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Prevalence, associated risk factors and its public health significance of *Baccilus cereus* from bovine raw milk in selected dairy farms in and around Wolaita sodo, SNNPRS, Ethiopia.

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Abstract

A cross sectional study was conducted on 384 lactating dairy cows to determine the prevalence and load of *B. cereus in* raw milk samples. In addition, risk factors associated with *B. cereus* load, antibiotic susceptibility of the isolates and potential public health implications were assessed from selected dairy farms in and around Wolaita Sodo, between November 2013 and June 2014 using CMT test, bacteriology and questionnaire survey. The prevalence of Bacillus cereus in raw cow milk was 16.14% at cow level. The Bacillus cereus count ranges from $1.04x10^3$-$1.06x10^6$CFU/ml. From positive samples, 67.74% (42/62) of total samples have significant counts (>10^5 CFU/ml) which was above legal limit in raw milk intended for human consumption. Bacillus cereus was shown to be one of the causative agents of subclinical mastitis since the bacterium was isolated at rates of 22.66%, 2%, 4.88%, 4.76% of negative, +1, +2, +3 scores of CMT, respectively. As the result indicated muddy floor type, semi-intensive management system, mid lactation and early parity were highly contaminated with B. cereus. An attempt was made to assess public health implication and source of raw milk contamination by using semi- structured questionnaire survey. Furthermore, antibiotic susceptibility testing of the isolates showed that udder infections with Bacillus cereus may not be cured by treatment regimes penicillin G, kanamycin, tetracycline, ampicilin and polimyxin B; the isolates were found to be susceptible to chloramphenicol(86.36%) followed by clindamycine(77.27%). In conclusion, our study results indicated that raw samples were highly contaminated with Bacillus cereus, exceeding the legal limit set for raw milk (>10^5 $_{CFU/ml}$), suggesting the need for effective hygienic measures to be introduced in milk value chains during milk production, distribution and processing and food service establishments to avoid public health hazards.

Keywords: *Bacillus cereus*, Bacterial count, Ethiopia, Prevalence, Public health, raw milk, Wolaita Sodo.

Introduction

Milk is a food of good nutritional value which ensures benefits from its consumption. For people in world in general and Ethiopia in particular, especially the rural areas where milk represent a good source of protein, calcium and vitamin D that stimulate the growth and body functions [1, 2].

The safety of dairy products with respect to food-borne diseases is a great concern around the world. This is especially true in developing countries where production of milk and various dairy products take place under rather unsanitary conditions and poor production practices [3, 4]. Also, the composition and the neutral pH of milk makes it an optimum medium for the growth of microorganisms that may come from the interior of the udder, exterior surfaces of the animal, milk handling equipment and other miscellaneous sources such as milking environment [5]. Milk has nutrients that make it suitable for the rapid multiplication of bacteria that cause spoilage.

Milk does not contain any natural antimicrobials that would inhibit or kill microorganisms that might be present [6]. Poor handling and undesirable practices such as use of non potable water can also cause spoilage of milk [7]. Thus the safety of milk is threatened by various agents including pathogenic microorganisms, aflatoxins, pesticides and antimicrobial agents [8].

Pathogenic microorganisms constitute the most important food related to threat public health [8]. And these microorganisms may contaminate milk at various stages of milk procurement, processing and distribution. It is known that tropical conditions which have a hot, humid climate for much of the year are ideal for quick milk deterioration so pose particular problems because the temperature is ideal for growth and multiplication of many bacteria [9].

Raw milk serves a good medium for food borne pathogens such as *Listeria monocytogenes*, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum* and *Shigella* have been reported by several authors in raw milk samples [10, 11].

Bacillus cereus is the aerobic endospore former next in importance to *B. anthracis* as a pathogen of humans and other animals. It has been reported from a wide range of opportunistic infections both in immuno-compromised and in immunocompetent patients and causes two distinct foodborne illness syndromes and a wide range of opportunistic infections [12, 13].

Bacillus cereus is an emerging human food-borne pathogen. This pathogen is classified as the third most important cause of collective food-borne infections in Europe, after *Salmonella* and *Staphylococcus* [14]. *B. cereus*-induced gastroenteritis is generally mild, but bloody diarrhea and emetic poisoning leading to some fatal cases. *B. cereus* is also associated with severe local and systemic human infections, such as endophthalmitis, pneumonia, and meningitis, posing a public health problem [15]. This organism is also responsible for spoilage of different food products. As *B. cereus* is a spore former organism, there is a risk of its transmission through heat-treated and processed food products [16].

B. cereus is found frequently as a saprophyte in soil, water, vegetation and air, from where it is easily transferred to food, either from the original raw material or during the food processing. It is common in dried foodstuffs, spices, cereals, meat, eggs, milk and milk products, cooked and inappropriately kept food. The colonization of different ecological niches is enabled by its extremely good adaptability and resistance to various influences [17].

Bacillus cereus is a Gram positive, rod-shaped, foodborne pathogen and can cause two types of foodborne illnesses; diarrheal and emetic type outbreak [18, 19]. *Bacillus cereus* has been detected and implicated in several contaminated food products and supplements since 1906, when Plazikowski associated the organism associated with food poisoning [20].

B. cereus produces endospores that are resistant to various disinfectants. It also forms enzymes such as lipases, proteases, xylanases and others. In milk and milk products, it decomposes casein into peptides and amino acids, and milk fat into free fatty acids, thus degrading the quality of milk products and shortening their shelf life [16].

B. cereus is also relatively resistant to heat that survive pasteurization due to the formation of spores; therefore, it grows easily during food storage and may be responsible for food poisoning [21]. Exactly, it produces toxins that can be found in the food, or be produced in the gut after the ingestion of *B. cereus* contaminated products; in both cases the result is a foodborne enteric intoxication [22].

B. cereus and some closely related species from the genus *Bacillus* have several features including the production of various biologically active metabolites like antibiotics, proteinases and bacteriocins that make them attractive candidates for biological control agents [23].

Even though the dairy industry is often confronted with severe implications caused by *Bacillus cereus*, there is no study done concerning *Bacillus cereus* which is the main causes of milk borne poisoning except the work done in Debre Zeit, *Ethiopia*, by Alemneh, [24] and in Alage TVET dairy farm by (Seblewengel, [25].

Therefore, the objectives of this study are:

- To determine the prevalence and load of *Bacillus cereus* in raw bovine milk samples collected from lactating cows in the study area.

- To assess risk factors associated with the prevalence of *Bacillus cereus* in raw milk and its public health significance.
- To conduct antibiotic sensitivity test to the isolate *Bacillus cereus*
- To assess the public health implication and source of raw milk contamination of Bacillus cereus through questionnaire survey in the study area.

Materials and Methods

Study area

The study was conducted in and around Wolaita Sodo, Southern Nation Nationalities People Regional State, southern, Ethiopia. Wolaita Sodo is located about 390km south of Addis Ababa. The town Sodo is located at latitude of 8°50°N and longitude of 37°45°E. Topographically, the area is marked by hilly, flat, steep slopes and gorges and a number of streams and mountains. The highest mountain is Damota, 2500 m above sea level, which is located near Sodo town [26]. The Altitude varies from 1100-2950 m.a.s.l. The area experiences mean annual temperature of about 20°C.The mean maximum temperature is 26.2°C and the average monthly minimum temperature is 11.4°C.The rainfall regimes over much of the area are typically bimodal with the big rainy season extending from June to September and a small rainy season occurring from February to April. The mean annual rain fall of the area ranges from 450-1446 mm with the lowest being in low land and highest in high land. The livestock population in the area is estimated to be 68,900 cattle, 1992 sheep, 382 goats, 121 horses, 131 mules, 488 donkeys and 55,191 chickens [27].

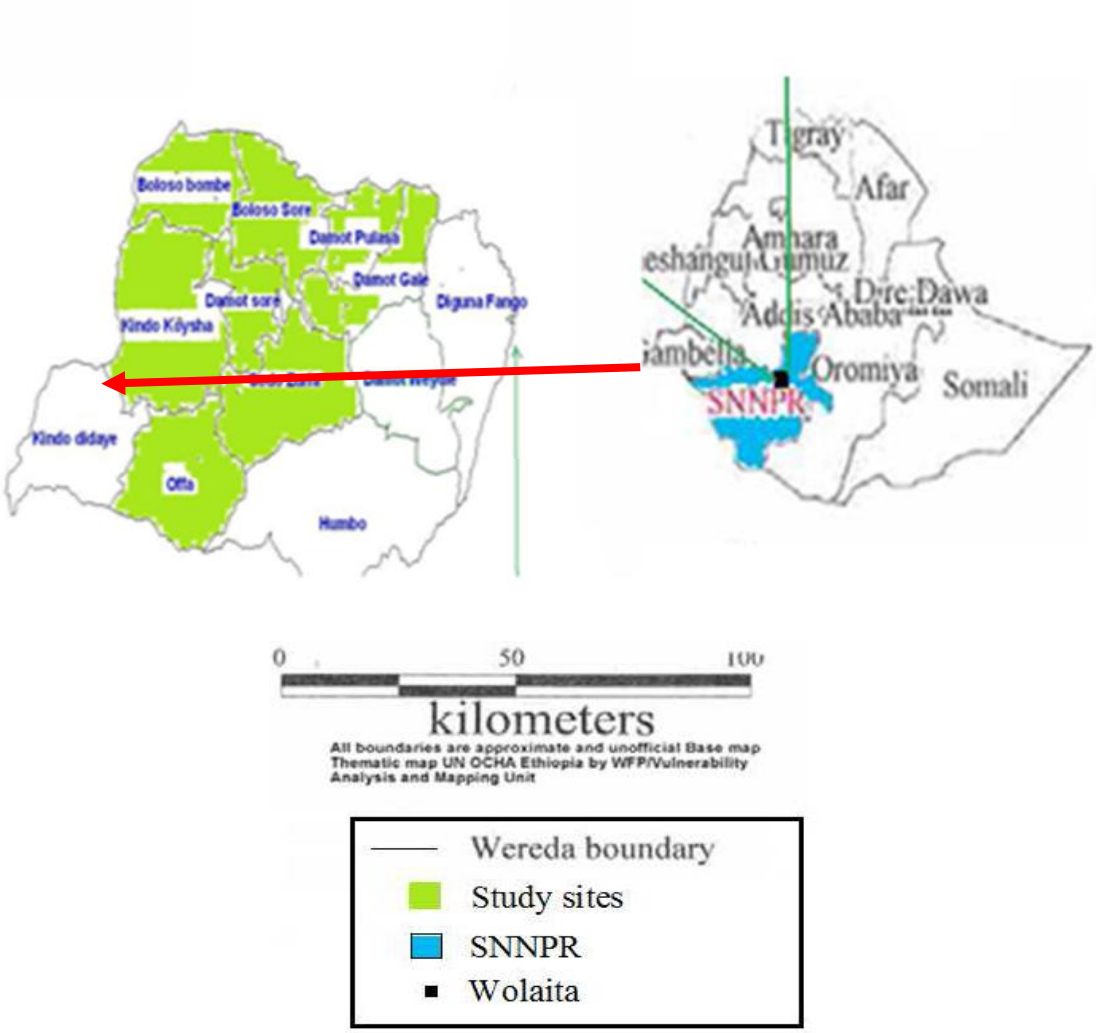

Figure 1: A map of Wolaita Sodo, SNNPR
Source: FEDB, 2009[28]

The study Design

A cross sectional study was carried out from November, 2013 to June, 2014 on bovine raw milk samples collected from purposively selected dairy farms in Wolaita Sodo. Census sampling method was applied in which all lactating cows in purposively selected farms were sampled. In addition, prior to sample collection, information was gathered from milkers by using semi-structured questionnaire survey which was designed to assess the risk factors for milk contamination with the organism like the situation of dairy farm management, milking procedures and hygienic status of the farm and from the consumer to assess public health significance of the organism. All respondents of questionnaire survey were selected purposively based on their voluntariness. Totally 110 voluntary personnel were interviewed.

Study population

The study populations were all lactating dairy cows found in purposively selected dairy farms in and around Wolaita Sodo.

Sample size determination

The total sample size for raw milk collection, isolation and enumeration of *B. cereus* was assigned according to statistical formula of Thrustfield, [29]. A 5% absolute precision at 95% confidence interval was used during determining the sample size. Since there was no previous work in the study area for *B. cereus* prevalence on raw milk, the expected prevalence of this bacterium on raw milk was taken as 50% according to Thrustfield, [29]. Therefore, the total sample size for this study was calculated as follows:

$$n = \frac{(1.96)^2 x\ P\ (1-P),}{d^2}$$

Where: n = the total sample size
P = expected prevalence (50%)
d= desired absolute precision (0.05) at 95% CI

$$n = \frac{(1.96)\ x\ (1.96)\ x\ (0.5)\ x\ (1-0.5)}{(0.05)\ x\ (0.05)} = 384$$

From the above equation, a total of 384 lactating cows from purposively selected dairy farms were sampled in this study.

Bacteriological analysis

Examination of the udder and milk

During sampling, observation was made about the condition of the udder for the presence of lesion or anatomical malformation or swelling. The milk was examined for its consistency, color and other visible abnormalities. Clinical mastitis was recognized by abnormal milk and signs of udder infection where as sub-clinical mastitis was recognized by apparently normal milk and an increase in leukocyte counts as evidenced by California Mastitis Test (CMT).

Sample collection and handling procedure

Raw bovine milk samples were collected from lactating cows found in the purposively selected dairy farms in the study areas. Samples were collected directly from the udder of apparently healthy animals. Procedure for collection of milk was according to [30]Quinn *et al*. (2002); strict aseptic procedures were adopted when collecting milk samples in order to prevent contamination with microorganisms present on the body of animal and from the barn environment.

Before sampling, the udder and teats were washed with potable water and disinfected with cotton soaked in 70% Ethanol wearing latex glove. Disinfectant soaked cotton ball was used individually to each teat. The first two to three streams of milk was streaked into ground and then representative milk samples (about 10 ml) was collected directly from teats (1 – 2 streams from each teat) into a sterile screw capped universal bottle of 15 ml. The cap was removed from the universal bottle without touching the inside and it was held in such way that the inner surface faces down to prevent sample contamination. The universal bottle was kept at 45^0 angles so that debris did not fall into it during sampling. The cap was immediately replaced after the sample was obtained.

Information on the cow parity, lactation stage, floor type and management system of the farms were also collected at the time of sampling using data recording sheet. Finally, the milk sample was immediately transported to Sodo regional laboratory and Microbiology Laboratory of Sodo University, in tightly closed ice box.

Sample Processing and Plating

Sample processing was done by diluting 1ml milk, from 10 ml milk sample, with 9 ml of 0.1% peptone water (CM0009, Oxoid Ltd) in safety cabinet. The diluted sample was mixed manually by moving gently about half arc 10–15 times. From this initial dilution (10^{-1}), serial dilutions from 10^{-2} to 10^{-4} were made in a sterile peptone water. Following this, 0.1 ml milk sample was spread on to solidified *Bacillus cereus* selective medium (CM0617; Oxoid Ltd, Basingstoke Hampshire, England) in duplicates; that is two plates were used for each dilution factor. For prevalence determination, 0.1 ml of each raw milk sample was plated without dilution. After inoculation plates were incubated aerobically at 30 ^{0}C for 18 – 24 hrs and checked for presumptive colony growth. If no colonies grew, the incubation was extended for another 24 hrs and rechecked for colony growth.

Total viable count by standard plate count technique

This was performed either by pour plate method or by surface spread method for enumerating the number of viable organisms in foods like the milk. It helps to get information both the degree of contamination of milk and pathogenic levels (doses) of microbes to affect consumers. As the original sample was highly contaminated with viable organisms, it was necessary to dilute the samples up to 10^{-3} to 10^{-6}. After incubation, organisms that grew on culture media as distinct colonies were counted with the help of colony counter. The number was expressed as colony forming units (cfu)/ml of the milk.

Total viable counts were measured according to the UK national standard method [31](HPA, 2004). Using peptone saline diluents (containing 1.0 g peptone, 8.5 g sodium chloride in 1 L distilled water) serial dilutions were undertaken. Each dilution was mixed for 1 min and 1ml was inoculated into three Petridishes. Subsequently, plate count agar was added, mixed with the inoculums, and incubated aerobically at 30 ◦c for 72 hrs. The plate colonies were then counted and the total viable count per milliliter was calculated. The plates having colonies below 15 may not serve as the true representatives of the sample and plates with more than 150 colonies caused difficulty in colony counting. The numbers of colonies per plate were only taken into account when the count laid between 15 and 150 and the number of viable microorganisms per milliliter of sample was calculated using the standard equation [32].

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

Where:
N = is total viable count
ΣC=is the sum of the colonies (C) counted from all plates ($15 \leq c \geq 150$ colonies)
n_1 = is the number of plates counted at the first dilution,
n_2 = is the number of plates counted at the second dilution
C= number of colonies lay between 15 and 150 on all plates
d =is the dilution from which the first counts will be obtained (i.e. least diluete).

Confirmatory and differential tests

For confirmation and differentiation from positive plates 2–3 presumptive colonies were picked and transferred to nutrient agar slants. These were incubated for 24 hrs at 30 ^{0}C aerobically. Using Gram staining *Bacillus cereus* group were identified as large Gram-positive rod shaped cells with short to long chains. Most biochemical tests are confirmatory; but they are common for *Bacillus cereus* group members namely, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis*, and *Bacillus anthracis* with identical characteristics; therefore, additional tests were required for differentiation.

The following characteristics were employed for identifying *B. cereus* from the other group members: on sheep blood agar (CM0854; Oxoid Ltd), *Bacillus cereus* colony grow as with flat and irregular shaped, 2–5 mm in diameter forming creamy to white color on a ground glass appearance with strong β-haemolysis. This colonial appearance was used for differentiating *Bacillus cereus*. Since *Bacillus anthracis* forms non haemolytic gray/white colonies where as *Bacillus mycoids* forms colonies with rhizoid/hairy like projections. Alternatively, *Bacillus cereus* was differentiated from other non motile group members forming diffuse growth in semisolid SIM medium (M181; HiMedia Ltd) except from *Bacillus thuringiensis*. In addition, from *Bacillus cereus* group only *Bacillus mycoids* can form rhizoid growth on pre-dried nutrient agar (CM0003; Oxoid Ltd) or blood agar (CM0854; Oxoid Ltd).

Rapid staining methods using warm 0.5% basic Fuchsin (212545; BD Difco BBL Stains), Malachite green (90903; Fluka) and Sudan Black B (199664;

Sigma-Aldrich), give characteristic morphology of pale green endospores without bulged sporangium and with no parasporal crystal bodies in red stained cytoplasm. This was used to differentiate *Bacillus cereus* from *Bacillus thuringiensis.*

Antibiotic Sensitivity Testing

Antimicrobial susceptibility tests were conducted on 22 isolates of *Bacillus cereus*. The isolates were tested for 7 commonly used commercially available antimicrobials using the Kirby-Bauer disk diffusion method by 0.5 McFarland Standard on Muller Hinton agar plates [33, 34]. The following antimicrobial discs (all from Oxoid, Basing stock, UK) with their corresponding concentration were used in this study: kanamycin(30µg), tetracycline (10µg), clindamycin(10µg), polimyxin B(300µg), Penicillin G(10u), ampicilin(10µg), and chloramphnicol(30µg).

The antibiotic discs were applied on to the surface of the inoculated Muller Hinton agar plates using aseptic technique. Each disc was pressed down to ensure complete contact with the agar surface. The discs were deposited with centers at least 24 millimeter apart [33].

The inhibition zone was reported as the diameter of the zone surrounding the individual disc in which bacterial growth was absent. Based on this, the isolates were defined as resistant, intermediate and susceptible according to the guide lines of the manufacturer manual and [34].

Data Management and Processing

The data collected through questionnaire survey and laboratory results of the collected samples were entered into databases using Microsoft Excel and analyzed using SPSS 20 statistical computer software programs. The log_{10}-transformed values of raw milk standard plate count (log_{10}CFU/ml) were computed using mean values as continuous variable and parities, lactation stage, laboratory result and CMT result as categorical variables. Descriptive statistics were used to describe the nature and the characteristics of the data. Comparison between prevalence of groups were analyzed by using Chi-square (x^2) test. For all statistics 95% CI with 5% degrees of freedom ($P < 0.05$) was considered to say significant.

Results

In the present study a total of 384 lactating cows were sampled for raw milk in selected dairy farms in and around Wolaita Sodo and milk samples were processed microbiologically for isolation and identification of *Bacillus cereus*. Variations in floor type, parity, lactation stage, CMT result, bacterial load and management system of the selected farms were used as risk factors for assessing contamination rates of milk by *Bacillus cereus.*

Prevalence of *Bacillus cereus*

The overall prevalence of *Bacillus cereus* in raw bovine milk samples was 16.14% at cow level. The *Bacillus cereus* load from raw milk samples ranged from 3.1401 to 6.1605 logarism of colony forming unit per milliliter (log CFU/ML). The bacterial loads in CFU/ml of most of milk samples were above legal limit (>10^5CFU/ml) in raw milk. From positive samples, counts above and below legal limit in cow raw milk intended for human consumption were 66.13% (41/62), 33.87%(21/62), respectively (Table 1).

Table 1: Overall prevalence and bacterial load above legal limits in raw bovine milk at Wolaita Sodo

	No of examined animals	No of positive animals	Prevalence (%)
Bacillus cereus	384	62	16.14%
Bacterial load above legal limit	384	41	66.13%
Bacterial load below legal limit	384	21	33.87%

Association of *Bacillus cereus* Contamination of raw milk

Analyses were made to look at the association of Bacillus cereus occurrence in raw milk with various host and management factors.

The Statistical analysis in table 2 below shows California mastitis test, management system and floor type in small house holder dairy farms were significantly associated with the positivity of Bacillus cereus. As the laboratory result revealed the prevalence B. cereus was significantly ($p<0.05$) higher as the management system declines, increasing the chance of contamination of raw bovine milk with Bacillus cereus. The contribution of floor type of the dairy farms as a source of *Bacillus cereus* was significantly associated with the positivity of *Bacillus cereus*. *Bacillus cereus* was isolated from concrete(cemented) and soil(muddy) floor types of the dairy farms at the rates of 22.88% and 13.16%, respectively ($p<0.05$). Statistically significant association was also observed between California mastitis test values and Bacillus cereus prevalence, whereas there was insignificant association of lactation stage with positivity of *Bacillus cereus* and parity with positivity of *Bacillus cereus* (P>0 .05)

Table 2: Association between risk factors and positivity of Bacillus cereus contamination of bovine raw milk using chi-square (χ2) at Wolaita Soddo town

Association of Bacillus cereus contamination with California mastitis test

Risk Factors	category	Observation	Positivity of *Bacillus cereus*	X^2	df	P-Value
Mastitis(CMT reaction)	Negative	256	58(22.66%)	24.340	4	.001
	Trace	16	0(0%)			
	+1	50	1(2%)			
	+2	41	2(4.88%)			
	+3	21	1(4.76%)			
Management System	Intensive	275	36(13.09%)	6.678	1	.010
	Semi-intensive	109	26(23.85%)			
Floor Type	soil(muddy)	118	27(22.88%)	5.708	1	.017
	Concrete(Cemented)	266	35(13.16%)			
lactation stage	1-3	164	27(16.46%)	1.071	3	.784
	4-6	110	20(18.18%)			
	7-9	67	10(14.92%)			
	>9	43	5(11.63%)			
Parity in number	1-3	199	34(17.08%)	0.467	2	.792
	4-6	139	22(15.83%)			
	>7	46	6(13.04%)			

Bacillus cereus contamination analyzed by logistic regression

Results of univariate logistic regression revealed that management system (OR=2.191, 95%CI: 1.056-4.546) had a significant impact on *Bacillus cereus* contamination. Semi-intensive management system has a higher chance of acquiring *Bacillus cereus* contamination than intensive system of management (Table 3).

Table 3: Univariate analysis of the association of risk factors with *Bacillus cereus* contamination

Category		N	Prevalence (%)	P-value	OR	CI	
Floor type	Muddy soil	118	27(22.88%)	0.607	1.211	0.584	2.511
	Concrete(Cement)	266	35(13.16%)				
Management	Semi-intensive	109	26(23.85%)	0.035	2.191	1.056	4.546
	Intensive	275	36(13.09%)				
Parity	1-3	199	34(17.08%)	0.641	1.272	0.462	3.504
	4-6	139	22(15.83%)	0.803	1.142	0.402	3.248
	7 and above	46	6(13.04%)				
Lactation stage	1-3	164	27(16.46%)	0.610	1.304	0.469	3.624
	4-6	110	20(18.18%)	0.700	0.814	0.286	2.317
	7-9	67	10(14.92%)	0.934	1.050	0.330	3.336
	10 and above	43	5(11.63%)				
Mastitis(CMT)	Negative	256	58(22.66%)	0.04			
	+1	50	1(2%)	0.009	0.069	0.009	0.519
	+2	41	2(4.88%)	0.04	0.158	0.036	0.692
	+3	21	1(4.76%)	0.053	0.129	0.016	1.029
	Trace	16	0(0%)	0.998	0.000	0.000	

Antimicrobial Susceptibility Profiles of *B. cereus* Isolates

From the total of 62 positive *Bacillus cereus* identified, 22 isolates were tested for antimicrobial susceptibility. Out of 22 *Bacillus cereus* isolates tested for antimicrobial susceptibility higher levels of resistance was found against penicillin G (81.81%), tetracycline (90.91%), kanamycine (81.81%), clindamycine(90.91%), ampicillin(86.36%), and polymixine B(100%);however, the isolates were highly susceptible to chloramphnicol (86.36%) (Table 4).

Table 4: Antibiotic susceptibility profiles of *Bacillus cereus* isolates

Antimicrobial agent	Zone diameter nearest whole mm (%)		
	Resistant	Intermediate	Susceptible
Penicillin G	20/22(90.91%)		2/22(9.09%)
Tetracycline	18/22(81.81%)	1/22(4.45%)	3/22(13.63%)
Chloramphincol	1/22(4.54%)	2/22(9.09%)	19/22(86.36%)
Kanamycin	18/22(81.81%)	-	4/22(18.18%)
Clindamycin	5/22(22.73%)	-	17/22(77.27%)
Ampicilin	19/22(86.36%)	-	3/22(13.63%)
Polymixin B	100%	-	-

Finding of the Questionnaire Survey on Public Health Implication

Questionnaire survey was done to assess risk factors for public health implication of *Bacillus cereus* from raw milk. Accordingly, the respondents' 41.6 % of milk consumers use once boiled milk without reheating from eleven up to sixteen hours. Only 36.6 % of milk consumers preserve milk by boiling, and the rest 28.7% and 34.7% were using refrigeration and simply putting in a plastic container, respectively. Milk were used as a common diet in the study area (44.6%) and 38.4% of the consumer consume raw milk and 23.1% and 17.3% consume in the form of yogurt and cheese, respectively (Table 5).

Table 5: Descriptive Statistics of public health questionnaire survey

Survey variable	Questionnaire respondents'			
	A	B	C	D
Consumption of milk	94.5Y	5.5N		
Form of milk consumption	38.4R	23.1YG	17.3CH	21.2B
Peoples whose family members ill after milk consumption	58.2Y	41.8N		
Clinical signs observed	22.2FA	42.9D	34.9V	
Knowledge of any MBD	60.7Y	39.3N		
Description of MBD	38.1IP	17.5DV	20.6AP	23.8TB
Time used once boiled milk without re-heating	9.9OF	27.7FT	41.6ES	20.8GS
Frequency of milk consumption	20.8FB	44.6CD	16.8RA	17.8FQ
Method of milk preservation	36.6B	28.7RF	34.7PC	

AP-Abdominal Pain, B- Boiled, CD-Common Diet, CH-Cheese, D-Diarrhea, DV-Diarrhea and Vomiting, ES-Eleven up to Sixteen hours , FA-Fever and Abdominal pain, FB-For Breakfast, FT- Five up to Ten hours, FQ-Frequently, GS-Greater than Sixteen hours, IP-Internal Parasite, N-No, OF-One up to Four hours, PC-Plastic Container, RA-Rarely, RF-Refrigeration, V-Vomiting, Y-Yes.

Farm Questionnaire Survey

As observed during the current study, all the respondents reported of washing their hands before milking; however, 94.7% did not wash their hands between each milking. Washing of milking utensils was practiced by all of the respondents but only 36.8% of the farms use detergents. Most of the respondents (89.5%) wash the cow's udder and teats before milking. Most farms (68.4%) had not have towels for cleaning purposes of cow's udder (Table 6).

Table 6**:** Descriptive statistics of farm milk contamination

Survey variable	Questionnaire respondents'		
Cleaning of milk utensils before milking	100Y	0N	
Washing the hand before milking	100Y	0N	
Cleaning hand between each milking	5.3Y	94.7N	
Supply of towel	26.3 C	5.3S	68.4N
Uses of detergents	36.8Y	63.2N	
Washing of udder and teats before milking	89.5Y	10.5N	

C-Common, N-No, S- Separate, Y-Yes

Discussion

The present study was conducted on raw bovine milk samples so as to determine *Bacillus cereus* prevalence, evaluate the bacterial load in raw milk and assess risk factors for raw milk contamination by *Bacillus cereus* and its public health implication.

The prevalence of *Bacillus cereus* was 16.14% at cow level in raw bovine milk in selected dairy farms in and around Wolaita Sodo and its bacterial load was 3.1401 to 6.1605 logarism of colony forming unit per milliliter (log CFU/ML). This result is a little higher than the works of Alemneh. [24], Seblewengel. [25] and Gilles *et al.* [35], who reported isolation rates of 15.4%, 15.86%, 15.4% and respectively.

However, reports of higher contamination were reported by Yobouet *et al.* [36], Adesina *et al.* [37], Hassan *et al.* [38], Haughton *et al.* [39], Rezende-lago *et al.* [40], Ombui and Nduhiu. [41], EI- Shinawy. [42], Ayuob *et al*. [43], Schelegelova *et al.*[44] and (Te Giffel and Beumer. [45], at rates of 27%, 46.7%, 50%, 59%, 50%, 35.2%, 62%, , 26.7%, 31% and 35%, respectively. This may be because of certain climatic variation or farm conditions which is different from the present study.

In the present study, majority of raw milk samples had higher *Bacillus cereus* load (66.13 % (41/62)) than acceptable limit of raw milk (>10^5CFU/ml) which is higher than the result reported by Seblewengel. [25] 38.98%. This may be due to the differences in farm management systems between the current studies conducted at Wolaita Sodo and that of Seblewengel. [25] conducted in Alage; good hygienic practices may be present at Alage farm as compared to dairy farms in Wolaita Sodo.

In this study, the CFU/ml of *Bacillus cereus* ranged from 1.04x10^3-1.06x10^6CFU/ml (5.25x10^5ave.). This finding is lower when compared with the work of DeGraff *et al.* [46] (3.88x10^7CFU/ml), Godifay and Molla [9] (1.9x10^8CFU/ml), Bonfoh *et al*. [47] from Senegal (10^7CFU/ml) and Esther *et al.* [48] (3x10^7CFU/ml); however, it is in the same range with the finding of Seblewngel [25] (4.29×10^5) and Alemneh [24] (2.8x10^5) from Ethiopia, Rai and Dawvedi [49] from India (7.7x10^5CFU/ml), Kurwijilla *et al*. [50] from Tanzania (10^5CFU/ml), Ombui *et al.* [51] from Kenya (5x10^4CFU/ml), and Bonfoh [47] from Mali (10^6CFU/ml) from raw bovine milk.

The positivity of *Bacillus cereus* was not significantly different with in lactation stage in raw milk ($p>0.05$), but there is higher prevalence around early and mid stage of lactation and this indicates the middle stage of lactation is very important for the occurrence of *Bacillus cereus.* Therefore, consumers and milk producers need to be careful in handling milk originating from cows at their early and mid lactation stages since such cows shade higher load of *Bacillus cereus* [25].

The positivity of *Bacillus cereus* was not significantly different with parity in raw milk ($p>0.05$),but there is higher prevalence around early and mid parity and this may due to high milk production and aggressive behavior of the animals at young stage which increase the chance of contamination during milking and bedding. This finding is similar to Seblewengele [25].

The presence of *Bacillus cereus* were significantly variable with CMT scores (<0.05). It indicates *Bacillus cereus* is one of the causative agents for the occurrence of subclinical mastitis and is responsible for 1.61%, 0%, 3.22%, 1.61% of +1, trace, +2, +3 scores of CMT, respectively. The present finding supports the work done by Seblewngel [25], Girma *et al.* [52] and Gizaw [53] from Ethiopia, Jones and Turnbull [54] from British and Schiefer *et al*. [55] from Canada and Horváth *et al.* [56] from USA. Their report describes *Bacillus cereus* as the cause of gangrenous mastitis in cows. Therefore, frequent monitoring and treatment of dairy cows for presence of subclinical mastitis is very crucial to control shading of the bacteria into raw milk.

The contribution of management systems between intensive and semi-intensive of the dairy farms as a source of *Bacillus cereus* was significantly associated ($p<0.05$) with the positivity of *Bacillus cereus*. This was similarly reported by Slaghuis *et al.* [57], Christiansson *et al*. [58] and Magnusson *et al*. [59]. This difference may be due to many reasons like variations in shed floor construction, hygiene of the farms, milking procedures and farm managements and when cows were at pasture, the teats might be predominantly contaminated with the soil.

The contribution of floor type between concrete (cemented) and muddy soil of the dairy farms as a source of *Bacillus cereus* was significantly associated with the positivity of *Bacillus cereus* ($p<0.05$). The present study is similar with work of Magnusson [59].

The hygienic quality of milk has serious implications on its economic value and more importantly on its public health safety. The questionnaire results mainly gave a broad understanding of the milking and hygiene practices and also the feeding habits of milk in the study area. Maintaining the sanitary condition of milking area is important for the production of good quality milk [60]. Therefore, it is likely that raw milk might be contaminated from soiled bedding and soil [61].

As observed during the present study, most of the respondents (89.5%) wash the cow's udder before milking. However most of the dairy cow owners (63.2%) did not use detergent for cleaning milking utensils, which may lead to insufficient cleaning and hence could serve as a major cause of milk contamination. Most of the dairy farms (68.4%) did not use towel and only 26.3% used one towel for all cows. The reuse of towel for cleaning and sanitizing may result in recontamination of the udder [1]. Furthermore, milkers wash their hands at the beginning of milking but did not wash between each milking. This might contribute to the high level of contamination of milk [62].

From public health questionnaire survey conducted, only 33.7 % of milk consumers in the study area preserve milk by boiling and the rest 28.7% and 37.6% used refrigeration and simply putting in a plastic container as a way of milk preservation respectively. 44.6% of respondents in the study area were used milk as common diet. Accordingly, the respondents' 41.6 % of milk consumers were used once boiled milk without reheating from eleven up to sixteen hours. Consumption of boiled milk without reheating for long period of time may facilitate recontamination of the milk by the bacteria [25].

Antibiotic resistant bacteria pose a growing problem of concern worldwide since the bacteria can be easily circulated in the environment. Effectiveness of current treatments and ability to control infectious diseases in both animals and humans may become hazardous [63].

In the present study the *Bacillus cereus* isolates were resistant to penicillin G (90.91%), tetracycline (81.81%), Ampiciline (86.36%), kanamycin (81.81%) and 100% resistant to polimyxine B. The isolates were found highly susceptible to chloramphenicol (86.36%), clindamycin (77.27%). The present study is similar with the works of Drobniewski [64], Seblewengel [25] and Agwa *et al.* [65]. Therefore, proper communication of such results to the relevant bodies is important to import chloamphenicol containing intramammary infusions so as to prevent the public from health problems that may originate from *Bacillus cereus* and its toxin.

Conclusion and Recommendations

In this study the presence of *Bacillus cereus* in raw cow milk on Wolaita Sodo selected dairy farms and the corresponding bacterial loads were confirmed using standard bacteriological procedures. The majority of *Bacillus cereus* positive cows had bacterial load beyond the legal limits ($p<0.05$) for human consumption. Milking equipments and milking procedure were also act as source of milk contamination with the bacteria. The consumers had little knowledge about the impact of *Bacillus cereus*. *Bacillus cereus* causes food poisoning in human and mastitis in cows. Contamination rate of raw bovine milk with *B. cereus* was associated with risk factors like management system and floor type, udder cleaning frequencies, habits of using towel and cleaning detergents ($p< 0.05$). To treat the disease caused by *Bacillus cereus*, Chloramphinicol is the drug of choice.

Therefore, based on the above findings the following recommendations are forwarded:

- The hygiene status in dairy farms should be improved to reduce *B. cereus* load to acceptable level and prolong the keeping quality of raw milk.
- HACCP should be Implemented to minimize the *Bacillus cereus* load in the dairy farm below acceptable limit ($<10^5$) for public consumption and prolong the keeping quality of raw milk.
- Milk for public consumption should be properly boiled at appropriate temperature and time.
- Treatments of *Bacillus cereus* infected cows should be done based on drug susceptibility testing.
- Further study should be conducted in determining the status of *B. cereus* and its toxins in raw milk in the country so as to design proper preventive measures.

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