cereus

The study revealed that the prevalence of subclinical mastitis in cow was 45.1% (41/91). The CMT test result was significantly associated ($p < 0.05$) with *B. cereus* isolation (Table 3).

Table 3. *Bacillus cereus* prevalence and association with CMT reaction

Bacillus cereus status	CMT status			X^2 (*p-value*)
	Positive (%)	Negative (%)	Total	
Positive	18 (64.3)	10 (35.7)	28	6.042 (0.014)
Negative	23 (36.5)	40 (63.5)	63	
Total	41 (45.1)	50 (54.9)	91	

In-Vitro Antimicrobial Susceptibility of Isolates

The result showed that, *B. cereus* isolates were variably resistant to the tested antimicrobials (Figure 1). Accordingly, all isolates showed 100% sensitivity to enrofloxacin and majority of isolates were sensitive to doxycycline (89.5%), vancomycin (78.9%) and erythromycin (73.7%). Conversely, *B. cereus* isolates showed resistance to penicillin (100%), ampicillin (100%), cefoxitin (94.7%) and amoxicillin (89.5%).

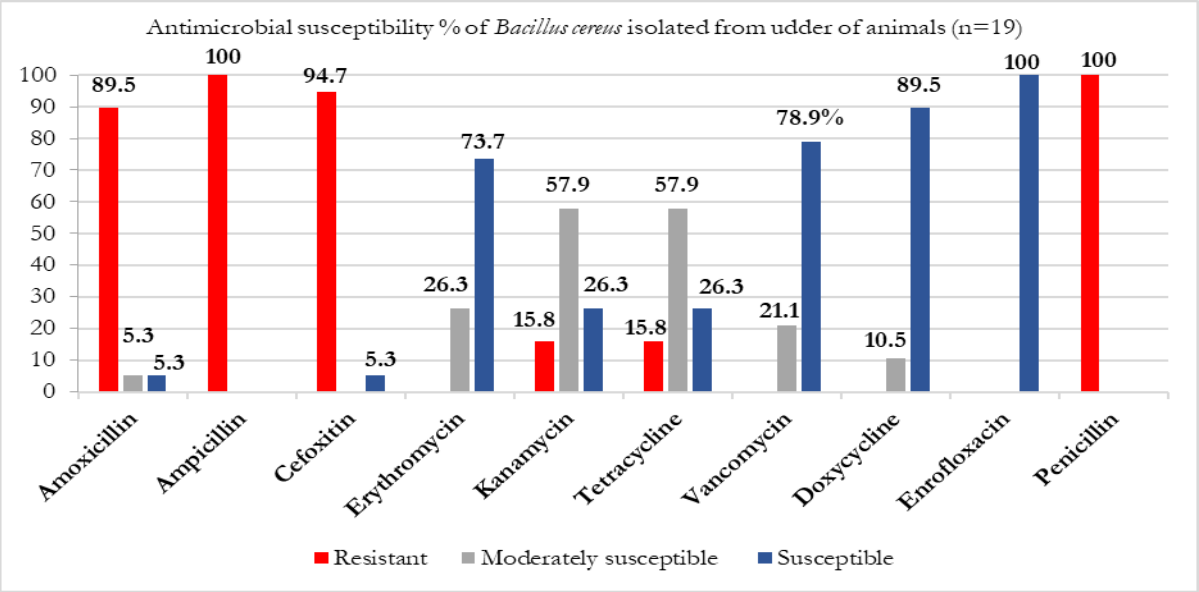

Figure 1. *In-vitro* antimicrobial susceptibility of the isolated *Bacillus cereus*.

All the isolates were resistant to different combinations of 3 up to 5 antibiotics (Table 4). A most frequent multidrug resistance pattern consisting of five drugs was exhibited for kanamycin, ampicillin, penicillin, amoxicillin and cefoxitin with a resistance by 15.8% (3/19) of the isolates. Moreover, majority of the isolates 12/19 (63.2%) showed resistance to ampicillin, penicillin, amoxicillin and cefoxitin.

Table 4. Multidrug resistance patterns of *Bacillus cereus* isolated from cows' milk (n=19)

Antimicrobial	Resistant to:	Resistant isolates	
		Number	%
One drug	-	0	0
Two drugs	-	0	0
Three drugs	AMP, P, TE	1	5.3
	AMP, P, FOX	1	5.3
Four drugs	AMP, P, FOX, AML	12	63.2
Five drugs	TE, P, AML, FOX, AMP	2	10.4
	K, AMP, P, AML, FOX	3	15.8
	None	0	0
Total		19	100

AMP= Ampicillin; P= Penicillin; TE= Tetracycline; FOX= Cefoxitin; AML= Amoxicillin; K= Kanamycin.

***Bacillus cereus* Count**

Based on *B. cereus* count of milk samples, 83.3% of ovine, 44.4% of caprine, and 42/8% of bovines had higher count in reference to the legal limit (above accepted limit). Meanwhile, 60% of cow samples from Aweday had higher count in reference to the legal limit (above accepted limit) (Table 5). However, the load of *B. cereus* did not significantly vary ($p > 0.05$) among animal species and study sites.

Table 5. *Bacillus cereus* load of raw milk samples and association with different factors

Variables	Category	Number of samples tested	*Bacillus cereus* load in reference to legal limit (%)		X^2	*p*-value
			Above	Below		
Species	Bovine	28	12 (42.8)	16	3.3278	0.263
	Ovine	6	5 (83.3)	1		
	Caprine	9	4 (44.4)	5		
	Total	43	21 (48.8)	22		
Study site*	Aweday	10	6 (60)	4	1.9882	0.665
	Haramaya	8	3 (37.5)	5		
	HU	10	3 (30)	7		
	Total	28	12 (42.8)	16		

** Means only cows are considered for comparison; HU= Haramaya University.*

Discussion

The present study revealed that, the overall prevalence of *B. cereus* in raw milk of farm animals (ewes, does, cows) was 29.5%, with an overall prevalence of 30.8 % in raw cow's milk. This finding is similar with the results of Hassan *et al.* (2010) and Yobouet *et al.* (2014) at proportions of 30% (Egypt) and 27 (Côte d'Ivoire), respectively. Moreover, it is comparable with the reports of Te-Giffel *et al.* (1996) from Netherlands (35%) and Hempen *et al.* (2004) from Guinea (33.3%) and Senegal (35.2%). However, the current findings on the prevalence of *B. cereus* in raw milk samples (29.5%) is far lower than values reported in some other countries. For instance, Ombui and Nduhiu (2005), Rezende-Lago *et al.* (2007) and Adesina *et al.* (2011), reported proportions of 75% (Kenya), 50% (Brazil) and 46.7% (Nigeria), respectively.

The current finding from cow's teat (30.8%) is far higher than previous reports in Ethiopia by Alemneh (2012), Yared (2014) and Shibabaw (2014), who reported 15.4%, 16.1% and 22.4%, respectively. However, lower occurrences were reported by Ahmed *et al.* (1983) from U.S. State of Wisconsin (9%), Parkash *et al.* (2007) from India (6.7%) and Hempen *et al.* (2004) from the Gambia (17%). The differences in the prevalence of *B. cereus* could be related to variation in udder management (hygienic practices) and housing conditions for lactating dairy cows (Radostits *et al.*, 2006). In the present study, the high prevalence of *B. cereus* could be related to the poor hygienic practices. Animals in the small household farms were milked on unhygienic floor. More importantly, the animals spent most of their time under unhygienic environment. Therefore, it is likely that the udder is at high risk of being infected with *B. cereus*. For instance, Youssif *et al.* (2020) showed that there was a strong correlation between hygiene of the farm environment and prevalence of *B. cereus* isolated form subclinical mastitic cows.

The present study revealed that 42.8% of milk samples from cow had *B. cereus* count above recommended limit for human consumption. This higher rate of contamination is supported by the report of Shibabaw (2014) (75%) and Yared (2014) (66.12%) from bovine teat in the country. This makes raw milk consumption unsafe in the vicinity of the study areas.

The prevalence report of *B. cereus* on sheep and goat is first of its kind in the country. So, it is difficult to discuss its situation in the country. Necidova *et al.* (2014) reported that *B. cereus* is one of the pathogens responsible for mastitis in sheep and goats. Moreover, Pirzada *et al.* (2016) indicated that, *B. cereus* was isolated from 10.52% of caprine sub-clinical mastitis in Tandojam, Pakistan. This showed that, the organism can reside in the udder of these animals and may lead to mastitis and pose risk for people who consume raw milk.

Statistical analysis revealed that, the prevalence and count of *B. cereus* did not significantly vary between different factors. This could be due to similar exposure rate and similar patterns in the occurrence of the organism in the environment. Moreover, it should be noted that, *B. cereus* is ubiquitous organism in the environment with wide host range (Radostits *et al.*, 2006; Bottone, 2010).

The study revealed that the occurrence of *B. cereus* was significantly higher ($p < 0.05$) in CMT positive milk. This finding is in agreement with previous reports in the country by Yared (2014) and Shibabaw (2014), who showed statistically significant association between *B. cereus* positivity and CMT score in bovine milk samples.

Disc diffusion assay showed that, majority of *B. cereus* isolates were sensitive to doxycycline (89.5%), vancomycin (78.9%) and erythromycin (73.7%). Similarly, Shibabaw (2014) reported that 92% of *B. cereus* isolates were susceptible to vancomycin. In addition, Luna *et al.* (2007) showed that majority of *B. cereus* isolates were susceptible to erythromycin (81%) and vancomycin (100%). Moreover, in the present study *B. cereus* isolates showed high rate of resistance to penicillin (100%), ampicillin (100%), cefoxitin (94.7%) and amoxicillin (89.5%). This is supported by the findings of Shibabaw (2014) and Yared (2014), who reported that majority of *B. cereus* isolates showed resistance to penicillin (76% and 90.1%, respectively) and ampicillin (80% and 83.4%, respectively). According to Luna *et al.* (2007), majority of *B. cereus* isolates showed resistance to ampicillin (95%) and penicillin (95%). In this study, tetracycline and kanamycin showed moderate activity against most of the isolates. Contrary to our findings, Shibabaw (2014) and Yared (2014) reported that majority of the *B. cereus* isolates were resistant to tetracycline (88% and 81.8%, respectively) and kanamycin (72% and 81.8%, respectively). This variation on the efficacy of tetracycline could be due to use of tetracycline discs with different potency (10μg) by Shibabaw (2014) and Yared (2014) as compared to the tetracycline (30 μg) used in the present study.

Conclusion

The results of this study have demonstrated a higher prevalence of *Bacillus cereus* in milk samples collected from lactating cows, sheep and goats. The significant association of *B. cereus* with subclinical mastitis gives an insight on the possible involvement of the organism in the udder infection. Moreover, the bacterial load in the milk samples, which was above the tolerable limit for human consumption suggest the potential risk of consuming raw milk and dairy products. The degree of resistance of the bacteria to antibiotics and moderate susceptibility to the majority of the tested antimicrobials would be a challenge in the treatment of infections associated with the bacteria. Hygienic milking practices need to be adapted to reduce infection of the udder and contamination of milk. As the size of the sample was small in the present study, a study with large samples size was suggested in the future to robustly determine the effects of different risk factors associated with *B. cereus* in the infection of udder of dairy cows, sheep and goats.

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Conflict of Interests

The authors declare that they have no competing interests.

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