ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

STUDY ON THE PREVALENCE OF *BACILLUS CEREUS* AND ASSOCIATED RISK FACTORS IN BOVINE RAW MILK IN DEBRE ZEIT, ETHIOPIA

By

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ABBRREVIATIONS

µm	micro meter
ANOVA	Analysis of Variance
APHA	American Public Health Association
B. cereus	*Bacillus cereus*
CDC	Centers for Disease Control
CFU	Colony Forming Units
CI	Confidence Interval
CSA	Central Statistics Agency
df	degrees of freedom
DVM	Doctor of Veterinary Medicine
E	East
E.C	Ethiopian Calendar
FDA	Food and Drug Administration
g	gram
GLM	Generalized Linear Model
HACCP	Hazard Analysis and Critical Control Point
HPA	Health Protection Agency
hrs	hours
ICMSF	International Commission on Microbiological Specifications for Food
IDF	International Dairy Federation
Inc	Incorporation
ISO	International Standardization Organization
IU	International Unit
Km	Kilometer
Km^2	Square kilometer
L	Liter
Ltd	Limited
mg	milligram
ml	millilitre
mm	millimeter
MPN	Most Probable Number

MSc	Master of Science
MYPA	Manitol-Egg Yolk Polymyxin Agar
N	North
OSPBH	Opinion of Scientific Panel on Biological Hazards
PEMBA	Polymyxin Egg-yolk Manitol Bromothymol Blue Agar
pH	power of Hydrogen
PhD	Doctor of Philosophy
PMO	Pasteurized Milk Ordinance
Pp	Pages
ppm	parts per million
P-Value	Probability Value
rRNA	ribosomal Ribonucleic Acid
Sig	Significant
SIM	Sulfur-Indol-Motility
SPC	Standard Plate Count
UK	United Kingdom
USA	United States of America
USDA	United States Drug Administration
V/V	Ratio of Volume to Volume
VP	Voges Proskauer
W/V	Ratio of Weight to Volume
WHO	World Health Organization

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ABSTRACT

The objectives of this study were to determine the prevalence of *B. cereus*, evaluate the load of *B. cereus* and assess risk factors associated with *B. cereus* load and its public health implication in raw milk samples collected from dairy farms in Debre Zeit, Ethiopia. A total of 384 raw milk samples were collected from eleven randomly selected dairy farms. The overall prevalence of *B. cereus* in raw milk sample was 15.4% (59). The *B. cereus* count ranges from 2.3 x 10^4 - 5.4 x 10^5 CFU/ml. From positive samples, 58 (15.1%) of total samples have significant counts (> 10^5 CFU/ml) which was above legal limit in raw milk intended for human consumption. The mean of *B. cereus* counts differed significantly between farms and within parities ($p < 0.05$). An attempt was made to assess hygienic keeping quality of milk at farm level by using semi structured questionnaire survey. Our study results indicated that raw milk samples collected from both zero-grazing and semi-intensive farm management systems were highly contaminated with *B. cereus*, exceeding the legal limit set for raw milk (> 10^5 CFU/ml), suggesting effective hygienic measures should be introduced in milk value chains during milk production, distribution and processing and food service establishments to avoid public health hazards.

Keywords: *Bacillus cereus, Count, Ethiopia, Prevalence, Public health, Raw Milk*

1. INTRODUCTION

Bacillus cereus is Gram-positive, rod-shaped, β-hemolytic, endospore forming aerobic or facultatively anaerobic bacterium which is widely distributed in nature and the most important pathogen found in dairy products (Christiansson *et al.,* 1999).

Bacillus cereus causes many problems in the dairy industries hampering the quality of raw milk and its products producing spoilage enzymes such as proteases, lipases and lecithinases (Fagerlund *et al.,* 2004). The most important milk spoilages because of *B. cereus* are 'bitty cream' (floating clumps of fat) due to lecithinase activity, 'sweet curdling' (curdling without acidification) and bitter-rotten off-flavours due to protease activity and fruity-rancid off-flavours due to lipolytic activity (Stenfors *et al.,* 2008).

In dairy industries, *B. cereus* and its spores have very diverse contamination sources like soil, bedding, feed, dust, air, feces, dirty teats and milking equipment (Christianson *et al.,* 1999; Fagerlund *et al.,* 2004; Stenfors *et al.,* 2008). Aerobic spore forming *B. cereus* contaminates raw milk through both the vegetative form and the heat resistant spore form. It is specifically this spore configuration that poses great problems. Once these spores have intruded into the raw milk, they cannot be destroyed by conventional heating processes, to assure safety of the product such as pasteurization for 15 seconds at 72 ^{0}C and ultra high temperature (UHT) treatment for 2 – 5 seconds at 140 – 145 ^{0}C (Scheldeman *et al.,* 2006). The production heat resistant spores in raw milk, and its adherence to metal surfaces makes *B. cereus* an unwelcome, but very frequent contaminant of dairy products (Faille *et al.,* 2001).

Another important threats of *B. cereus* is its ability to grow at the storage temperature of milk (4 – 7 ^{0}C), which mainly determines the shelf life of pasteurized milk and derived milk products as well as toxins production causing public health hazard (Griffiths and Schraft, 2002; Chen *et al.,* 2003). *Bacillus cereus* causes two kinds of foodborne intoxications, an emetic (vomiting) intoxication due to the ingestion of a toxin (cereulide) pre-formed in the food and a diarrheal infection due to the ingestion of bacterial cells or spores (enterotoxins) in the small intestine (Dierick *et al.,* 2005). The diarrheagenic enterotoxin, which is heat labile (sensitive to heat), is produced by vegetative form of *B. cereus* during growth in the small intestine and in the food (Reyes *et al.,* 2007). The emetic toxin is heat stable (resistant to heat

126 ^{0}C for 90 min) and resistant to long Ph ranges 2–11, and enzymatic digestion (From *et al.,* 2005).

In general, the study of *B. cereus* in relation to dairy industries has gained significance in the nature of its ability to form heat resistant spores in milk, the capacity to grow in a wide varieties of dairy products and its ability to produce toxins adapting chemicals, heat treatments and cold environment posing risks to public (Schlegelova *et al.,* 2003; Ouoba *et al.,* 2008). *Bacillus cereus* is particularly challenging to control in dairy industries due to possibilities of milk contamination at numerous points in production and processing units causing quality defects, limiting the shelf life of the milk and posing public health.

Different research papers over the world reported that *B. cereus* is the predominant microorganism in dairy farms and raw milk via diverse contamination sources and have a negative impact on milk quality and safety; however, no previous research has been done in Ethiopian context, on the prevalence and microbial loads of *B. cereus* in raw bovine milk.

Therefore, the objectives of this study were

- To determine the prevalence of *Bacillus cereus* in raw bovine milk.
- To evaluate *Bacillus cereus* load in raw bovine milk.
- To assess risk factors associated with *Bacillus cereus* load in raw milk and its public health significance

2. LITERATURE REVIEW

2.1. Characteristics of *Bacillus cereus*

Bacillus cereus is Gram-positive, motile, facultative anaerobic, spore-forming and mesospheric bacterium, with growth temperature ranging from 10 – 48 ^{0}C with optimal growth between 28 ^{0}C and 35 ^{0}C, at Ph values of 4.9 to 9.3 and water activities of 0.92 to 1.0 (OSPBH, 2005). The organism does not ferment mannitol and has a very active phospholipase and lecithinase enzymes (Nieminen *et al.,* 2007). *Bacillus cereus* vegetative cell has dimensions of about 1.0-1.2 μm by 3.0-5.0 μm and forms ellipsoidal spores in a central or terminal position without swelling the sporangium (Stenfors *et al.,* 2008).

2.2. Taxonomy of *Bacillus cereus*

Since the first edition of the Bergey's manual of systematic bacteriology, the structure and content of the Genus *Bacillus* have been substantially modified (Xu and Côté, 2003). The *Bacillus* species belonging to the "*B. cereus* group" have 99-100% similarities in 16S Rrna sequence and are polyphyletic (Bavykin *et al.,* 2004); however, additional sequencing of the gyrase A and gyrase B genes (Chun and Bae, 2000; Wang *et al*., 2007) aid the useful key to discriminate some strains of these species. The "Hypercat" database provides a global view of the phylogenetic structure of *B. cereus* group combining several approaches; Multi Locus Sequence Typing, Amplified Fragment Length Polymorphism and Multi Locus Enzyme Electrophoresis (Tourasse *et al.,* 2010), based on the seven phylogenetic *B. cereus* cluster defined by Guinebretière *et al.* (2008). As result, the current taxonomy of *B. cereus* group consists (*B. cereus senso xperi, B. thuringiensis, B. anthracis, B. pseudomycoides and B.weiihenstephansis* (Stenfors *et al.*, 2008).

Morphological and biochemical test approaches also showed a high diversity in phenotypic and genotypic within isolates of *B.* cereus group (Lopez and Alippi, 2007); however, *Bacillus* species can be broadly divided into three groups based on the morphology of the spores and their sporangium (HPA, 2007). According to HPA, (2007), those clustered at group1 consists Gram positive, possessing licithinase system, producing central or terminal ellipsoidal/cylindrical spores without distend sporangium are (*B. cereus, B. anthracis, B. megaterium, B. mycoides* and *B. thuringiensis*); and clusters in group$_2$ consists Gram variable,

possess ellipsoidal spores with swollen sporangia and cluster group$_3$ consists Gram-variable, with bulged sporangia having terminal/sub-terminal spherical spores.

2.3. *Bacillus cereus* sporulation

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 *B. cereus* spores can survive heating, drying, radiation, freezing, and pasteurization temperature (Kotiranta *et al.,* 2000).

Spore germination is commonly in response to L-alanine which stimulates germination events including hydrations of spores, loss of Ca^{2+} and dipicolinic acid, and metabolism (Pol *et al.,* 2001) (Figure 1).

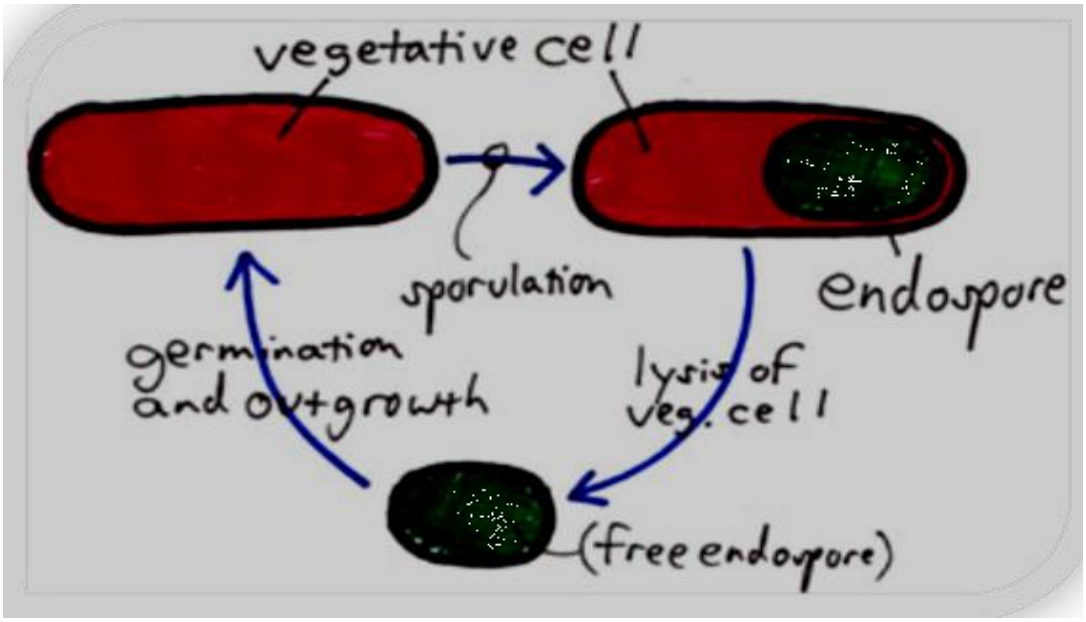

Figure 1: *Bacillus cereus* spore cycle
Source*:* ***http://www.splammo.net/bact102/102bacillus.html Accessed on 8/3/2011***

2.4. Worldwide importance and outbreak history

The genus *Bacillus* was coined in 1835 by Christian Gottfried Ehrenberg who coined genus *Bacterium* seven years prior and later amended by Ferdinand Cohn to spore-forming, gram-positive/variable, rod-shaped bacteria as genus *Bacillus* and *B. cereus* was added fifteen years later (Cohon, 1872). In 1887, *B. cereus* was isolated from air in cow's shed by Frankland, and since 1950, many outbreaks from a varieties of foods were reported (WHO, 2001).

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). 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 Netherlands, during the period 1993-1998, 2% of reported outbreaks were caused by *B. cereus* (Brandsema *et al.,* 2004). 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). In France, from 1998 to 2000, *B. cereus* represented 4 to 5 % of foodborne poisoning outbreaks of known origin (Haeghbaert *et al*., 2002).

2.5. Occurrence of *Bacillus cereus* in milk

Bacillus cereus species can be found in both the spore and vegetative form in raw and pasteurized milk (Singleton, 2004). *Bacillus cereus* spores have been reported as the main source of contaminants of raw milk and its products (Lin *et al.,* 1998).

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

Authors	Prevalence	Samples types	Methods	Countries
Christiansson *et al.* (1999)	10 – 880 spores/L	Dairy products	Spore count	Sweden
Schlegelova *et al.* (2003)	31% (66/215)	Dairy products	SPC	Czech Republic
Hempen *et al*. (2004)	35.2% (206)	Raw and Sour milk	SPC	Senegal
	33.3% (268)	Raw milk	SPC	Guinea
	17% (378)	Raw milk	SPC	Gambia
El-Tabiy *et al.* (2009)	25% (10/40)	Skim milk	SPC	Egypt
	47.5% (19/40)	Pasteurized full cream	SPC	Egypt
Muhamed *et al.* (2010)	10% (8/80)	Raw milk sample	SPC	South India

2.5.1. Risks and sources of *Bacillus cereus* milk contamination

Since *B. cereus* is a ubiquitous bacterium, living in a soil and in foods of plant and dairy origin (Granum and Baird-Parker, 2000). The concentration of spores in air, in feed, feces, in soil deep sawdust bedding of housed animals and milking equipment are important sources of raw milk contamination (Magnusson *et al.,* 2007). The spore content of raw milk is strongly associated with degrees of soil contamination with teats, udder (Christiansson *et al.,* 1999).

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. 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 carries microbes with dust in the atmosphere. For these reasons, dust is the main source of contamination for utensils and equipments (Christiansson *et al.,* 1999). Pails, strainers, milking machines, cans, pipes, bottles, and other equipment used for the handling of milk are sometimes not properly washed and sanitized. In dairy farm, water from surface supplies is contaminated by dust, animals, plants, people, and other agents (Magnusson *et al.,* 2007).

2.5.2. Public health and economic significances of *Bacillus cereus*

Bacillus cereus produces many types of toxins, two of which are most frequently associated with food poisonings. The thermo-labile enterotoxins that are destroyed when food is heated and usually associated with foods that were insufficiently cooked or contaminated after cooking whereas, the emetic toxin, which is not inactivated by heating of food and usually associated with highly cooked and fried or boiled and rapidly cooled foods (Quinn *et al.*, 1999; Granum, 2007). The diarrheal syndrome is associated with meat and dairy products while the emetic toxin is linked to starchy products such as cereals and rice (Jay *et al.,* 2005).

The most common is a diarrheal illness caused by a heat-labile toxin, accompanied with abdominal pain with incubation period of 4-16 hrs and symptoms lasting 12-24 hrs. The emetic illness: characterized by vomiting and nausea that usually occurs within 1-5 hrs of contaminated food ingestion (FDA, 2007). *Bacillus* foodborne illnesses occur due to survival of the bacterial endospores when food is improperly cooked (Turnbull, 1996). 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 enterotoxins contaminating the raw food are likely to be detoxified by heat, but no method is known for detoxifying the emetic toxin (cereulide) in food (Elina, 2008) (Figure 2).

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. Most psychrophiles produce extracellular enzymes in large quantities and some of these are mainly lipases and heat stable proteases which affect the storage stability of milk. The protease attack proteins and are responsible for the development of bitter flavor or gelatin in milk (Quinn *et al.,* 1999). The rancid flavor produced in milk and in milk products through the action of microbial lipases is mainly the liberation of butyric acid from triglycerides by the lipases (Walstra *et al*., 1999).

Mesophilic bacteria are the groups which grow best at normal temperature ranging from about 20 to 40 ^{0}C and known by attacking the milk sugar lactose and convert it to lactic acid. Their uncontrolled growth gives rise to the souring and even curdling of milk stored at ambient temperatures (Harding, 1999). Thermophilic organisms, although they are less harmful to health, are responsible for changes in organoleptic properties of milk products and can endure heat of pasteurization (Harding, 1999).

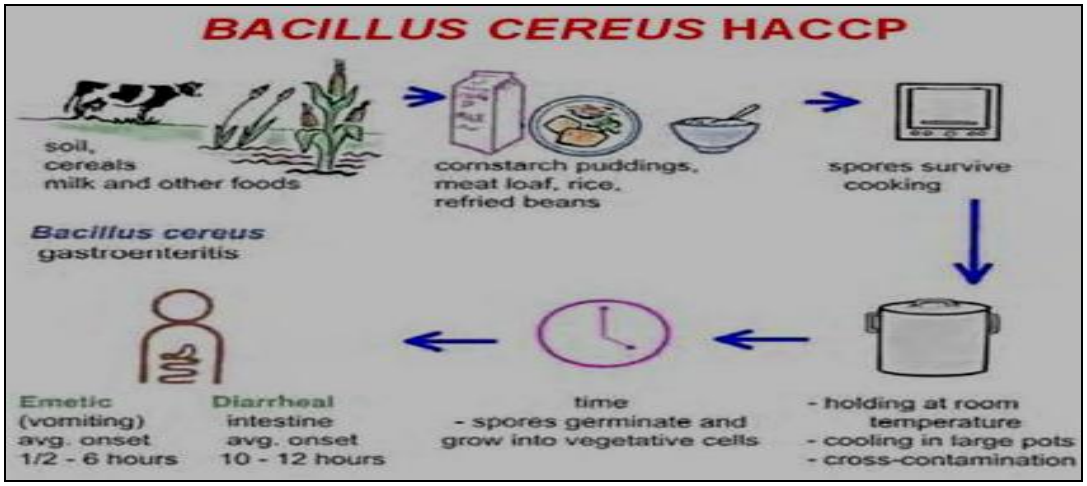

Figure 2: The public health significance of *Bacillus cereus* species
Source: ***www.fda.gov/downloads/Food/FoodSafety//Operators/UCM077957***

2.6. Virulence factor in *Bacillus cereus*

The virulence factor of *B. cereus* encounters problems when food is improperly refrigerated, that is allowing the endospores to germinate (Davis *et al.*, 2008). Bacterial growth results in production of enterotoxins, and ingestion leads to two types of illness: diarrheal and emetic syndrome (Ehling-Schulz *et al.*, 2004). The diarrheal type is associated with a wide-range of foods, has an 8 to 16.5 hrs incubation time and causes confusion to differentiate from poisoning caused by *Clostridium perfringens* (Guinebretière *et al.*, 2002). The emetic form is commonly caused by rice that is not cooked for a time and temperature sufficient to kill any spores and then improperly refrigerated as result toxin (cereulide) is produced, which is heat stable. This form leads to nausea and vomiting 1–5 hrs after consumption creating confusion to distinguish from *Staphylococcus aureus* food poisoning (Hoton *et al.*, 2005).

The diarrheic syndromes in patients are thought to stem from the chromosomal genes (nhl/hbe/cytK) of *B. cereus* leading to three toxins: Hemolysin BL (Hbl), Nonhemolytic Enterotoxin (Nhe) and Cytotoxin K *(CytK)* toxins (Guinebretière *et al.*, 2002). The proteins exhibit a structure called "beta-barrel" that can insert into cellular membranes due to a hydrophobic exterior, thus creating pores with hydrophilic interiors. The effect is loss of cellular membrane potential and eventually cell death. CytK is a pore-forming protein more related to other hemolysins (Ehling-Schulz *et al.*, 2004). The emetic syndrome is caused by a toxin called cereulide that is found only in emetic strains. Cereulide contains 3 repeats of 4

amino acids produced by non ribosomal peptide synthesis. Cereulide is believed to activate 5-HT_3 (serotonin) receptors to increased afferent vagus nerve stimulation (Hoton *et al.,* 2005).

2.7. Control at food production and from foodservice establishments

Control of *B. 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 *B. cereus*. The combination of decreasing Ph, increasing salt concentration and setting storage temperatures below 12 ^{0}C is sufficient to inhibit *B. cereus* growth after heat treatment at 90 ^{0}C for 10 minutes (Martinez *et al.*, 2007).

A combination of electrolyzed water and 1% citric acid exhibits synergistic effect on the inactivation of *B. cereus* vegetative cells and spores (Park *et al.,* 2009). Using acetic acid-based disinfectant is better than amphoteric surfactant based disinfectant (Ernst *et al.,* 2006). *Bacillus cereus* counts tremendously decreased by 3.5 log numbers at 1.0 ppm ozone concentration for 360 minutes ozone treatment and up to 2 log reductions in the number of *B. cereus* spores were observed above 1.0 ppm ozone concentration at the end of 360 minutes of ozonation (Akbas and Ozdemir, 2008).

In general, food production and foodservice establishments must use heating methods that destroy vegetative cells and most spores. Cooked food should not be stored at room temperature. Foodservice personnel should be trained to use good personal hygiene and proper methods of hand washing. Methods that adequately clean and sanitize surfaces, equipment and utensils should be used. As a routine practice, clean all bench tops, cutting boards and utensils with detergent and follow up with a sanitizer which kills bacteria. ***www.marion.sa.gov.au. Accessed on August, 10/2011.***

2.8. Isolation and enumeration of *Bacillus cereus*

2.8.1. Isolation media for *Bacillus cereus* strains

Some common media for enumeration, identification and isolation of *B. cereus* in food (Mossel, *et al.,* 1967; Holbrook and Anderson, 1980) are Bacillus cereus selective agar base (CM0167), Bacillus cereus selective agar (PEMBA), Mannitol-Egg-Yolk Polymyxine (MYP), Bacillus cereus rapid agar (BACARA®), COMPASS Bacillus cereus agar.

Polymyxin Egg-yolk Mannitol Bromothymol Blue Agar (PEMBA) is described as selective medium for *B. cereus* isolation and enumeration. Principally in medium, Peptone provides nitrogen and amino acids. Sodium chloride maintains the osmotic balance of the medium; Mannitol is the carbon source that serves as the fermentable carbohydrate, fermentation of which can be detected by the Ph indicator. Mannitol fermenting organisms like *B. megateruim* yield yellow colored colonies.

When chromogenic mixture present in the medium is cleaved by the enzyme β-glucosidase found in *B. cereus* resulting in the formation of blue colonies. Magnesium sulphate provides ions; disodium phosphate and mono-potassium phosphate constitute the buffer system of the medium. Sodium pyruvate stimulates the growth of microorganisms. Bromothymol blue is the Ph indicator. Agar is the solidifying agent. Polymyxin B is the selective agent and has a bactericide activity against Gram-negative microorganisms. The egg yolk emulsion is incorporated to detect the proteolytic activity (ICMSF, 1996).

After 18-24 hours of incubation at 30°C in aerobic conditions, *B. cereus* shows crenated, colonies about 5 mm in diameter, turquoise blue in color, surrounded by a distinct opaque zone of egg yolk precipitation of the same color as the colonies. Bacillus cereus selective agar (CM0617, Oxoid) is described based on the highly specific diagnostic and selective PEMBA medium, developed by Holbrook and Anderson (1980), for the isolation and enumeration of *B. cereus* in foods. It meets the requirements for a medium that is sufficiently selective to be able to detect small numbers of *B. cereus* cells and spores in the presence of large numbers of other food contaminants. The medium is also sufficiently diagnostic that colonies of *Bacillus cereus* are readily identified and confirmed by microscopic examination.

In the formulation of Bacillus cereus selective agar, a peptone level of 0.1% and the addition of sodium pyruvate improve egg yolk precipitation and enhance sporulation. Bromothymol

blue is added as a Ph indicator to detect mannitol utilization. The medium is made selective due to Polymyxin B Supplement (SR99) which gives a final concentration of 100IU of polymyxin B per ml of medium. Polymyxin B, as a selective agent for the isolation of *B. cereus* has been previously suggested by Donovan (1958) and found to be satisfactory (Mossel *et al.*, 1967). It is recommended that, where large numbers of fungi are expected in the inoculums, cycloheximide (SR222) is added to the medium at a final concentration of 40mg/l.

2.8.2. Presumptive *Bacillus cereus* colony identification

The primary diagnostic features on the medium are colonial morphology, precipitation of hydrolyzed lecithin around colonies and the failure of *B. cereus* to utilize mannitol sugar. The typical colony of *B. cereus* on Bacillus cereus selective agar (CM617, Oxoid) is crenate, about 5mm in diameter and have a distinctive turquoise to peacock blue color surrounded by egg yolk precipitation of the same color. These features distinguish *B. cereus* group from other *Bacillus* species.

Other egg yolk reacting organisms which can grow on the medium, including *Staphylococcus aureus, Serratia marcescens* and *Proteus vulgaris,* are distinguished from *B. cereus* group by colony form and color. These organisms also produce an egg yolk-clearing reaction in contrast to egg yolk precipitate produced by *B. cereus* group. Microscopic examination for presence of lipid globules in the vegetative cells is recommended as a rapid and confirmatory test for *B. cereus* and replaces the need for biochemical testing (Holbrook and Anderson, 1980). They confirmed that only *B. cereus* from *Bacillus* species possess lipid globule in its vegetative cell. One further advantage of this test is that strains of *B. cereus* that react only weakly or not at all with egg yolk can be detected and confirmed microscopically after staining.

To interpret results, bacteria that ferment mannitol produce acid products and form yellow colonies. Bacteria that possess lecithinase hydrolyze lecithin and a zone of white precipitate forms around the colonies; therefore, *B. cereus* is known by mannitol non fermentation (blue colonies) and lecithinase system (forming zone of precipitation around colonies) producing colonies which are flat, crenate to slightly rhizoid, turquoise to peacock blue in color and having a ground glass surface appearance. Therefore, enumeration of presumptive *B. cereus* colonies can be carried out according to ICMSF. (1996) by surface plating technique (0.1 ml

of prepared dilutions) onto polymyxin pyruvate egg yolk mannitol bromothymol blue agar incubating at 30 ^{0}C for 24 hrs. This may be transferred to nutrient agar and blood agar for further confirmatory and differential tests according to APHA. (1992).

2.8.3. Confirmatory and differential tests for *Bacillus cereus*

a. Rhizoid growth test: to perform this confirmatory test, 4-6 licithinase positive typical colonies are selected. Each of these colonies is sub-cultured on a pre-dried nutrient agar plate and to test for rhizoid growth, inoculates several well isolated areas of a pre-dried nutrient agar plate. A 3 mm inoculating loop is used to make a point of contact inoculation. The plate is incubated in an upright position at 30 °C for 24-48 hrs. If hair-like projections (rhizoids) develop outward from these colonies, the isolate is *B. cereus* var. *mycoides* which is not human pathogen (USDA, 1998).

b. Haemolysis test: to do this differential test, sheep blood agar plate that has been divided into 4 – 6 segments is used for inoculation. A 2 mm loop should be used to deposit the inoculums in the center of the segment. Then, size of the hemolytic zone (and whether it is partial or complete) is noted (USDA, 1998). *Bacillus cereus* has β-haemolysis on blood agar.

c. Motility test: this differential test can be done on motility medium by making a center line stab inoculation with a 3 mm loop and incubating the tube at 30 °C for 18-24 hrs. A motile organism is indicated by observing diffuse growth into the medium away from the stab. Alternatively a microscopic motility test may be used. The slide motility test is done by adding 0.2 ml of sterile water to a nutrient agar slant and then inoculating the aqueous phase with a 3 mm loopful of a 24 hrs slant culture, and incubating for 6-8 hrs at 30 °C. Place a loopful of the liquid culture on a glass slide and overlay with a cover slip. *B. cereus* and *B. thuringiensis* are actively motile while *B. anthracis* and the rhizoid strains of *B. cereus* are non-motile (USDA, 1998).

d. Protein toxin crystal stain: to do this differential stain, a smear is made on a microscope slide with sterile water from a 2-3 day old nutrient agar plate or slant. The slide is allowed to air dry and then gently heat fix. After cooling, the slide is flooded with methanol, wait for 30 seconds and poured off. Then the slide is flooded with 0.5% aqueous solution of basic

fuchsine; the slide heated gently until steam is observed. After the removal of the heat, wait for 1-2 minutes and repeat the procedure. Then the slide is cooled and rinsed well with water, examined under oil immersion for free spores and darkly stained, diamond shaped toxin crystals. Toxin crystals should be present if the cells have lysed and free spores are observed. The presence of toxin crystals is strongly indicative that the organism is *B. thuringiensis*. If further biochemical testing is warranted, consultation should be made to either Bergey's Manual of Systematic Bacteriology or the Compendium of Methods for the Microbiological Examination of Foods (Borge *et al*., 2001).

Finally, the bacterium which is lecithinase positive, strongly hemolytic on blood agar, actively motile, does not produce rhizoid colonies and protein toxin crystals as parasporal body, non manitol fermenter, Gram positive, rod shaped producing endospore can be confirmed as *B. cereus* (USDA, 1998).

2.8.4. Enumeration of *Bacillus cereus* colonies

Enumeration of *B. cereus* like other bacteria can be done using viable count or total count by direct/indirect methods. Direct viable count, for example Standard Plate Count (SPC) involves counting cells that can be cultured and/or are metabolically active. This can be done by diluting a sample in saline water and spread on solid media then count colonies. Finally, calculate the number of cells in original sample from counts and dilutions. Direct total count, for example Fluorescent staining method involves staining the cells with fluorescent dyes to make them visible. Then, enumeration is done by counting cells using a fluorescent microscope (USDA, 1998).

Indirect viable count, for example Most Probable Number (MPN) technique is done by making statistically estimated of cells by their patterns of growth in liquid culture. Indirect total count; for example Spectroscopy which uses measuring the amount of light that passes through a liquid culture using a spectrophotometer and estimate the number of cells/ml based on amount of light that passes through (USDA, 1998).

2.8.5. Standard plate count (SPC)

Standard Plate Count (SPC) is direct viable count used to determine the number of viable bacterial cells per unit of a sample using agar plate media. For example, if we are interested in

determining the number of viable bacterial cells per milliliter of milk we should transfer a fixed volume of milk to a plate and spread on it and count the colony forming units (CFU) after incubation. To avoid producing too many colony forming units that is difficult to count accurately on the plate, sample should first be diluted serially so that a countable number of colonies will appear.

The highest dilutions will produce the lowest number of colony forming units and the lowest dilutions will produce the highest number of colony forming units. The plate with the countable number of colonies should be selected to count. When using standard size Petri dishes a countable plate would be one with between 30 and 300 colony forming units. Dilutions with fewer than 30 colonies are easy to count, but often produce inaccurate results since one or two contaminating colonies can cause a significant overestimate of the cell count. After the colonies are counted the concentration of cells in the plated dilution can be determined by dividing the amount plated.

The specific dilution determined the concentration in the original sample and can be calculated by dividing the total dilution. A sample could be serially diluted as follows: Transfer one milliliter from the sample in to 9 ml of sterile dilution media in tube A and mixed. This is a 1/10 serial dilution. Then, one milliliter from tube A will be transferred into 9 ml of sterile dilution media in tube B and mixed. This is another 1/10 serial dilution. The total dilution up to this point is 1/100.

http://www.fda.gov/Food/ScienceRes/Lab.Methods/BAM/ucm063346.htm. Last updated: 07/15/2011

3. MATERIALS AND METHODS

3.1. Study area

The study was done in Debre Zeit town which is located 47 Km East of Addis Ababa. Debre Zeit is found in the East Shewa Zone of the Oromia Regional State, in central Ethiopia at latitude and longitude of 8°45′N 38°59′E with an elevation of 1,920 meters above sea level (Figure 3). The total area of Debre Zeit is about 14,000 hectare of land (140 km^2). The average annual temperature range is 14 ^{0}C to 26 ^{0}C with relative humidity of 61.3%. Based on figures from the Central Statistical Agency (CSA, 2007), the national census reported that a total population of Debre Zeit was 99,928, of whom 47,860 were men and 52,068 were women.

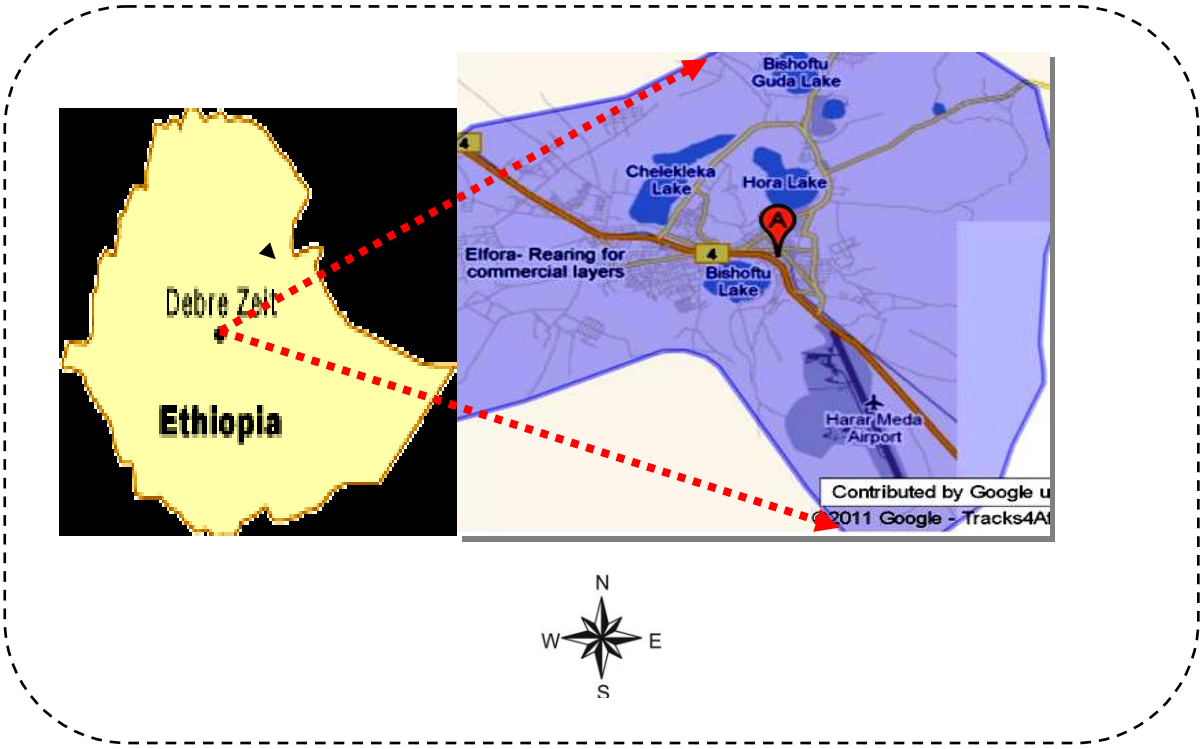

Figure 3: Map of the study area
Source: ***http://www.maplandia.com/ethiopia/ oromiya/east-shewa /debre-zeit***

3.2. The study design

A cross sectional study was carried out from October, 2011 to April, 2012 on bovine raw milk samples collected from selected dairy farms. Dairy farms were selected by using simple random sampling method from volunteered farms. Dairy cows in a farm were selected by using census sampling method that is all lactating cows in a farm were sampled. In addition, prior to sample collection, information were gathered from milkers by using semi-structured questionnaire survey which was designed to assess the risk factors for milk contamination like the situations of dairy farm managements, milking procedures and hygienic status of the farms. In addition, the questionnaire format was designed in such way that helped to assess public health implication of raw milk consumption. All respondents of questionnaire survey were selected purposively based on their voluntariness and farm employees from where milk samples were collected.

3.2.1. The study population and sample size determination

The study populations were lactating and apparently healthy dairy cows used for milking in volunteer dairy farms. The total sample size for raw milk collection was assigned according to the statistical formula (Thrusfield, 2005). A 5% absolute precision at 95% Confidence Interval (CI) was used during determination of the sample size. Since there is no previous work in the study area for *B. cereus* prevalence on raw milk, the expected prevalence of this bacterium in raw milk was taken as 50% according to Thrusfield (2005). Therefore, the total sample size for this study was calculated as follows:

$$N = \frac{(1.96)^2 \times P_{ex}(1-P_{ex})}{(d^2)}$$

Where: N = the total sample size
P_{ex} = expected prevalence (50%)
d = absolute precision (0.05) at 95% CI

$$N = \frac{(1.96) \times (1.96) \times (0.5) \times (1-0.5)}{(0.05) \times (0.05)} = \underline{384 \text{ samples}}$$

3.2.2. Sampling procedures

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 were 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. For detail procedures refer Annex-F.

The universal bottles were clearly labeled by water proof ink using coding system for each dairy farm and ear tag identity cards for individual cows. Additional secondary data were documented in to pre-prepared tables. Finally, the milk samples were immediately transported to the Microbiology Laboratory of the School of Veterinary Medicine, Addis Ababa University, in tightly closed ice box.

3.3. Sample processing and plating

Sample processing was done by diluting 1 ml milk from 10 ml milk sample and pipetting this into sterile universal bottle filled 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 surface spread on to solidified Bacillus cereus selective medium (CM0617; Oxoid Ltd, Basingstoke Hampshire, England) in duplicate, that was two plates for each dilution factors. For prevalence purpose from each raw milk samples 0.1 ml without dilution was also plated. During some situations, samples which could not be processed immediately were stored at 4 ^{0}C refrigerator only for about 24 hrs. 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.

3.4. Presumptive *Bacillus cereus* colony count

The presumptive *B. cereus* colonies were identified based on colony color, morphology and precipitation of egg yolk. After 18 – 24 hrs of incubation at 30 ^{0}C in aerobic conditions, *B. cereus* shows crenate colonies about 5 mm in diameter, turquoise blue in color, surrounded by a distinct opaque zone of egg yolk precipitation of the same color as the colonies.

Based on morphological characteristics on selective media, presumptive colonies were counted. As a rule, the plates having colonies below 30 were not serving as the true representatives of the sample and recorded as too small to count whereas, plates with more than 300 colonies are difficult for counting and reported as too numerous to count. However, in this study there was no plate with colonies of too numerous to count unlike colonies of too small to count. In addition, from plates with too small to count no plates were confirmed as *B. cereus*. Therefore, the actual numbers of CFU in this study was colonies from plates falling within 30 and 300 colony counts. Finally, the number of CFU/ml of samples was calculated using the standard equation (Nicoletta and Royston, 2008).

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

Where: N = total viable colony count

Σc = sum of colonies counted from all plates

n_1 = number of plates counted at first dilution

n_2 = number of plates at second dilution

C= number of colonies counted

d = dilution factor from which the first counts obtained (least counted dilution)

3.5. 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 (212539, BD Difco^{TM} BBL^{TM} Stains) *B. cereus* group appears as large Gram-positive rod shaped with short to long chains. Most biochemical tests were confirmatory but not differential that are common for *B. cereus* group members namely,

B. cereus, B. mycoides, B. thuringiensis, and *B. anthracis* with identical characteristics (Annex, E); therefore, differentiation required additional tests.

By inoculating isolates on sheep blood agar (CM0854; Oxoid Ltd), *B. cereus* colony grew 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 helped for differentiating *B. cereus* from its group members. Since *B. anthracis* forms non haemolytic gray/white colonies where as *B. mycoids* forms colonies with rhizoid/hairy like projections. Alternatively, *B. cereus* was differentiated from other non motile group members forming diffuse growth in semisolid SIM medium (M181; HiMedia Ltd) except from *B. thuringiensis*. In addition, from *B. cereus* group only *B. 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), showed that characteristic morphology of pale green endospores without bulged sporangium and with no parasporal crystal bodies in red stained cytoplasm. This helped to differentiate *B. cereus* from *B. thuringiensis.* (Annex, H).

3.6. Data analysis

The data were entered into Microsoft Excel (MS-Excel, 2007). These data were analyzed using Statistical Package for Social Science (SPSS version 16.0, Inc., 2007, Chicago, Illinois) software. The $\log_{10}$-transformed values of raw milk standard plate count ($\log_{10}$CFU/ml) were computed using mean values as continuous variable and farms, parities, farm managements and questionnaire data as categorical variables. The descriptive statistics called Pearson Chi-Square test was used to see statistical significance for categorical data. GLM (Generalized Linear Models) was used for mean comparison among farms and parities. One way ANOVA test was used for testing mean differences between farm managements. For all statistics 95% CI with 5% degrees of freedom ($P < 0.05$) was considered to say significant.

4. RESULTS

A total of 384 raw bovine milk samples were collected and processed from 11 randomly selected dairy farms. Variations in farm managements, parities, cow sheds' floor type and hygienic status in the farms and milking procedures were used as risk factors for assessing contamination rates of raw milk by *B. cereus*.

The overall prevalence of *B. cereus* in raw bovine milk samples was 15.4% (59/384). The *B. cereus* load from raw milk samples ranges from $2.3 \times 10^4 - 5.4 \times 10^5$ colony forming unit per-milliliter (CFU/ml). Majorities of positive colony forming units per milliliter were above legal limit ($> 10^5$ CFU/ml) in raw milk. Samples collected from farms coded with 2, 3, 4, 5, 6, and 8 were less contaminated with *B. cereus* (0 – 1%), whereas those collected from farms coded with 1, 7, 9, 10 and 11 were relatively highly contaminated with *B. cereus* (1.8 – 3.6%), (Table 2).

Milk samples collected from both farms managed by zero-grazing and semi-intensive systems were highly contaminated. *B. cereus* load in both farm management systems was beyond ranges of legal limit ($> 10^5$ CFU/ml) in raw milk with ranges $2.0 \times 10^5 - 5.4 \times 10^5$ CFU/ml and $2.3 \times 10^4 - 5.4 \times 10^5$ CFU/ml (Table 2).

Samples collected from cows found in 1^{st}, 5^{th} and 6^{th} parities were less contaminated with *B. cereus* than samples collected from cows found in 2^{nd}, 3^{rd} and 4^{th} parities. The lower limits of CFU/ml in 1^{st}, 5^{th} and 6^{th} parities were fewer than those in 2^{nd}, 3^{rd} and 4^{th} parities (Table 2).

Table 2: Prevalence of *Bacillus cereus* in raw bovine milk

Variables	Categories	Ranges of CFU/ml	Positive*	Negative**
Farms Code	Farm-1	$3.2 \times 10^5 - 5.4 \times 10^5$	7 (1.8%)	38 (9.9%)
	Farm-2	4.1×10^5- 5.2×10^5	4 (1.0%)	28 (7.3%)
	Farm-3	$4.7 \times 10^5 - 5.1 \times 10^5$	2 (0.5%)	14 (3.6%)
	Farm-4	$3.5 \times 10^5 - 4.1 \times 10^5$	3 (0.8%)	14 (3.6%)
	Farm-5	$2.3 \times 10^4 - 5.3 \times 10^5$	4 (1.0%)	20 (5.2%)
	Farm-6	0	0 (0%)	20 (5.2%)
	Farm-7	$3.5 \times 10^5 - 5.4 \times 10^5$	8 (2.1%)	51 (13.3%)
	Farm-8	$2.0 \times 10^5 - 3.3 \times 10^5$	2 (0.5%)	28 (3.3%)
	Farm-9	$2.4 \times 10^5 - 5.3 \times 10^5$	14 (3.6%)	49 (12.8%)
	Farm-10	$4.1 \times 10^5 - 5.4 \times 10^5$	5 (1.3%)	13 (3.4%)
	Farm-11	$2.0 \times 10^5 - 5.3 \times 10^5$	10 (2.6%)	50 (13%)
Farm Managements	Zero-grazing	$2.0 \times 10^5 - 5.4 \times 10^5$	43 (11.2%)	279 (72.7%)
	Semi-intensive	$2.3 \times 10^4 - 5.4 \times 10^5$	16 (4.2%)	105 (27.3%)
Parities	Parity-1	$2.3 \times 10^4 - 5.4 \times 10^5$	7 (1.8%)	17 (4.4%)
	Parity-2	$3.3 \times 10^5 - 5.3 \times 10^5$	14 (3.6%)	43 (11.2%)
	Parity-3	$2.5 \times 10^5 - 5.4 \times 10^5$	14 (3.6%)	85 (22.1%)
	Parity-4	$3.3 \times 10^5 - 5.4 \times 10^5$	17 (4.4%)	77 (20.1%)
	Parity-5	$2.0 \times 10^5 - 4.1 \times 10^5$	3 (0.8%)	69 (18%)
	Parity-6	$2.4 \times 10^5 - 5.4 \times 10^5$	4 (1%)	34 (8.9%)
Prevalence			59 (15.4%)	325 (84.6%)

Positive*: Samples with confirmed *Bacillus cereus* colony forming units

Negative**: Samples with no confirmed *Bacillus cereus* colony forming units

CFU/ml: Colony forming units per milliliter

Pair-wise comparisons of marginal means of Log_{10} CFU/ml *B. cereus* among farms were computed each other. By using statistics Generalized Linear Models (GLM) on Log-transformed means of CFU/ml, farms coded with 6, 8, 9 and 10 had statistically significant difference ($P< 0.05$). The pair-wise mean comparisons revealed statistically significant difference between ($farm_6$*$farm_9$), ($farm_6$*$farm_{10}$) and ($farm_8$*$farm_9$) with p-values 0.020, 0.025 and 0.036, respectively (Tables 3 and 4). The GLM means comparison was computed for all eleven farms, however, those non significant farms' pair-wise comparisons were not shown here due to inconvenience of demonstrating those lengthy unmanageable tabulations.

Table 3: GLM pair-wise comparison of mean differences among farms

(I) Farms	(J) Farms	Mean Difference (I-J)	Std. Error	df	Sig.	95% Wald CI for Difference	
						Lower bound	Upper bound
Farm-6	Farm-1	-1.0365	.6022	1	.085	-2.2168	.1439
	Farm-2	-.8131	.6389	1	.203	-2.0653	.4391
	Farm-3	-.7853	.6399	1	.220	-2.0395	.4690
	Farm-4	-.9603	.6328	1	.129	-2.2006	.2800
	Farm-5	-.8582	.5967	1	.150	-2.0278	.3114
	Farm-7	-.8662	.5791	1	.135	-2.0013	.2689
	Farm-8	-.4788	.5806	1	.410	-1.6166	.6591
	Farm-9	-1.3303*	.5740	1	.020	-2.4553	-.2053
	Farm-10	-1.4009*	.6269	1	.025	-2.6297	-.1721
	Farm-11	-1.0558	.5775	1	.068	-2.1877	.0760
Farm-8	Farm-1	-.5577	.4445	1	.210	-1.4290	.3136
	Farm-2	-.3343	.4891	1	.494	-1.2930	.6243
	Farm-3	-.3065	.4911	1	.533	-1.2691	.6560
	Farm-4	-.4815	.4832	1	.319	-1.4286	.4656
	Farm-5	-.3794	.4343	1	.382	-1.2307	.4718
	Farm-6	.4788	.5806	1	.410	-.6591	1.6166
	Farm-7	-.3874	.4111	1	.346	-1.1931	.4182
	Farm-9	-.8515*	.4062	1	.036	-1.6476	-.0554
	Farm-10	-.9221	.4791	1	.054	-1.8612	.0169
	Farm-11	-.5771	.4123	1	.162	-1.3852	.2310

*The mean difference is significant at the 0.05 level

Table 4: GLM pair-wise comparison of mean difference among farms

(I) Farms	(J) Farms	Mean Difference (I-J)	Std. Error	df	Sig.	95% Wald CI for Difference	
						Lower bound	Upper bound
Farm-9	Farm-1	.2938	.4380	1	.502	-.5647	1.1522
	Farm-2	.5172	.4864	1	.288	-.4361	1.4704
	Farm-3	.5450	.4869	1	.263	-.4093	1.4993
	Farm-4	.3700	.4783	1	.439	-.5674	1.3073
	Farm-5	.4720	.43101	1	.273	-.3727	1.3168
	Farm-6	1.3303*	.5740	1	.020	.2053	2.4553
	Farm-7	.4641	.4058	1	.253	-.3312	1.2594
	Farm-8	.8515*	.4062	1	.036	.0554	1.6476
	Farm-10	-.0707	.4721	1	.881	-.9960	.8547
	Farm-11	.2744	.4033	1	.496	-.5161	1.0649
Farm-10	Farm-1	.3644	.5097	1	.475	-.6346	1.3635
	Farm-2	.5878	.5490	1	.284	-.4881	1.6638
	Farm-3	.6156	.5464	1	.260	-.4554	1.6866
	Farm-4	.4406	.5356	1	.411	-.6090	1.4903
	Farm-5	.5427	.4963	1	.274	-.4300	1.5154
	Farm-6	1.4009*	.6269	1	.025	.1721	2.6297
	Farm-7	.5347	.4800	1	.265	-.4060	1.4754
	Farm-8	.9221	.4791	1	.054	-.0169	1.8612
	Farm-9	.0707	.4721	1	.881	-.8547	.9960
	Farm-11	.3451	.4789	1	.471	-.5936	1.2837

*The mean difference is significant at 0.05 levels

During comparisons of mean of farm managements, the GLM pair-wise mean comparison was not done due to variables being less than three categories. Due to this other statistics, One Way ANOVA mean comparison was used to test difference between farm managements. The mean comparison of the two farm managements by descriptive statistics (Table 5) and one way ANOVA (Table 6) were done. By using descriptive statistics, means of zero-grazing and semi-intensive farm managements showed nearly the same values. Zero-grazing farms had means 0.8673 $\pm$ 2.0359, whereas semi-grazing farms had means 0.8435 $\pm$ 2.0029. The mean counts of dairy farms in zero-grazing and semi-intensive farm management systems were not statistically significant ($P > 0.05$) (Table 6).

Table 5: Descriptive statistics for means of two farm management systems

Farm Managements	N	Mean	Std. Deviation	Std. Error	95% CI for Mean	
					Lower Bound	Upper Bound
Zero-grazing	279	.8673	2.03592	.12189	.6273	1.1072
Semi-intensive	105	.8435	2.00297	.19547	.4559	1.2311
Total	384	.8608	2.02438	.10331	.6577	1.0639

Table 6: One way ANOVA test of mean difference between farm managements

ANOVA Test	Sum of squares	df	Mean Square	F	Sig.
Between groups	.043	1	.043	0.010	0.919
Within groups	1569.538	382	4.109		
Total	1569.581	383			

The mean pair-wise comparison of first parity with 3rd, 4th, 5th and 6th parities was statistically significant. The second parity pair-wise means comparison with 3rd, 5th and 6th parities was statistically significant. The pair-wise mean comparisons of 4th with 5th parities were statistically significant ($p< 0.05$) (Table 7).

Table 7: GLM pair-wise mean differences comparison within parities

(I) Parities	(J) Parities	Mean Difference (I-J)	Std. Error	df	Sig.	95% Wald Confidence Interval for Difference	
						Lower Bound	Upper Bound
parity-1	parity-2	.3956	.46968	1	.400	-.5250	1.3161
	parity-3	1.1305*	.43756	1	.010	.2729	1.9881
	parity-4	.8691*	.44048	1	.048	.0058	1.7325
	parity-5	1.5546*	.45986	1	.001	.6533	2.4559
	parity-6	1.2852*	.53616	1	.017	.2344	2.3361
parity-2	parity-1	-.3956	.46968	1	.400	-1.3161	.5250
	parity-3	.7350*	.32737	1	.025	.0933	1.3766
	parity-4	.4736	.33423	1	.157	-.1815	1.1287
	parity-5	1.1590*	.35714	1	.001	.4590	1.8590
	parity-6	.8897*	.44968	1	.048	.0083	1.7710
parity-3	parity-1	-1.1305*	.43756	1	.010	-1.9881	-.2729
	parity-2	-.7350*	.32737	1	.025	-1.3766	-.0933
	parity-4	-.2614	.28263	1	.355	-.8153	.2926
	parity-5	.4241	.30969	1	.171	-.1829	1.0311
	parity-6	.1547	.41318	1	.708	-.6551	.9645
parity-4	parity-1	-.8691*	.44048	1	.048	-1.7325	-.0058
	parity-2	-.4736	.33423	1	.157	-1.1287	.1815
	parity-3	.2614	.28263	1	.355	-.2926	.8153
	parity-5	.6855*	.31377	1	.029	.0705	1.3004
	parity-6	.4161	.41598	1	.317	-.3992	1.2314
parity-5	parity-1	-1.5546*	.45986	1	.001	-2.4559	-.6533
	parity-2	-1.1590*	.35714	1	.001	-1.8590	-.4590
	parity-3	-.4241	.30969	1	.171	-1.0311	.1829
	parity-4	-.6855*	.31377	1	.029	-1.3004	-.0705
	parity-6	-.2694	.43183	1	.533	-1.1157	.5770
parity-6	parity-1	-1.2852*	.53616	1	.017	-2.3361	-.2344
	parity-2	-.8897*	.44968	1	.048	-1.7710	-.0083
	parity-3	-.1547	.41318	1	.708	-.9645	.6551
	parity-4	-.4161	.41598	1	.317	-1.2314	.3992
	parity-5	.2694	.43183	1	.533	-.5770	1.1157

***The mean difference is significant at ($P< 0.05$).

Questionnaire survey analysis was done to assess risk factors for milk contamination like hygienic status of milk and public heath implication of raw milk. The questionnaire survey and corresponding mean count of Log_{10} CFU/ml for each farm was computed.

There was statistically significant difference of *B. cereus* mean count among farms that used cleaning common towels, individual towels and those not use towel at all ($p< 0.05$) (Table 8).

Constructing farm floor with concrete and gravel sand had statistical significant difference on mean count of *B. cereus.* There was statistical significant difference in mean counts of *B. cereus* between farms that were cleaned every day and twice a day ($p< 0.05$) (Table 8).

The mean counts of *B. cereus* between farms that used udder cleaning disinfectants and those did not use disinfectants were statistically significant ($p< 0.05$) (Table 8).

There was no statistically significant difference of *B. cereus* mean counts among respondents that consumed raw milk, yogurt milk and boiled milk; however, there was statistically significant difference of *B. cereus* count among respondents who used boiled milk after storing for 6 hrs, 8 hrs and 12 hrs ($p< 0.05$) (Table 8).

Table 8: Descriptive statistics of questionnaire survey

Survey Variables		Questionnaire Respondents According to their Farms											Asymptote Sig. (2-sided)
		F1M	F2M	F3M	F4M	F5M	F6M	F7M	F8M	F9M	F10M	F11M	
Proportions		10.3% (4/39)	7.7% (3/39)	5.1% (2/39)	5.1% (2/39)	7.7% (3/39)	5.1% (2/39)	15.4% (6/39)	7.7% (3/39)	15.4% (6/39)	5.1% (2/39)	15.4% (6/39)	
Mean Counts of Farms		.8777	.7064	.7112	.9858	.8828	0.0000	.7700	.3607	1.2478	1.5805	.9308	
Farm management	Zero-grazing	10.3%	7.7%	-	-	-	5.1%	15.4%	-	15.4%	-	15.4%	0.000
	Semi-intensive	-	-	5.1%	5.1%	7.7%	-	-	7.7%	-	5.1%	-	
Sheds' floor type	Concrete	10.3%	7.7%	-	-	-	5.1%	15.4%	7.7%	15.4%	5.1%	15.4%	0.000
	Gravel sand	-	-	5.1%	5.1%	7.7%	-	-	-	-	-	-	
Sheds cleaning	Every day	-	-	5.1%	5.1%	7.7%	5.1%	15.4%	-	-	-	-	0.000
	Twice a day	10.3%	7.7%	-	-	-	-	-	7.7%	15.4%	5.1%	15.4%	
Udder cleaning	Every day	-	7.7%	-	-	7.7%	-	-	-	-	-	-	0.000
	Twice a day	10.3%	-	5.1%	5.1%	-	5.1%	15.4%	7.7%	15.4%	5.1%	15.4%	
Udder washing	With disinfectants	10.3%	-	-	-	-	5.1%	15.4%	-	15.4%	-	15.4%	0.000
	Without disinfectants	-	7.7%	5.1%	5.1%	7.7%	-	-	7.7%	-	5.1%	-	
Towel use	Common	10.3%	7.7%	-	-	-	-	-	7.7%	15.4%	-	15.4%	0.000
	individual	-	-	-	-	-	5.1%	15.4%	-	-	-	-	
	Not at all	-	-	5.1%	5.1%	7.7%	-	-	-	-	5.1%	-	
Milk container	Aluminum	-	-	-	-	-	5.1%	15.4%	7.7%	15.4%	5.1%	-	0.000
	Plastic	10.3	7.7%	5.1%	5.1%	7.7%	-	-	-	-	-	15.4%	
Consuming milk as	Raw milk	2.6%	-	2.6%	-	-	-	5.1	2.6	2.6%	2.6%	-	0.458
	Yogurt milk	-	-	-	-	5.1%	2.6%	2.6	5.1%	2.6%	2.6%	5.1%	
	Boiled milk	7.7%	7.7%	2.6%	5.1%	2.6%	2.6%	7.7	-	10.3%	-	10.3%	
Store once boiled milk	For 6hrs	7.7%	7.7%	2.6%	5.1%	7.7%	5.1%	15.4%	7.7%	15.4%	-	10.3%	0.003
	For 8hrs	-	-	2.6%	-	-	-	-	-	-	-	5.1%	
	For 12hrs	2.6%	-	-	-	-	-	-	-	-	5.1%	-	

F1-11**M**: **F**-Dairy farms coded with 1-11, **M**- milkers (respondents) of questionnaire survey

5. DISCUSION

The present study was conducted on raw bovine milk samples setting the following objectives; to determine *B. cereus* prevalence, evaluate *B. cereus* load in raw milk and assess risk factors for raw milk contamination by *B. cereus* and its public health implication.

The prevalence of *B. cereus* was 15.4% (59/384) in raw milk from eleven dairy farms in Debre Zeit, with its load ranging from $2.3 \times 10^4 – 5.4 \times 10^5$ CFU/ml.

In previous studies, *B. cereus* was less frequent contaminant of raw milk as reported by Ahmed *et al.* (1983), Mosso *et al.* (1989), Parkash *et al.* (2007) and Muhamed *et al.* (2010); they found that, 9%, 0%, 6.5%, and 10% respectively.

This finding is similar to Gilles *et al.* (2002) and Hempen *et al.* (2004) who reported that 15.4% and 17% of examined raw milk samples were contaminated with *B. cereus*, respectively.

However, higher contamination frequencies were reported by Abdel-Khalek and El-Sherbini (1996), Te Giffel and Beumer (1998), Ayoub *et al.* (2003), El-Shinawy (2004), Rezende-lago *et al.* (2007) and Adesina *et al.* (2011), as they reported that; 40%, 35%, 26.7%, 62%, 50%, and 46.7% of raw milk samples were contaminated with *B. cereus*, respectively.

The higher prevalence in some findings may be due to high raw milk contamination. Since *B. cereus* is widely distributed in the environment, the organism can be introduced into raw milk from the soil, air, water source, bedding, feedstuff, milk handlers, udder infection, feces of cows and milking equipments (Te Giffel *et al.* 1996; Clarence *et al.*, 2009).

In this study, majority of raw milk samples collected from different herds was contained with higher *B. cereus* load than acceptable limit of raw milk (> 10^5 CFU/ml). The CFU/ml recorded from 58 (98.1%) positive isolates ranged from $2.0 \times 10^5 – 5.4 \times 10^5$ CFU/ml, which was two to five folds above legal limit set for raw milk. The microbial standard for grade ‘A’ raw milk is 1×10^5 CFU/ml for individual producer milk (from raw milk at farm level) and 3×10^5 CFU/ml as commingled milk (ICMSF, 1996; PMO, 2001; FDA, 2005).

In this study, the CFU of *B. cereus* ranged from 2.3 x 10^4 – 5.4 x 10^5 CFU/ml. This findings when compared with previous works on milk bacteriology was lower than total aerobic plate counts of raw milk of DeGraaf *et al*. (1997) (3.88 x 10^7 CFU/ml), Godifay and Molla (2000) (1.9 x 10^8 CFU/ml), Bonfoh *et al*. (2003) (10^7 CFU/ml) and Esther *et al*. (2004) (3 x 10^7 CFU/ml).

However, this finding falls in the same ranges of aerobic plate counts reported by Rai and Dawvedi (1990) from India (7.7 x 10^5 CFU/ml), Kurwijilla *et al.* (1992) from Tanzania (10^5 CFU/ml), Ombui *et al.* (1995) from Kenya (5 x 10^4 CFU/ml), and Bonfoh (2003) from Mali (10^6 CFU/ml) from raw milk.

The difference of reports may be due to cosmopolitan nature of *B. cereus* along milk value chains and may also be the time variation from milk collection till inoculation. This variation is common since; bacterial populations in raw milk may be as high as 5Log CFU/ml before commingling (at farm level) and 5.4Log CFU/ml after commingling (in collection tank) (FDA, 2005).

Pathogenic bacteria like *B. cereus* in milk have been a major factor for public health concern since the early days of the dairy industry. The health of dairy herd and milking conditions basically determine the milk quality. Another source of contamination by microorganisms is unclean teats. The use of unclean milking and transportation equipments also contributed to the poor hygienic quality of milk (Parekh and Subhash, 2008).

The pair wise mean comparison among farms had statistically significant difference from farms coded by ($farm_6$*$farm_9$), ($farm_{6*}farm_{10}$) and ($farm_8$*$farm_9$) with p-values 0.020, 0.025 and 0.036, respectively (Tables 3 and 4). Milk samples collected from farm-9 was highly contaminated with *B. cereus* than those samples collected from farm-6 and farm-8. Raw milk samples collected from farm-10 was highly contaminated than milk samples collected from farm-6 (Tables 3 and 4).

This difference may be due to many reasons like variations in shed floor construction, hygiene of the farms, parities of dairy cows, milking procedures and farm managements. When cows are at pasture, the teats are predominantly contaminated with the soil, whereas teats of cows housed in the barn are mainly contaminated with feces and bedding material (Christiansson *et al*., 1999; Magnusson *et al*., 2007).

Raw milk from dairy farms managed by zero-grazing was highly contaminated with *B. cereus* than those managed by semi-intensive systems; however, these variations were not statistically significant ($p > 0.05$). Milk samples collected from dairy cows that differ in parities were varying significantly in their B. *cereus* load in raw milk ($p< 0.05$). *Bacillus cereus* load in raw milk increases gradually as parities increase from 1^{st} to 4^{th} parities and declines from 5^{th} to 6^{th} (Table 7). This may be due to host clearing mechanisms that operated at the 5^{th} and 6^{th} parities as compared to the first and mid parities that lead to colonization with bacteria.

From questionnaire survey results, constructing dairy house from concrete floor, cleaning cow shed and udder frequently as well as keeping hygiene of personals had strong effects on *B. cereus* count in raw milk ($p< 0.05$) (Table 8).

The recorded high count (above limit) of *B. cereus* in raw milk indicates poor hygienic production of milk. This is hazard alarm to public health implication. It is more effective to exclude microorganisms than to try to control microbial growth once they entered into milk (Adesina *et al.,* 2011). The diarrheal toxin is caused by a high molecular weight, heat-labile enterotoxin produced in the intestines when *B. cereus* concentration reaches 7Log CFU/ml in food (Jay *et al.,* 2005). In addition, the emetic syndrome is produced by a low molecular weight, heat-stable toxin produced in the food product when *B. cereus* concentration reaches 6Log CFU/ml, (Jay *et al.,* 2005).

The presence of possible pathogenic organisms in the analyzed 59 cow raw milk with above acceptable limit should be of great concern to the producers, consumers and the concerned arms of Government structures, since food poisoning by *B. cereus* may happen as a result of consuming contaminated milk. Since a large number of viable cells (10^5-10^6 CFU/ml) of *B. cereus* are enough to cause illness (Quinn *et al.,* 1999). *Bacillus* foodborne illnesses occur due to survival of its endospores after cooking and heat resistant toxins (Turnbull, 1996; Elina, 2008).

6. CONCLUSIONS AND RECOMENDATIONS

Raw milk samples collected from 59 cows was highly contaminated with *B. cereus.* Contamination rate of raw milk with *B. cereus* varies between different farms. The frequencies of *B. cereus* in zero-grazing and semi-intensive systems varied; however, these variations of *B. cereus* load were not statistically significant ($p > 0.05$). The contamination rate of raw milk with *B. cereus* significantly varies within different parities ($p < 0.05$). Contamination rate of raw milk with *B. cereus* was associated with risk factors like types of housing and milking floor construction, cow shed and udder cleaning frequencies, habits of using towel and cleaning disinfectants ($p < 0.05$). *Bacillus cereus* load in majorities of dairy farms were beyond ranges of legal limit in raw milk intended for public consumption. This *B. cereus* load above acceptable limit in raw milk was an alarm for public health implication.

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

- The hygiene status in dairy farms should be improved to reduce *B. cereus* load to acceptable level and prolong the keeping quality of raw milk.
- Individual towel use, udder dipping by disinfectants and personal hygiene should be regularly practiced in dairy farms.
- Good milk processing procedures should be adopted in dairy processing plants to reduce *B. cereus* load from raw milk.
- Milk for public consumption should be properly boiled at appropriate temperature and time.
- 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. ANNEXES

Annex A: 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 and 45-55 mm length
3. Incubators, 30 ± 2°C and 35 ± 2°C
4. Manual colony counter chamber
5. Marking pen, (waterproof)
6. Bunsen burner
7. Wire loops, platinum wire, 2-3mm
8. Vortex mixer
9. Microscope, microscope slides
10. Culture tubes, 13 x 100 mm sterile
11. Test tubes, 16 x 125 mm
12. Bottles, 3 oz, sterile
13. Water bath, 48-50°C
14. Culture tube racks
15. Staining rack
16. Petri dishes, sterile, 15 x 100 mm
17. Dilution bottles, 6 oz (160 ml),
18. Borosilicate-resistant glass, with plastic screw caps
19. Thermometers (mercury) appropriate range
20. Plate count agar (Oxoid, MC0167)
21. Refrigerator, to cool and maintain samples milk, 0-4.4°C
22. Storage space, free of dust and insects

Annex B: Media and reagents

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%
10. Circulating water bath, for tempering agar, thermostatically controlled to 50 °C

Annex C: Questionnaires for Milk Contamination Assessment

1. How do you keep your dairy cows?

A. Extensively B. Semi-intensively C. Zero grazing system

2. Type of floor construction for housing dairy cows?

A. Concrete floor B. Soil floor

C. Straw bedded floor D. Floor covered with gravel sands

3. Type of floor for milking area?

A. Concrete floor B. Soil floor

C. Straw bedded floor D. Floor covered with gravel sands

4. Did you come across with the following abnormalities?

A. Udder swelling (yes/no) B. Teat defects (yes/no)

C. Rapid drop in milk (yes/no) D. Color change in milk (yes/no)

5. How often do you clean your cows' shed? A. Every day B. Two times a day

C. Weekly D. Two times a week, E. Not at all

6. Do you clean the cows' udder? A. Yes B. No

7. If yes, how often did you clean the cows' udder?

A. Every day B. Twice a day C. When it dirt D. Not at all

8. What kind of milking equipment do you use?

A. Aluminum cans B. Plastic container C. clay pots D. other type

9. How often do you clean your milk collecting equipments?

A. Every day B. Two times a day

C. Weekly D. As dirt seen E. Other options

10. How do you milk the cows?

A. After rubbing teat by towel B. After washing udder with soap water

C. Washing hands by pure water D. Without washing hands E. Using chemicals

11. Do you use cleaning towels? A. Yes (Common) B. Yes (single) B. No at all

12. When do you bring milks to collecting center?

A. Just within two hours after milking B. Four hours after milking

C. Six hours after milking D. mixing morning and evening

E. Next day of milking

Annex D: Questionnaires for Public Health Implication Assessment

1. How did you consume the milk at home?

   A. Raw B. Fermented products (yogurt)

   C. After boiling D. Other form

2. Did one of your family members become ill within 6 hours after consuming milk?

   A. Yes B. No

3. 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

4. What did you do when your milk become rejected as spoiled at collecting center?

   A. Fed to children B. Fed to calves

   C. Fed to dogs D. Made yogurt E. Discarded

5. Do you know any milkborne diseases?

   A. Yes B. No

6. If yes, list few signs……

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

A. For 2 hours B. For 4 hours C. For 6 hours D. For 8 hours E. For 12 hours

8. Which kinds of water sources do you and your cows use for drinking?

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

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

A. For breakfast B. As common diet C. Rarely D. Not at all

Annex E. 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	

+/–*, 50-50% of strains are positive.
-*, most strains are negative
Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998, Chapter 14. Updated February 2012

Annex F: 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.

I. To collect a composite sample (milk from all four quarters in the same tube), begin sample collection with the nearest teats and progress to the teats on the far side of the udder. One to 2 ml of milk should be collected from each quarter of the udder.
II. When samples are taken at the end of milking or between milkings, teats should be dipped in an effective germicidal teat disinfectant following sample collection.
III. Store samples immediately on ice or in some form of refrigeration. Samples to be cultured at a later date (more than 48 hours) should be frozen immediately.

Source: NMC publication: Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality, Pp 24 (4th-Ed. 2004).
http://nmconline.org/articles/corynnotes.htm

Annex G: Bacillus cereus selective agar base (CM0167, Oxoid), formulation for *B. cereus* isolation and enumeration

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	

Source: ***www.oxoid.com/UK/blue/prod/detail/prod_detail.asp?pr.***

Procedure: 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.

Annex H. Staining chemicals and procedures

A. Gram stain ***http://www.microbiologybytes.com/blog/about/***

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 O
	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.

9. Curriculum Vitae

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Education Background

1982-1990 E.C:	Zigem Elementary School, Kilaj
1990-1994 E.C:	Chagni High School
1995 E.C	Addis Ababa University, Science Faculty (Arat kilo)
1996-2000 E.C	Addis Ababa University, Faculty of Veterinary Medicine

Literature and research experience (Not yet published)

- DVM Seminar paper, review on 'Estrous Synchronization for Bovine and Swine species' (1999 E.C.).
- DVM thesis paper on topic 'Study on Gastrointestinal Helmenthosis in Camel in and around Dire Dawa, Ethiopia, (2000 E.C.).
- MSc Seminar paper, Review on Prevalence of *Campylobacter jejunii* and *coli* Retail Meat (2003 E.C.).
- MscThesis paper on 'Study on the prevalence of *Bacillus cereus* and associated risk factors in bovine raw milk in Debre Zeit, Ethiopia, (2004 E.C.).

Work Experience

As field Veterinarian and Technical Process owner two years xperience, in Metekel Ranch, Fogera Cattle Breeding and Breed Improving Center, in Chagni, under Bureau of Agriculture of the Amhara National Regional State (2001 to 2002).

10. SIGNED DECLARATION SHEET

I, the under signed, declare that the thesis is my original work and has not been presented for a degree in any university.

Name Alemneh kassa Terefe

Signature _____

Date of Submission _____

This has been submitted for examination with our approval as

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