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A STUDY ON PREVALENCE, PUBLIC HEALTH SIGNIFICANCE AND THE ASSSOCIATED RISK FACTORS OF *BACILLUS CEREUS* ON BOVINE RAW MILK IN SELECTED DAIRY FARMS IN AND AROUND WOLAITA SODO, SNNPR, ETHIOPIA

MSC THESIS

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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, IMMIUNOLOGY AND PUBLIC HEALTH

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BISHOFTU, ETHIOPIA

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A Thesis submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in veterinary public health

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Approval Sheet of Thesis

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Yared Dendana** entitled: **A study on prevalence, public health significance and the associated risk factors of *Bacillus cereus* on bovine raw milk in selected dairy farms in and around Wolaita Sodo, SNNPR, Ethiopia.** And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary public health.

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DEDICATION

This thesis manuscript is dedicated to my grandmother, W/o Tirunesh and to my mother W/o Aynalem and also to all my family and friends those who are behind my success.

SIGNED STATEMENT OF DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any University and that all sources of material used for the thesis have been duly acknowledged.

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TABLE OF CONTENETS

LIST OF ABBREVIATIONS

a_w	Water Activity
CFU	Colony Forming Units
CMT	California Mastitis Test
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
FEDB	Finance and Economic Development Bureau
HBL	Haemolysin BL
5-HT3	5-Hydroxytryptamine
m.a.s.l	Meter Above Sea Level
MPN	Most Probable Number
MS- Excel	Microsoft Excel
MYB	Mannitol-egg Yolk-phenol red polymyxin - agar
NHE	Non-Hemolytic Enterotoxin
NRPS	Non-Ribosomal Peptide Synthetase
PEMBA	Polymyxin pyruvate Egg yolk Mannitol Bromthymol Blue Agar
SPC	Standared Plate Count
SPSS	Statistical Package for Social Science
UK	United Kingdom
USA	United States of America
WHO	World Health Organization
ZNS	Ziehl-Neelsen Stain

LIST OF TABLES

LIST OF TABLES IN THE APPENDICES

LIST OF FIGURES

LIST OF APPENDICES

A STUDY ON PREVALENCE, PUBLIC HEALTH SIGNIFICANCE AND THE ASSSOCIATED RISK FACTORS OF BACCILUS CEREUS ON BOVINE RAW MILK IN SELECTED DAIRY FARMS IN AND AROUND WOLAITA SODO, SNNPR, ETHIOPIA

ABSTRACT

A cross-sectional study to assess the prevalence of Bacillus cereus, load of the bacteria in raw milk and associated risk factors, and its public health significance was conducted on 384 lactating cows in and around Wolaita Sodo dairy farms 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.

Key words: *Antibiotic susceptibility, Bacillus cereus, Bacterial count, CMT test, Prevalence, Public health, raw milk, Wolaita Sodo.*

1. 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 (Alehegn, 2004; Hoppe *et al.,* 2006).

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 (Zelalem and Faye, 2006 ; Miller *et al.,* 2007). 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 **(**Worku *et al.,* 2012). 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 (FDA, 2005b). Poor handling and undesirable practices such as use of non potable water can also cause spoilage of milk (Paul *et al*., 2004). Thus the safety of milk is threatened by various agents including pathogenic microorganisms, aflatoxins, pesticides and antimicrobial agents (Wang *et al.,* 2006).

Pathogenic microorganisms constitute the most important food related to threat public health (Wang *et al.,* 2006). 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 (Godefay and Molla, 2000).

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 (Chye *et al*., 2004 ; Millogo *et al.,* 2009).

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 (Bottone, 2010 ; Logan *et al.,* 2011) and causes two distinct foodborne illness syndromes and a wide range of opportunistic infections.

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* (Anonymous, 2009). *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 (Kamar *et al*., 2013). 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 (Das *et al.,* 2009).

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 (WHO, 2001).

Bacillus cereus is a Gram positive, rod-shaped, foodborne pathogen and can cause two types of foodborne illnesses; diarrheal and emetic type outbreak (Granum, 2005; Christiansson, 2011). *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 (Jay *et al.,* 2005).

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 (Das *et al.,* 2009).

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 (Katsuya, 2009). 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 (Brooks *et al.,* 2001).

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 (Torkar and Matijaši, 2003).

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 (2012) and in Alage TVET dairy farm by (Seblewengel, 2013).

Therefore, the objectives of this study are:

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

2. LITERATURE REVIEW

2.1. Etiology

The genus *Bacillus* is composed of rod-shaped, endospore forming bacteria that are members of the phylum Firmicutes. Owing largely to the fact that they are common inhabitants of soil and aquatic sediment, species within the genus are widespread in nature and are found in virtually every environment. While their main roles appear to involve carbon and nitrogen cycling, some species are well known human and livestock pathogens (e.g. *Bacillus anthracis* and *Bacillus cereus*) and insect pathogens (e.g. *Bacillus thuringiensis*) (Rooney *et al.*, 2009).

Bacillus cereus is a spore-forming, large Gram-positive rod which is facultatively anaerobic and catalase positive bacterium. The temperature for growth of this microorganism is oscillating between 25 ^{o}c and 75 °c with an optimum growth at 28 ^{o}c–35 °c and pHs between 4.5 and 9.3, even though it can survives at temperatures as low as –5°c. At pH lower than four the growth of *B. cereus* is inhibited. This species is highly lipolytic, saccharolytic and proteolytic and it is pathogenic for humans. *B. cereus* has a wide distribution in nature, frequently isolated from soil and growing plants and can growth in the intestinal tracts of insects and mammals (Stenfors *et al.,* 2008).

It is a saprophyte, widely-distributed in air, soil and water, and is a rare cause of mastitis in cattle (Quinn *et al.,* 1999). Toxin-producing *B. licheniformis* and *B. pumilus* have also been isolated from milk from mastitic cows in Finland and have been described as hazards to public health (Nieminen *et al.,* 2007). Spores are formed under conditions favorable for growth and are resistant to pasteurization. It can exist in two forms vegetative and spore.

2.1.1. Growth and survival of Bacillus cereus

B. cereus has an optimum growth temperature of 30–40 °C, with growth possible between 4° and 55 °C. Strains that grow at 7 °C or below are identified as psychrotrophic and will not grow above 43°C. Mesophilic *B. cereus* usually grows at temperatures of 15–50°or 55 °C. The optimum pH for *B. cereus* growth has been reported as 6.0–7.0, with generation times of approximately 23 minutes at 30 °C. The minimum pH for growth is 5.0 and the maximum is 8.8. In the presence of sodium chloride as humectants, *B. cereus* will not grow at a_w of 0.93. However, when glycerol was used as a humectant, growth was possible at a_w of 0.93, but not 0.92. Only few reports are available regarding the effects of organic acids and chemical preservatives on *B. cereus* (Riemann and Cliver, 2006).

2.1.2. Bacillus cereus spore

All *Bacillus* species can form heat stable endospores (Henriques and Moran, 2007). The bacterial endospore is a resting, dormant, tough, non reproductive structure and it is the most resistant living structure known (Atrih and Foster, 1999). Endospores formed by *Bacillus* and related aerobic endospore-forming Firmicutes are a strategy to survive during unfavorable conditions.

Sporulation involves asymmetric cell division with a copy of the genome partitioned into each of the sister cells. The smaller cell develops into the mature endospore and the mother cell contributes to the differentiation process of the endospore and then autolyses releasing the mature spore into the environment. It takes approximately six hours for the process of spore formation of *B. cereus* to complete (Henriques and Moran, 2007).

Several properties reported for the spores of *B. cereus* make them a problem for the food industry. *B. cereus* spores are highly resistant to adverse conditions such as heat, dehydration, desiccation, radiation, disinfectants and cleaning agents. The spores of *B. cereus* are hydrophobic and adhere to the processing equipment and subsequently form biofilm (Peng *et al.*, 2001).The resistance properties reported for the spores of *B. cereus* are also a problem for human health. *B.*

cereus spores are highly resistant to acidity in a range of media simulating the conditions in the human stomach after food ingestion. The decrease in the spore counts was less than 1.5 log CFU ml -1 after 6 h of incubation at pH 1 and 1.5 (Clavel *et al.*, 2004).

B. cereus spores, as their many different layers can protect the organism in extreme environments. Where raw milk does not generally support the germination of spores, a high temperature short time pasteurization treatment renders the milk as a good germination medium. This then gives the organism a good hold in the food product as a spore which can survive further treatments down the production line, such as desiccation (Becker *et al.*, 1994).

Bacillus cereus spore formation occurs when nutrients are scarce within the environment and germinates into vegetative cells once they are available (Wijnands *et al.*, 2006). Therefore, spore structure is the important part for the survival of this bacterium. The spore's coat is made of proteins, small amounts of lipids and carbohydrates which contribute great resistance to oxidizing agents and chemicals by blocking toxic molecules and its outer structure helps for heat and γ-radiation (Pol *et al.*, 2001). The highly resistant *Bacillus cereus* spores can survive heating, drying, radiation, freezing, and pasteurization (Kotiranta *et al.*, 2000).

2.1.3. Virulence and infectivity

The two types of gastrointestinal diseases caused by *B. cereus* are derived from two significantly different types of toxins. The emetic syndrome is caused by a preformed emetic toxin whereas the diarrheal syndrome is caused by one or more diarrheal enterotoxin produced in the intestine (Granum, 1994).

The emetic toxin or cereulide is produced by a non-ribosomal peptide synthetase (NRPS) complex (Horwood *et al*., 2004; Toh *et al*., 2004). The entire NRPS cluster has been characterised (Ehling-Schulz *et al*., 2006) resulting in a highly specific method for detection of cereulide producing *B. cereus* strains (Fricker *et al*., 2007).

Production of the emetic toxin has been shown to occur in skim milk within the temperature range of 12–37°C, with more toxin produced at 12 and 15°C compared to higher temperatures (Finlay *et al.*, 2000). The emetic toxin is highly resistant to environmental factors, showing stability from pH 2–11 and during heating to 100°C for 150 minutes (pH 8.7–10.6) (Jenson and Moir, 2003).

Three types of enterotoxins are associated with the diarrhoeal form of disease. These include the three component enterotoxin haemolysin BL (HBL), the three component non-haemolytic enterotoxin (NHE) and the single component enterotoxin cytotoxin K. After consumption of food containing *B. cereus*, the enterotoxins are released into the small intestine during vegetative growth following spore germination, and by any surviving vegetative cells (Wijnands *et al*., 2009).

The diarrhoeal enterotoxins can be produced in the temperature range of 10–43°C, with an optimum of 32°C. Production occurs between pH 5.5–10, with an optimum of pH 8. The diarrhoeal enterotoxins are stable at pH 4–11 and inactivated by heating to 56°C for 5 minutes (Jenson and Moir, 2003). Maltodextrin is known to stimulate growth of *B. cereus* and to aid diarrheal enterotoxin production in reconstituted and stored infant milk formulae. It has also been shown that *B. cereus* produces more HBL and NHE under conditions of oxygen tension (low oxygen reduction potential) that simulate the anaerobic, highly reducing fermentative conditions encountered in the small intestine (Zigha *et al.,* 2006).

Up to 26% of *B. cereus* vegetative cells can survive conditions that simulate passage through the stomach. The survival rate of the vegetative cells is dependent on the strain type, phase of vegetative cell growth and the gastric pH (Wijnands *et al*., 2009). As diarrhoeal enterotoxins are unstable at low pH and are degraded by digestive enzymes, any enterotoxins pre-formed in food would be destroyed during passage through the stomach and so not cause illness if ingested (Jenson and Moir, 2003).

In contrast, spores of *B. cereus* are able to pass unaffected through the gastric barrier. The spores contain receptors that need triggering by certain low molecular weight substances to commence

germination. These inducers may be present in the food as well as the intestinal epithelial cells. In the small intestine the spores germinate, grow and produce enterotoxins (Wijnands, 2008).

A crucial virulence factor required for causing the diarrhoeal symptoms is the ability of the vegetative cells and spores of *B. cereus* to adhere to the epithelial cell wall of the small intestine. The adhesion efficiency of spores and cells has been shown to be low, approximately 1% (Wijnands, 2008).

The ability of the enterotoxins to act as tissue-destructive proteins and damage the plasma membrane of the epithelial cells of the small intestine suggests a role for these enterotoxins in causing diarrhoea (Senesi and Ghelardi, 2010). Beecher *et al*. (1995) showed HBL causes fluid accumulation in ligated rabbit ileal loops, implicating a role in diarrhoea. However, direct involvement of NHE and cytotoxin K in causing diarrhoea is yet to be demonstrated (Senesi and Ghelardi, 2010).

Efficient horizontal DNA transfer systems are present within the *B. cereus* group, enabling plasmids to be transferred among strains of different species of this group (*B. cereus, B. anthracis* and *B. thuringiensis*). The plasmids are known to be important determinants of virulence properties of *B. cereus* strains, since they contain genes responsible for virulence such as the *ces* gene cluster required for cereulide formation and emetic disease (Arnesen *et al*., 2008). Furthermore, chromosomal DNA contains genes associated with the diarrhoeal disease, and is therefore present in all strains (Guinebretière *et al*., 2010).

2.2. Epidemiology

2.2.1. Worldwide distribution and outbreak of Bacillus cereus

B. cereus related food poisoning is not a notifiable disease in most countries, and therefore incidence data is extremely limited. It is recognised that there may be significant under reporting of *B. cereus* illness due to the generally mild, short duration and self-limiting symptoms, in

addition to it being infrequently tested for in routine laboratory analyses of stool samples (Hall *et al.*, 2005).

In USA, since 1969, the first well-characterized *B. cereus* outbreak was documented. One study estimated about 84,000 cases of *B. cereus* illness annually in US with an estimate cost of $430/case and a total of $36 million with reports of 37 outbreaks and 571 cases (OSPBH, 2005). Scallen *et al.* (2011) estimated that in the United States (US), *B. cereus* caused 0.7% of foodborne illness caused by 31 major pathogens. In England and Wales, during the period 1993-1999, over 1093 foodborne outbreaks with known causative agent, 2% were caused by *B. cereus* (WHO, 2000).

There was one reported outbreak of *B. cereus* foodborne illness in Australia in 2011 and one outbreak reported in 2010 (OzFoodNet, 2012a; OzFoodNet, 2012b). It has been estimated that *B. cereus* accounts for 0.5% of foodborne illness caused by known pathogens in Australia (Hall *et al.*, 2005). In New Zealand there was one foodborne *B. cereus* outbreak reported in 2011, there were no outbreaks reported in 2010 (Lim *et al.*, 2012).

In the European Union there were 0.04 reported cases of *B. cereus* foodborne illness per 100,000 population in 2011 (ranging from <0.01–0.24 per 100,000 population between countries). This was an increase from the 2010 case rate of 0.02 cases per 100,000 populations (EFSA, 2012; EFSA, 2013).

In Norway, during the period 1988 to 1993 around 33% of reported bacterial foodborne poisoning cases were linked to *B. cereus* (Granum and Baird-Parker, 2000). In France, from 1998 to 2000, *B. cereus* represented 4 to 5 % of foodborne poisoning outbreaks of known origin (Haeghbaert *et al*., 2002).

In Netherlands, during the period 1993-1998, 2% of foodborne outbreaks were caused by *B. cereus* (Brandsema *et al.*, 2004). *B. cereus* was also reported as a major causative agent of foodborne illness in the Netherlands in 2006 causing 5.4% of the foodborne outbreaks and in Norway in 2000 causing 32% of foodborne outbreaks (Wijnands 2008).

2.2.2. Reservoirs of Bacillus cereus

B. cereus is an ubiquitous environmental rod; exactly, its natural reservoir is represented by decaying organic matter and vegetables, fresh and marine waters, along with the intestinal tract of invertebrates from which soil and food may become contaminated (leading to human gut transient colonization) (Bottone, 2010). When Bacilli come into contact with organic matter or within an insect or animal host, they may lose their flagella, attach to the arthropod enteric epithelium, and sporulate (Bottone, 2010).

B. cereus also has a saprophytic life cycle where spores germinate in the soil, with the production of vegetative bacilli, that could then sporulate, keeping the cycle; following host defecation or death, cells and spores are released into the soil, where vegetative organisms may sporulate again, then surviving until the uptake by another host (Bottone, 2010).

2.2.3. Risk factors for contamination of milk

Temperature plays a major role in the diversification of microorganisms and spoilage of milk during transport, processing and storage. During storage under low temperature the milk undergoes spoilage due to proteinases and lipases released by psychrotrophic bacteria (Braun and Fehlhaber, 2002). *Bacillus cereus* is able to grow at refrigeration temperatures and alter the milk by producing heat resistant proteolytic enzymes which induce degradation of casein (Vyletelova *et al.,* 2000).

Feed and feces are sources of milk contamination (Magnusson *et al.,* 2007). Spores are present in high concentrations in deep sawdust bedding of housed animals (Magnusson *et al.,* 2007) and in the soil of grazed areas (Christiansson *et al.,* 1999). The concentration of spores in air, in feed, feces and milking equipment are important sources of raw milk contamination. Foremilk had a much higher spore concentration than mid or late-stream milk (Magnusson *et al.,* 2007). The spore content of milk in cows is strongly associated with degrees of soil contamination with teats, dirtiness of the cow and its concentration (Christiansson *et al.,* 1999).

The strains and concentrations of microorganisms transmitted from the farm environment to milk via the exterior of teats depend on the composition of the attached dirt and microbial concentration in the dirt. When cows are at pasture, the teats are predominantly contaminated with soil, whereas teats of cows housed in the barn are mainly contaminated with faeces and bedding material (Christiansson *et al.,* 1999; Magnusson *et al.,* 2007). The contamination of teats with soil during the grazing period is considered to be the main cause of elevated concentrations of spores of *B. cereus* in bulk tank milk (Vissers *et al.,* 2007).

Milk from a cow with an infected udder is likely to contain a large number of organisms. Mastitis, which is a disease causing inflammation of the udder, contributes considerable number of organisms, sometimes even blood cells into the milk. The hair, dirt and dust often fall from the animal body into the milking pails or the teat cups of milking machines. Dried dirt and filth is picked up by all movements and carried about as dust in the atmosphere. For this reason, dust may be the source of almost every kind of contamination (Christiansson *et al.,* 1999).

Utensils and equipments are known to be the greatest sources of contamination. They may account for as much as 100, 000 to a billion organisms per milliliter. Pails, strainers, milking machines, cans, pipes, bottles, and other equipments used for the handling of milk are sometimes not properly washed and sanitized. All persons involved in the milking process must be in good health and careful in their personal cleanliness. Probably the majority of milk borne epidemics of disease was started by workers who were carriers, or who had mild cases or who were in close contact with others so affected. Water from surface supplies is contaminated by dust, animals, plants, people, and other agents (Magnusson *et al.,* 2007).

2.2.4. Host factors that influence disease

Animals and humans, specifically those who are immunocompromised, intravenous drug users, or neonates (murray *et al*., 2007; Logan *et al*., 2006; Rosovitz *et al*.,1998) are described as being of special risk for *Bacillus cereus* food borne disease. However, individuals with lowered stomach acidity, for example elderly people, may be more susceptible to *Bacillus cereus*

diarrhoeal disease, because a larger number of cells are expected to survive gastric acid (Kotiranta *et al.*, 2000).

All people are believed to be susceptible to *B. cereus* food poisoning. However, some individuals, especially young children, are particularly susceptible and may be more severely affected. Individuals vary in their response to cereulide dosages; this may be associated with differences in the number of 5-HT3 receptors in the stomach and small intestine of individuals (Wijnands, 2008).

The risk of illness after ingestion of vegetative cells is influenced by the strain, composition of the food, the liquid nature of the food and the age of the individual. Liquid foods are transported faster to the small intestine and therefore are protected from the influence of gastric conditions, providing more opportunity for survival of the pathogen (Wijnands, 2008).

2.2.5. Occurrence of Bacillus cereus in Milk

B. cereus is resistant to pasteurization (Granum, 2005) and has been successfully isolated from pasteurized, raw milk and other dairy products worldwide (Larsen and Jorgensen, 1999) (Table 1). *Bacillus cereus* can cause foodborne illness through the production of toxins that cause gastroenteritis (Jay *et al.,* 2005)and both the diarrhoeal strains (In't Veld *et al.*, 2001), and the emetic toxin producing strains of *B. cereus* (Svensson *et al.*, 2006) have been found in milk. *B. cereus* is a widespread contaminant of raw milks (Banykó and Vyletelová, 2009; Shaheen *et al.*, 2010)

Table 1: Occurrence of *Bacillus cereus* in milk and its products

Authors and year	Prevalence	Samples types	Methods	Countries
(Christiansson *et al.*, 1999)	10-880 spores/L	Dairy products	Culture and spore count	Sweden
(Schlegelova *et al.*, 2003)	31% (66 /215)	Dairy products	Culture and SPC	Czech Republic
(Mogessie, 2006)	$>10^9$ cfu/ml Mesophilic bacteria	Fermented dairy and beverages products	Culture filtrates SPC	Ethiopia
(Hempen *et al.*, 2004)	35.2% (206)	Raw and sour milk	Standard Plate Count (SPC)	Senegal
	33.3% (268)	Raw and sour milk	Standard Plate count	Guinea
	17% (378)	Raw and sour milk	Standard Plate count	The Gambia
(El-Tabiy *et al.*, 2009)	25% (10/40)	skim milk	Culture and SPC	Egypt
	47.5% (19/40)	pasteurized full cream	Culture and total viable count	Egypt
(Muhamed *et al.*, 2010)	10% (8/80)	Raw milk sample	Culture and total viable count	South India
(Alemnh,2012)	15.4%(59/384)	Raw milk sample	Culture and total viable count	Ethiopia
(Seblewegel,2013)	15.86%(59/372)	Raw milk sample	Culture and total viable count	Ethiopia

Source: (Seblewongel, 2013)

2.2.6. Mode of transmission

B. cereus food poisoning can be caused by either ingesting large numbers of bacterial cells and/or spores in contaminated food (diarrheal type) or by ingesting food contaminated with pre-formed toxin (emetic type). Transmission of this disease results from consumption of contaminated foods, improper food handling/storage and improper cooling of cooked foodstuffs (Schneider *et al*., 2004).

2.2.7. Clinical symptoms of disease caused by Bacillus cereus

B. cereus causes two types of food borne illness – emetic (vomiting) and diarrheal syndromes. The emetic syndrome is an intoxication that is caused by ingestion of a cyclic peptide toxin called cereulide that is pre-formed in the food during growth by *B. cereus*. This syndrome has a short incubation period and recovery time. The symptoms of nausea, vomiting and abdominal cramping occur within 1–5 hours of ingestion, with recovery usually within 6–24 hours (Schoeni and Wong, 2005; Senesi and Ghelardi, 2010).

The diarrheal syndrome is caused by enterotoxins produced by *B. cereus* inside the host. The incubation period before onset of disease is 8–16 hours and the illness usually lasts for 12–14 hours, although it can continue for several days. Symptoms are usually mild with abdominal cramps, watery diarrhea and nausea (Granum, 2007).

In a small number of cases both types of toxin are produced, and emetic and diarrhoeal symptoms occur (Montville and Matthews, 2005). Neither form of illness is considered life-threatening to normal healthy individuals, with few fatal cases reported (Jenson and Moir, 2003). *B. cereus* has been associated with non-food related illness, although this occurs rarely. The bacterium has been found in postsurgical and traumatic wounds and can cause opportunistic infections, especially in immunocompromised individuals, such as septicaemia, meningitis and pneumonia. *B. cereus* has

also been known to occasionally cause localised eye infections in humans (Schoeni and Wong, 2005).

2.2.8. Public health and economic significance of Bacillus cereus

Bacillus cereus potential to cause systemic infections is of current public health and biomedical concern. The bacterium has been found in postsurgical and traumatic wounds and can cause opportunistic infections, especially in immunocompromised individuals (Schoeni and Wong, 2005). The spectrum of infections apart from gastrointestinal infections caused by *B. cereus* include Respiratory tract infections, Nosocomial infections, Eye infections, CNS Infections, Cutaneous infections, Endocarditis, Osteomyelitis and Urinary tract infections.

Apparently healthy people with neither immunocompromission nor underlying predisposing conditions can develop *B. cereus* pneumonia, as well, although that of welders seems to be a category of subjects that show an increased professional risk to acquire *B. cereus* airway infections (Hoffmaster, 2006).

Severe and lethal *Bacillus cereus* infections have been described in newborn infants, with higher frequency among premature infants. The types of *B. cereus* infections in newborns included central nervous system, respiratory tract, primary bacteremia, and sepsis (Lebessi *et al.,* 2009 *and* Manickam *et al.,* 2008). Nosocomial outbreaks of *B. cereus* implicating hospital linens, manual ventilation balloons, contaminated diapers, and contaminated ventilator equipment have also been reported (Sasahara *et al.,* 2011 and Kalpoe *et al.,* 2008).

Multiplication of *B. cereus* in dairy products is not only concern of public health hazard but also a cause of economic losses through spoilage of contaminated products. The role of *B. cereus* in outbreaks of foodborne illness is well documented. A large number of viable cells (10^5-10^6 cfu/ml) of *B. cereus* are required to cause illness (Quinn *et al.,* 1999). Thermo-labile *Bacillus cereus* enterotoxins contaminating the raw materials of food are likely to be detoxified by heat, but no method is known for detoxifying the emetic toxin (cereulide) in food (Elina, 2008).

2.3.Diagnosis of *Bacillus cereus* Food Poisoning

2.3.1. Isolation and Identification of Bacillus cereus

Selective media is primarily used to isolate *B. cereus* by direct plating. MYP (Mannitol-yolk-polymyxin) and PEMBA (Polymyxin-pyruvate-egg yolk-mannitol bromothymol blue agar) are the most widely used selective media for isolating *B. cereus*. Both the media are based on the diagnostic features of *B. cereus* of lecithin hydrolysis and inability to ferment Mannitol. *B. cereus* forms peacock blue and pink colonies on PEMBA and MYP respectively, surrounded by a halo of lecithin hydrolysis. Polymyxin acts as the selective agent by inhibiting the growth of competitive organisms Pyruvate in the medium improves the egg-yolk precipitation reaction and a low level of peptone enhances sporulation. Colonies of *B. cereus* can be confirmed by a microscopic procedure combining a spore stain with an intracellular lipid stain. Spores appear green in a cell with red vegetative cytoplasm and containing black lipid globules. For further identification and confirmation several biochemical reactions are in use like glucose fermentation, nitrate reduction and Voges Proskauer reaction, hemolytic activity, motility, growth characteristics (rhizoidity), and protein toxin crystal identification may be prescribed (Borge *et al*., 2001).

Biochemical confirmation can be based on an isolate's ability to produce acid from glucose but not from mannitol, xylose and arabinose (Adams and Moss, 2008).

2.3.2. Enumeration of Bacillus cereus colonies

Viable counts involve counting cells that can be cultured and/or are metabolically active. Total counts involve counting all cells including dead or inactive cells. Direct methods of enumeration involve counting actual cells or colonies and indirect methods involve estimating the number of

cells based on cell mass by scattering light through a culture (spectroscopy) or by a statistical method called the MPN (most probable number) technique (USDA, 1998).

Plate count of *Bacillus cereus*

This is done by preparing serial dilutions from 10^{-2} to 10^{-6} by transferring 10 ml homogenized sample (1:10 dilution) to 90 ml dilution blank, mixing well with vigorous shaking and continuing until 10^{-6} dilution is reached. Inoculate duplicate MYPA plates with each diluted sample (including 1:10) by spreading 0.1 ml onto surface of each plate with sterile glass spreading rod. Incubate plates for 24 hrs at 30 °C and observe for colonies surrounded by precipitate zone, which indicates that lecithinase is produced by microbes. *B. cereus* colonies are usually a pink color that becomes more intense after additional incubation (USDA, 1998).

Select plates that contain an estimated 15-150 eosin pink lecithinase producing colonies. Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies that are typical of *B. cereus*. This is the presumptive plate count of *B. cereus*. Pick 5 or more presumptive positive colonies from MYPA plates and transfer to nutrient agar slants for confirmation as *B. cereus*. Calculate number of *B. cereus* cells/ml of sample, based on percentage of colonies confirmed as *B. cereus*. For example, if average count obtained with 10^{-4} dilution of sample is 65 and 4/5 colonies tested are confirmed as *B. cereus*, the number of *B. cereus* cells/ml of milk is 65 x 4/5 x 10,000 x 10 = 5,200,000 *B. cereus*. Multiplying by ten is used due to dilution factor which is tenfold higher than original sample; dilution of only 0.1 ml (not 1 ml) sample was tested. For this dilution, plates containing too many colonies (>150) and too small colonies (<15) should be discarded as a rule (USDA, 1998).

Most probable number (MPN) of Bacillus cereus

The MPN technique is recommended for enumerating *B. cereus* in foods that are expected to contain fewer than ten *B. cereus* organisms/ml. Inoculate 3-tube MPN series in trypticase soy-polymyxin broth, using 1 ml inoculum of 10^{-1}, 10^{-2} and 10^{-3} dilutions of sample with 3 tubes for each dilution. (Additional dilutions may be used if *B. cereus* colonies expected to exceed 10^{3}/ml). Incubate tubes 48 ± 2 hrs at 30 °c and observe for dense growth which is typical of *B. cereus*. Streak cultures from positive tubes onto separate MYP agar plates and incubate for 24-48 hrs at 30 °c. Pick one or more eosin pink, lecithinase positive colonies from each MYP agar plate and transfer to nutrient agar slants for confirmation as *B. cereus* and calculate MPN of *B. cereus* cells/ml of sample based on the number of tubes at each dilution in which the presence of *B. cereus* was confirmed (FDA, 2013).

2.3.3. Tests used to confirm and differentiate B. cereus

Motility test, rhizoid growth on nutrient agar, test for hemolytic activity and test for protein toxin crystal production were applied to differentiate typical strains of *B. cereus* from other members of the *B. cereus* group, including *B. mycoides*, *B. thuringiensis*, and *B. anthracis* also non-motile (FDA, 1998).

Rhizoid growth: Pour 18-20 ml nutrient agar into sterile 15 × 100 mm petridishes and allow agar to dry at room temperature for 1-2 days. Inoculate by gently touching surface of medium near center of each plate with 2 mm loopful of 24 h culture suspension. Allow inoculums to be absorbed and incubate plates 48-72 h at 30°C. Examine for development of rhizoid growth, which is characterized by production of colonies with long hair or root-like structures that may extend several centimeters from site of inoculation. Rough galaxy-shaped colonies are often produced by *B. cereus* strains and should not be confused with typical rhizoid growth, which is the definitive characteristic of *B. mycoides*. Most strains of this species are also non-motile.

Test for hemolytic activity: Mark bottom of a plate into 6-8 equal sections with felt marking pen, and label each section. Inoculate a premarked 4 cm sq area of trypticase soy-sheep blood agar plate by gently touching medium surface with 2 mm loopful of 24 h culture suspension. (Six or more cultures can be tested simultaneously on each plate.) Incubate plates 24 h at 35°C. Examine

plates for hemolytic activity. *B. cereus* cultures usually are strongly hemolytic and produce 2-4 mm zone of complete (β) hemolysis surrounding growth. Most *B. thuringiensis* and *B. mycoides* strains are also β-hemolytic. *B. anthracis* strains are usually nonhemolytic after 24 h incubation.

Motility test: Inoculate *Bacillus cereus* motility medium by stabbing down the center with 3 mm loopful of 24 h culture suspension. Incubate tubes 18-24 h at 30°C and examine for type of growth along stab line. Motile organisms produce diffuse growth out into the medium away from the stab. Non-motile organisms produce growth only in and along stab. Alternatively, add 0.2 ml sterile distilled water to surface of nutrient agar slant and inoculate slant with 3 mm loopful of culture suspension. Incubate slant 6-8 h at 30°C and suspend 3 mm loopful of liquid culture from base of slant in a drop of sterile water on microscope slide. Apply cover glass and examine immediately with microscope for motility. Report whether or not isolates tested were motile. Most strains of *B. cereus* and *B. thuringiensis* are motile by means of peritrichous flagella. *B. anthracis* and all except a few strains of *B. mycoides* are non-motile. A few *B. cereus* strains are also non-motile.

Test for protein toxin crystals: Inoculate nutrient agar slants with 3 mm loopfuls of 24 h culture suspensions. Incubate slants 24 h at 30°C and then at room temperature 2-3 days. Prepare smears with sterile distilled water on microscope slides. Air-dry and lightly heat-fix by passing slide through flame of Bunsen burner. Place slide on staining rack and flood with methanol. Let stand 30s, pour off methanol, and allow slide to air-dry. Return slide to staining rack and flood completely with 0.5% basic fuchsin or TB carbolfuchsin ZN stain. Heat slide gently from below with small Bunsen burner until steam is seen.

Wait 1-2 min and repeat this step. Let stand 30 s, pour off stain, and rinse slide thoroughly with clean tap water. Dry slide without blotting and examine under oil immersion for presence of free spores and darkly stained tetragonal (diamond-shaped) toxin crystals. Crystals are usually somewhat smaller than spores. Toxin crystals are usually abundant in a 3- to 4-day-old culture of *B. thuringiensis* but cannot be detected by the staining technique until lysis of the sporangium has occurred. Therefore, unless free spores can be seen, cultures should be held at room temperature for a few more days and re-examined for toxin crystals. *B. thuringiensis* usually produces protein toxin crystals that can be detected by the staining technique either as free crystals or parasporal

inclusion bodies within the exosporium. *B. cereus* and other members of the *B. cereus* group do not produce protein toxin crystals.

On the basis of the test results, identify as *B. cereus* those isolates which are actively motile and strongly hemolytic and do not produce rhizoid colonies or protein toxin crystals. Nonmotile *B. cereus* strains are also fairly common and a few strains are weakly hemolytic. These nonpathogenic strains of *B. cereus* can be differentiated from *B. anthracis* by their resistance to penicillin and gamma bacteriophage. A crystalliferous variant of *B. thuringiensis* and non rhizoid strains derived from *B. mycoides* cannot be distinguished from *B. cereus* by the cultural tests (FDA, 2013).

2.4. Treatment of *Bacillus cereus*

Therapeutic options against *B. cereus* diseases usually revolve around the antibiotic susceptibility profile of the isolated strain, although species-specific criteria for testing and interpreting the *in vitro* response to drug have not been defined, yet. In the setting of a suspected *B. cereus* infection, however, empirical antimicrobial treatment may be indispensable pending the antibiotic sensitivity testing results (Bottone, 2010). In general, most *B. cereus* isolates are resistant to penicillins and cephalosporins as a consequence of β-lactamase production; in particular, it is likely that resistance to penicillin, ampicillin and cephalosporins should be considered to be constant, nowadays, like that to trimethoprim (Brown, 2012).

Among betalactams, then, *B. cereus* strains have been found to potentially express resistance to all among ampicillin, penicillin G (and penicillins in general), ampicillin-sulbactam, amoxicillin-clavulanic acid, oxacillin, as well as to cephalosporins (including third and fourth generation compounds such as ceftriaxone, cefotaxime, and cefepime) (Katsuya, 2009; Frankard, 2004; Bottone, 2010; Savini, 2009).

Resistances may also include those to cotrimoxazole, clindamycin, erythromycin, tetracyclines, and carbapenems (imipenem, meropenem), so it may be difficult to choose a proper empirical treatment; nonetheless, *B. cereus* has been found to be alternatively susceptible to these

compounds (Katsuya, 2009; Frankard, 2004; Miller, 2012; Bottone, 2010; Brown, 2012; Savini, 2009). Molecules to which isolates may be susceptible also include fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), chloramfenicol, aminoglycosides (amikacin, gentamicin) and glycopeptides (vancomycin) (Katsuya, 2009; Frankard, 2004), and a recent *in vitro* study showed 100% sensitivities of 42 strains to rifampin, daptomycin, and linezolid (Bottone, 2010); in agreement, strains from our laboratory collection showed low MICs for daptomycin , as well. Unusual data were reported instead by Brown, who found combined vancomycin and daptomycin resistance in six isolates cultivated from an organ preservation fluid; such a finding clearly suggested differences in membrane composition of those organisms compared with susceptible strains described by other authors throughout the literature (Brown, 2012). Hence, it is hard to foresee *B. cereus* behaviour under antibiotic exposure, and susceptibility testing is needed (case-by-case) in order to provide patients with adequate therapies. In general, however, it seems that susceptibility to clindamycin, erythromycin, chloramphenicol, ciprofloxacin, vancomycin, aminoglycosides, and tetracycline is frequent, today, and vancomycin, clindamycin, imipenem and aminoglycosides have been alternatively recommended as treatment options against severe *B. cereus* diseases (Katsuya, 2009; Strauss, 2001; Bottone, 2010); instead, broad-spectrum cephalosporins and ticarcillin-clavulanate should be avoided in the empirical treatment when an infection by this orgamism is suspected (Bottone, 2010). However, it must be noted that serious infectious processes may require the use of a combined antibiotic therapy (Frankard, 2004).

Because antibiotic therapy typically destroys the planktonic *Bacilli* but not the biofilm, prolonged antibiotic therapy is indicated in high-risk patients, along with the removal of the infected devices. For deep-seated visceral abscesses (e.g., cerebral abscesses), surgical drainage often is needed in addition to antimicrobial treatment (Sankararaman and Velayuthan, 2013).

2.5. Prevention and Control Methods

The predominating contributing factors associated with *B. cereus* gastroenteritis, in order of relative importance, are improper holding temperature, contaminated equipment, inadequate cooking, and poor personal hygiene. The heat treatment normally used in food preparation, except for pressure cooking, may not destroy *B. cereus* spores.

Widespread occurrence of *B. cereus* in the natural environment ensures continued recovery of this organism from milk and other dairy products during all stages of production. Unlike other milk borne pathogens, heat-resistant *B. cereus* spores readily germinate as a result of pasteurization with outgrowth and enterotoxin production occurring in products stored at temperatures near refrigeration. However, because *B. cereus* populations greater than 10^5cfu/g are invariably needed to induce illness, dairy-related outbreaks of *B. cereus* poisoning are readily prevented by minimizing contamination of raw milk at the farm level and storing both fluid and reconstituted milk at temperatures less than or equal to 4°C. Active starter cultures also minimize growth of this organism during manufacture of fermented dairy products (Langeveld *et al*., 1996).

Control of *Bacillus cereus* during food processing can be achieved by heating up to proper temperature in an appropriate time. Decreasing pH values to ≤4 and increasing levels of sodium chloride to ≥1.0% decreases growth rate and increased the lag phase of *Bacillus cereus*. The combination of decreasing pH, increasing salt concentration and setting storage temperatures below 12 ^{0}C is sufficient to inhibit *Bacillus cereus* growth after heat treatment at 90 ^{0}C for 10 min (Martinez *et al.*, 2007).

The most important control measure is to keep food at a temperature at which the spores do not germinate and cells do not grow. This can be achieved by uniform quick chilling of the food to 4-5°C or holding the food above 60°C. Quick chilling can best be accomplished by storing a food in a shallow container, no more than 5 to 6 cm thick. Because *B. cereus* cells, given sufficient time, can grow and produce toxins at refrigerated temperature (≥4°C), a food should not be stored at low temperatures for long periods of time. This means that preparation of a food well in advance should be avoided. Because cells can get in a food through cross-contamination, proper

sanitary measures should be adopted while handling a food. Finally, as live cells are necessary for the symptoms, there should be uniform reheating a suspected food to above 75°C before serving. However, heating may not destroy heat-stable toxins associated with emetic symptoms (Ray, 2005). *Bacillus cereus* group strains shows expanded diversity, storing foods below 10°C prevents growth of strains that produce emetic toxin. Hence, reducing the storage temperature reduces the diversity of *B. cereus* population able to multiply below 10 centigrade (Granum, 2005).

Because of the ubiquitous nature of this organism, general hygiene measures related to feeding, milking and milk storage areas are required to reduce contamination of cow teats or the bulk milk tank directly. Pre-milking teat cleaning methods reduced spore count following experimental challenge. The most effective methods for reducing milk spore content (96% reduction) were use of a moist washable towel, with or without soap, followed by drying with a dry paper towel, for a total time of 20 s per cow (Magnusson *et al.*, 2007).Cleaning of teats prior to milking with an individual wet paper towel also halved the concentration of spores in milk (Christiansson *et al.*, 1999).

3. MATERIALS AND METHODS

3.1. 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 (Tamirat, 2007). 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 (Wolaita Zone Agricultural Office, 2011).

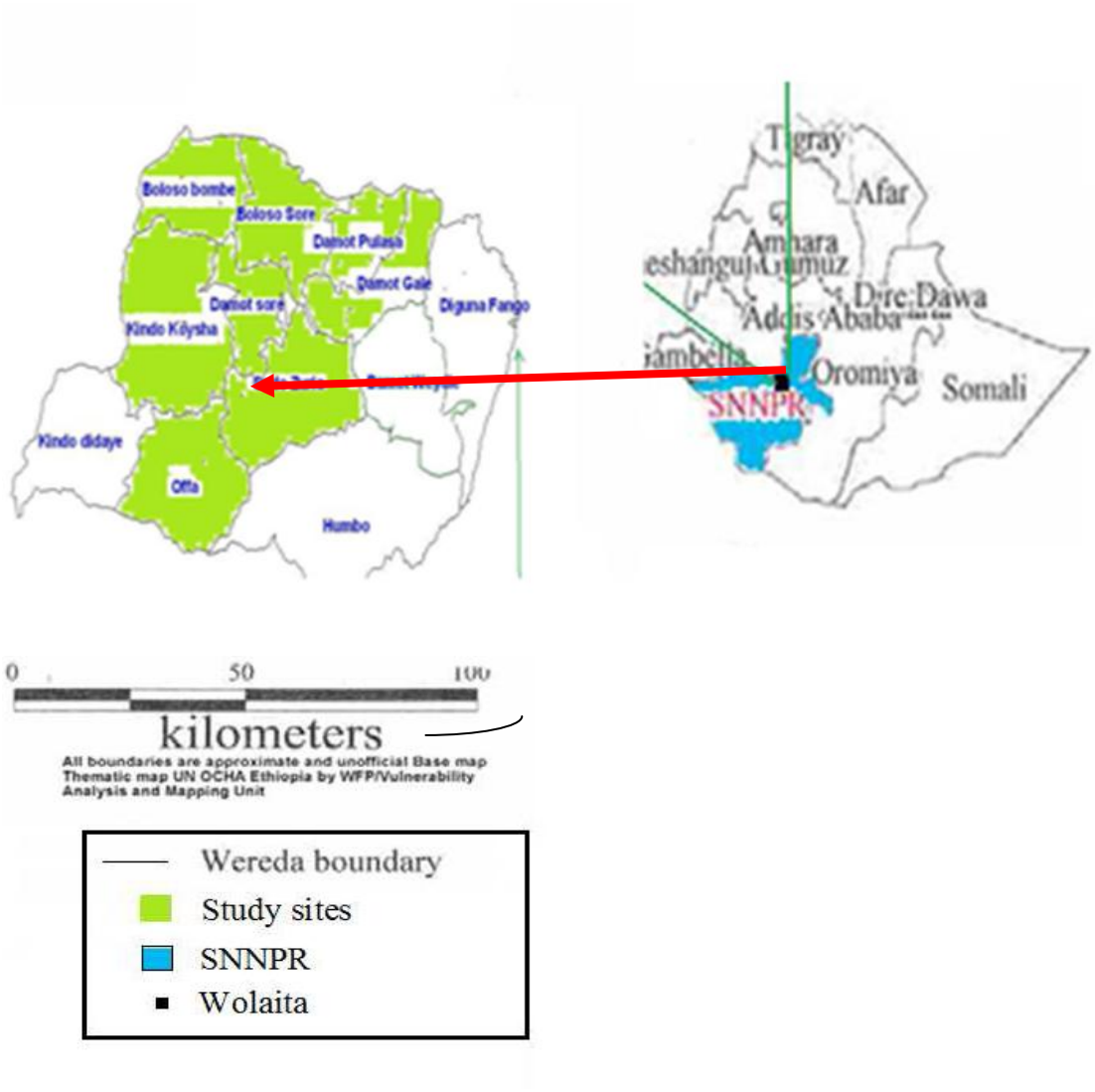

Figure 1: *A map of Wolaita Sodo, SNNPR*

Source*: FEDB, 2009*

3.2. 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.

3.3. Study population

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

3.4. 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 (2005). 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 (2005). 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

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

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

3.5. Bacteriological analysis

3.5.1. 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).

3.5.2. 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 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.

3.5.3. 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.

3.5.4. 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 (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 (Tallent and Rhodehamel, 2012).

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

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).

3.5.5. 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.*

3.6. 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 (NCCLS, 2003; Quinn *et al.,* 2004).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 (Quinn *et al.*, 2004).

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 NCCLS (1997).

3. 7. 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.

4. 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.*

4.1. 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 legal limit in cow raw milk intended for human consumption were 67.74% (42/62) of the cows' udder were affected with *Bacillus cereus* (Table 2).

4.2. Association of *Bacillus cereus* Contamination

Analyses were made to see at the association of *Bacillus cereus* occurrence in raw milk with various host and management factors and the findings are presented as follows:

4.2.1. CMT score

The contribution of mastitis as a source of *Bacillus cereus* in raw milk was determined by associating *Bacillus cereus* isolation rates with various CMT scores. *Bacillus cereus* was isolated from cows with CMT score of negative, trace, +1, +2 and +3 at the rates of 22.66%, 0%,

2%, 4.88% and 4.76%,respectively ($p<0.05$); therefore, presence of mastitis is an important contributor of occurrence of the bacteria in milk (Table 2).

Table 2: 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%)			

4.2.2. Management system

The contribution of management systems of the dairy farms for occurrence of *Bacillus cereus* in milk was significantly associated with the positivity of *Bacillus cereus. Bacillus cereus* was isolated from cows in semi- intensive and intensive dairy farms at the rates of 23.85% and 13.09%, respectively ($p<0.05$) (Table 3)

Table 3: Association of *Bacillus cereus* contamination with management

Risk Factors	Category	Observation	*Bacillus Cereus* Positivity		X^2	df	P-Value
			Positive	Negative			
Management System	Intensive	275	36(13.09%)	239	6.678	1	.010
	Semi-intensive	109	26(23.85%)	83			

4.2.3. Bacillus cereus load

The *Bacillus cereus* load from raw milk samples ranged from $1.04x10^3$ to $1.05x10^6$ colony forming unit per milliliter (CFU/ml). The bacterial loads in CFU/ml of most of milk samples were above legal limit ($>10^5$CFU/ml) in raw milk. From positive samples of cow raw milk that was found above the legal limit was 66.12%**.** *Bacillus cereus* load count was highly significant with the positivity of *Bacillus cereus* ($p<0.05$) (Table 4).

Table 4: Association of *Bacillus cereus* contamination with bacterial load

Risk Factors	category	Observation	*Bacillus Cereus* Positivity	X^2	df	P-Value
CFU	Above legal limit(>10^5)	41	41	384.000	2	.000
	Below legal limit(<10^5)	21	21			

4.2.4. Floor type

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 ($p<0.05$) (Table 5).

Table 5: Association of *Bacillus cereus* contamination with floor type

Risk Factors	**Category**	**Observation**	***Bacillus Cereus* Positivity**	**df**	**X^2**	**P-Value**
Floor Type	soil(muddy)	118	27(22.88%)	1	5.708	.017
	Concrete(Cemented)	266	35(13.16%)			

4.2.5. Lactation stage

The contamination of raw milk with *Bacillus cereus* observed in animals with lactation stage 1-3, 4-6, 7-9 and lactation stage of more than nine were 16.46%, 18.18%, 14.92%, and 11.63%, respectively. However, there was insignificant association of lactation stage with positivity of *Bacillus cereus* (P>0 .05) (Table 6).

Table 6: Association of *Bacillus cereus* contamination with lactation stage

Risk Factor	Category	Observation	*Bacillus Cereus* Positivity	df	X^2	P-value
lactation stage	1-3lactation	164	27(16.46%)	3	1.071	.784
	4-6lactation	110	20(18.18%)			
	7-9lactation	67	10(14.92%)			
	10 and above lactation	43	5(11.63%)			

4.2.6. Parity

The contamination of raw milk with *Bacillus cereus* observed in animals with parity 1-3, 4-6 and parity of more than six were 17.08%, 15.83% and 13.04%, respectively. However, there was insignificant association of parity with positivity of *Bacillus cereus* (P>0.05) (Table 7).

Table 7: Association of *Bacillus cereus* contamination with parity

Risk Factors	Category	Observation	*Bacillus Cereus* Positivity	X^2	df	P-Value
Parity	1-3 parity	199	34(17.08%)	0.467	2	.792
	4-6 parity	139	22(15.83%)			
	7 and above parity	46	6(13.04%)			

4.2.7. 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 8).

Table 8: 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	1-3	164	27(16.46%)	0.610	1.304	0.469	3.624
stage	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	

4.3. 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 9).

Table 9: 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%	-	-

4.4. 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 10).

Table 10: 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.

4.5. 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 11).

Table 11: 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

5. 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 (2012), Seblewengel (2013) and (Gilles *et al., 2002*) who reported isolation rates of 15.4%, 15.86%, 15.4% and respectively.

However, reports of higher contamination were reported by (Yobouet *et al.*, 2014),(Adesina *et al.* , 2011), (Hassan *et al.*, 2010), (Haughton *et al.*, 2010), (Rezende-lago *et al.*, 2007), (Ombui and Nduhiu, 2005), (EI- Shinawy ,2004), (Ayuob *et al*., 2003), (Schelegelova *et al.*, 2003) and (Te Giffel and Beumer, 1998) 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, 2013), 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, 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 $1.04x10^3$-$1.06x10^6$CFU/ml ($5.25x10^5$ave.). This finding is lower when compared with the work of (DeGraff *et al.*, 1997) ($3.88x10^7$CFU/ml), (Godifay and Molla, 2000) ($1.9x10^8$CFU/ml), (Bonfoh *et al*., 2003) from Senegal (10^7CFU/ml) and (Esther *et al.*, 2004) ($3x10^7$CFU/ml); however, it is

in the same range with the finding of (Seblewngel, 2013) (4.29×10^5) and (Alemneh, 2012) ($2.8x10^5$) from Ethiopia, (Rai and Dawvedi, 1990) from India ($7.7x10^5$CFU/ml), (Kurwijilla *et al*., 1992) from Tanzania (10^5CFU/ml), (Ombui *et al.*,1995) from Kenya ($5x10^4$CFU/ml), and (Bonfoh, 2003) 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* (Seblewengel, 2013).

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, 2013).

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, 2013), (Girma *et al.,* 2012) and (Gizaw, 2004) from Ethiopia, (Jones and Turnbull, 1981) from British and (Schiefer *et al*., 1976) from Canada and (Horváth *et al.,* 1986) 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.,* (1997),

Christiansson *et al*., (1999) and Magnusson *et al*., (2007). 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, 2007).

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 (Yilma, 2012). Therefore, it is likely that raw milk might be contaminated from soiled bedding and soil (Connor, 1995).

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 (Alehegne, 2004). 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 (Hayat, 2013).

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 (Seblewengel, 2013).

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 (Normanno *et al.*, 2007).

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, 2014), (Seblewengel, 2013) and (Agwa *et al.*, 2012). 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.

6. **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:

1. 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.

2. 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.

3. Treatments of *Bacillus cereus* infected cows should be done based on drug susceptibility testing.

4. Milk for public consumption should be properly boiled at appropriate temperature and time.

5. Epidemiological investigation should be undertaken to know the distribution of the bacteria in dairy cows in Ethiopia

6. Since it is an emerging disease to many countries different types of laboratory tests should be applied for the detection of toxin from *Bacillus cereus* in our country.

7. 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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8. APPENDICES

Appendix I: Questionnaires

A. Questionnaires for Public Health Implication Assessment

Respondent name _____Date_____

Address/kebele _____

1. Do you consume milk?

   A. Yes B. No

2. How do you consume the milk at home?

   A. Raw B. Fermented products (yogurt, cheese)

   C. After boiling D. Other form E. C and B

3. Did one of your family members become ill after consuming the milk?

   A. Yes B. No

4. If yes, which of the following signs did he or she showed?

   A. Fever and abdominal pain B. Diarrhea

   C. Vomiting/ Abdominal cramp D. All of these

   E. None F. if other sign, list here----------

5. What did you do when your milk become spoiled?

   A. Fed to children B. Made yogurt E. unobserved

   C. Fed to dogs D. Discarded F. just simply drinks it

6. Do you know any milk borne diseases?

   A. Yes B. No

7. If yes, list few signs……

8. For how much time did you use once boiled milk without re-heating?

A. 1-2 hours C. For 6 hours E. For 12 hours

B. 2- 4 hours D. For 8 hours F. if other----------

9. Which kinds of water sources do you use for drinking?

A. River water B. Well water C. Tap water D. Stagnant water

10. How often do you and your family consume the milk and its products?

A. For breakfast B. As common diet C. Rarely

D. frequently E. as required F. Not at all

11. Is there any time gap between taking & using the milk? A. yes B. No

12. If yes how much is the average time gap? _____

15. How and where do you preserve milk

16. How did you clean your equipment to take the milk from the distributer? Using

A. Boiled water only B. Boiled water & detergent

C. tap water only D. tap water & detergent

18. Cleaning the milk utensil before milking

Yes

No

18. Washing the hand before milking

Yes

No

19. Source of water used for washing milking utensils

B. Questionnaires for Milk Contamination Assessment

1. Cleaning the milk utensil before milking

Yes [ ]

No [ ]

2. Washing the hand before milking

Yes [ ]

No [ ]

3. Source of water used for washing milking utensils

A. River water B. Well water C. Tap water D. Stagnant water

4. Which kinds of water sources do you use for drinking the cows?

A. River water B. Well water C. Tap water D. Stagnant water

5. Hygienic status of the barn

A. Poor B. Moderate C. Good

6. Cleaning of hands between each milking

A. Yes B. No

7. Supply of towel

A. No B. Common C. Separate

8. Uses of detergent

A. Yes B. No

9. Washing of udder and teat before milking

A. Yes B. No

Appendix II: Equipment and materials

1. Pipettes, 1, 5, and 10 ml, graduated in 0.1 ml units
2. Glass spreading rods (hockey stick) 3-4 mm diameter with 45-55 mm spreading area
3. Incubators, 30 ± 2°C and 35 ± 2°C
4. Colony counter
5. Marking pen, black felt type
6. Bunsen burners
7. Wire loops, No. 24 nichrome or platinum wire, 2 mm and 3 mm
8. Vortex mixer
9. Microscope, microscope slides, and cover slips
10. Culture tubes, 13 x 100 mm, sterile
11. Test tubes, 16 x 125 mm, or spot plate
12. Bottles, 3 oz, sterile
13. Anaerobic jar, BBL GasPak, with H_2 + CO_2 generator envelopes and catalyst
14. Water bath, 48-50°C
15. Culture tube racks
16. Staining rack
17. Petri dishes, sterile, 15 x 100 mm
18. Dilution bottles, 6 oz (160 ml),
19. borosilicate-resistant glass, with rubber stoppers or plastic screw caps
20. Thermometers (mercury) appropriate range
21. Plate count agar (standard methods) (M1242)
22. Refrigerator, to cool and maintain samples milk, 0-4.4°C
23. pH meter
24. Storage space, free of dust and insects and adequate for protection of equipments

Appendix III: Media and reagents used for the study

1. Bacillus cereus selective agar base (MC0167, Oxoid, Ltd.)
2. Egg yolk emulsion, 50% (M514)
3. Nutrient agar (CM0003)
4. Blood Agar (CM0854; Oxoid Ltd)
5. Motility medium (SIM) (M181)
6. dilution water (R1117) sterilized, with 450 ± 5 ml and 90 ± 2 ml
7. Gram stain reagents (R3219)
8. Basic fuchsin staining (R320)
9. Methanol 95%, Ethanol 70%

Appendix III: Composition and preparation of media used for the study

A. Blood agar (Oxoid, England)

Typical formula (g/l):

'Lab-Lemco' powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0

Final pH 7.3 ± 0.2 at 25^0C

Instructions for use:

Suspended 40g in 1 litre of demineralised(distilled) water. Bring to the boil to dissolve completely. Sterilized by autoclaving at 121^0C for 15 munites. Cool to 45-50^0C and add 7% sterile defibrinated blood and display on sterile petridish

B. Nutrient agar (Oxoid, England)

Typical formula (g/l):

'Lab-Lemco' powder	1.0

Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

Final pH 7.4 ± 0.2 at 25^0C

Instruction for use:

Suspend 28g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes and display on sterile petridish.

C. Muller Hinton Agar

Formula

Beef extract	2 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g

Instruction for use:

Suspended 38 g of the powder in 1 liter of purified water mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121 ^{o}c for 15 minute. Do not over heat. Display on sterile petridish.

D. *Bacillus cereus* selective agar base (CM0167, Oxoid),

Typical Formula of *Bacillus cereus* Agar base

Typical formula	g/L
Peptone	1.0
Mannitol	10.0
Sodium Chloride	2.0
Magnesium Sulfate	0.1
Disodium Phosphate	2.5
Potassium dihydrogen Phosphate	0.25
Sodium Pyruvate	10.0
Bromothymol Blue	0.12
Agar	15.0
Supplement Polymyxin B (100,000IU)	0.015
Supplements Egg Yolk Emulsion	50 ml
Final Ph 7.2 $\pm$ 0.2 at 25 ^{0}C	

Instruction for use:

Suspended 20.5g in 475ml of distilled water and brought gently to the boil to dissolve completely. Sterilized by autoclaving at 121^{0}C for 15 minutes. Cooled to 50 ^{0}C and aseptically add the contents of supplements reconstituted as directed. Mix well and poured into sterile Petri dishes.

Appendix III: Detailed sampling procedures

A. Label tubes prior to sampling by (date, farm, cow, any changes in milk)
B. Brush loose dirt, bedding, and hair from the udder and teats and thoroughly wash
C. Discard several streams of milk from the teat (strict foremilk) and observe milk
D. Dip all quarters in an effective pre-milking teat disinfectant

E. Dry teats thoroughly with an individual towel.

F. Beginning cleaning teats on the far side of the udder, scrub teat ends vigorously

G. Begin sample collection from the closest teat and move to teats on the far side of the udder.

H. Remove the cap from the tube or vial but do not set the cap down or touch the inner surface of the cap.

I. Always keep the open ends of the cap facing downward. Maintain the tube or vial at approximately a 45 degree angle while taking the sample. Do not allow the lip of the sample tube to touch the teat end. Collect one to three streams of milk and immediately replace and tightly secure.

Appendix IV: chemicals and procedures for staining

A. Gram stain

Reagents: 1% aqueous Crystal violet
Gram's Iodine
Safranin
Distilled H_20
Alcohol (70%)

Procedures:

1. Prepare thin fixed smears of culture by adding 2 – 3 loopfuls of tap water onto slides, then using a flamed loop aseptically transfer a small amount of culture to a slide. Emulsify the bacterial cells in the water over an area of approximately 1square cm. Allow smears to dry and fix heat fix gently passing on flame.

2. Place the prepared smears on the staining rack – apply crystal violet to just cover the smear – usually one or two drops. Leave the dye on for one minute, then rinse off in a gentle stream of water. Shake the slides to remove excess water.

3. Flood the smears with the gram's iodine solution; allow standing for one minute, washing with water.

4. Add alcohol to the smear and gently rock the slide, tip off alcohol and repeat. Contact time with the alcohol should be approximately for 30 seconds by this time most of the blue coloration should be removed. Wash in water.

5. Stain with safranin for 10 seconds, wash in water, shake off excess water and allow airing dry or gently blotting dry with tissue paper taking care not to remove the cells.

6. Examine microscopically, and record cell morphology and gram reaction.

B. Rapid Confirmatory Staining Procedure

This staining method was developed by Holbrook and Anderson combining the spore stain of Ashby and the intracellular lipid stain of Burdon (Holbrook and Anderson, 1980).

Fuchsin solution preparation:

0.3 g of basic fuchsin

10 ml of ethanol, 95% (v/v)

5 ml of phenol, heat-melted crystals

95 ml of distilled water

Dissolve the basic fuchsin in the ethanol; then add the phenol dissolved in the water. Mix and let stand for 2-3 days. Filter before use.

Decolorizing solvent (alcohol) is 97 ml of ethanol, 95% (v/v)

Malachite green stain:

(0.5% (wt/vol) aqueous solution:

0.5 g of malachite green

100 ml of distilled water

Sudan black B is a lysochrome (fat soluble dye) predominantly used for demonstrating triglycerides in frozen sections. It is also valuable for demonstrating some protein bound lipids in paraffin sections. It may also stain other materials, not being completely restricted to lipids as the other dyes used.

Safranin counterstain:

Stock solution	(2.5% (wt/vol) alcoholic solution)
	2.5 g of safranin
	100 ml of 95% ethanol
Working solution	10 ml of stock solution
	90 ml of distilled water

Procedure:

1. Prepare films from the centre of a 1 day old colony or from the edge of a 2 day colony.
2. Air-dry the film and fix with minimal heating.
3. Flood the slide with aqueous 5% w/v malachite green and heat with a flaming alcohol swab until steam rises. Do not boil.
4. Leave for 2 minutes without re-heating.
5. Wash the slide with running water and blot dry.
6. Flood the slide with 0.3% w/v Sudan black in 70% ethyl alcohol. Leave for 15 minutes.
7. Wash the slide with running xylene from a wash bottle for 5 seconds.
8. Blot dry using filter paper.
9. Flood the slide with aqueous 0.5% w/v safranin for 20 seconds.
10. Wash under running water.
11. Blot dry and examine under the microscope using the oil immersion lens. A blue filter may be used to accentuate the appearance of the lipid granules but this will give a blue color cast to the red of the cytoplasm.

Differential characteristics of large-celled Group *B. cereus* members

Appendix VII: Differential characteristics of large-celled Group I *Bacillus* species

Appendix table 1: Differential characteristics of large-celled Group I *Bacillus* species

Feature	*B. Cereus*	*B. thuringiensis*	*B. mycoides*	*B. weihenstephanensis*	*B.anthracis*	*B. megaterium*
Gram reaction	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Motility	+	+	-	+	-	+/-*
Nitrate reduction	+	+	+	+	+	–
Tyrosine decomposing	+	+	+/–	+	–	+/–
Lysozyme resistance	+	+	+	+	+	–
Egg yolk reaction	+	+	+	+	+	–*
Anaerobic Glucose use	+	+	+	+	+	–
VP reaction	+	+	+	+	+	–
Ferment mannitol	–	–	–	–	–	+
Hemolysis on Sheep Blood agar	+	+	+	-	–	–
Para-sporal crystal formation	-	+	-	-	-	-

Known characterstics	Enterotoxin production	Endotoxin crystal (insecticide) production	Rhizoidal growth	Growth at 6 ^{0}C ; no growth at 43^{0}C	Pathogenic to animals and humans

-*, most strains are negative +/−*, 50-50% of strains are positive.

Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998, Chapter 14. Updated February 2012

Appendix VIII: Standards for interpretation

Appendix table 2: Zone Size Interpretive Chart for Antimicrobials

Antimicrobial agents	disc potency	resistance	intermediate	susceptible
Clindamycin	10µ	≤ 15	16-18	≥19
Tetracycline	10µ	≤ 14	15-18	≥19
Penicillin for staph	10 U	≤ 20	21-28	≥ 29

Penicillin for others	10 U	≤ 11	12-14	≥ 22
Vancomycin	30 mcg	≤ 9	10-11	≥12
Chloramphenicol	30 mcg	≤12	13-17	≥18
Gentamycin	10 mcg	≤12	-	≥13
Kanamycin	30 µ	≤13	14-17	≥18
Ampicillin	10µ	≤13	14-16	≥17
Polymixin B	300u	≤11	-	≥12

Source: NCCLS, (1998) and C LSI, (2012)

Appendix table 3: Interpretation of CMT Scores and Corresponding SCC values

CMT Score	**Visible reaction**	**SCC range (Cells per ml)**	**Somatic cell score**	**Approximate SCC midpoint**

Negative	Mixture remains liquid – no evidence of precipitate	0 – 200,000	0 1 2 3 4	12,500 25,000 50,000 100,000 200,000
Trace	Slight precipitate, best seen by tipping, disappears with continued movement	150,000 – 500,000	5	400,000
1	Distinct precipitate but no tendency toward gel formation	400,000-1,500,000	6	800,000
2	Mixture thickness immediately, moves toward center	800,000 – 5,000,000	7 8	1,600,000 3,200,000
3	Gel forms and surface becomes convex	>5,000,000	9	6,400,000

Source: NCCLS, (1998)

Appendix IX: Data collection sheet for each small household.

Appendix table 4: Data collection sheet on dairy farms

No	**D**	**ON**	**A**	**AC**	**P**	**LL**	**Floor type**		**Management system**			**MQA**
							Concrete(Cemented)	**Natural soil**	**Intensive**	**Semi-intensiv**	**Extensive**	

										e		

Key: A-Address, AC-Animal Code, D-Date, LL-Lactation Length, MQA-Milk Quality Assessment, ON-Owner name and P-Parity.