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# Review of potential sources and control of thermoduric bacteria in bulk-tank milk

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**Bacteria that contaminate milk include thermoduric bacteria that can survive pasteurisation and subsequently grow in the pasteurised milk or contaminate product. Elimination of thermodurics at milking is not feasible. Therefore, knowledge of their source and strategies for their reduction are important. The major sources of thermodurics in milk are contamination of the teat skin from soil and bedding, and subsequent contamination from deposits that can build up on milking equipment surfaces. Hygiene at milking can reduce the number of bacteria contaminating milk. Teat preparation at milking and a recommended plant cleaning procedure are critical to the prevention of the contamination of milk with thermoduric bacteria.**

***Keywords:*** ***Bacillus cereus;*** **bulk-tank milk; thermodurics; thermophiles; sulphite-reducing clostridia**

## Introduction

Milk quality is greatly influenced by the microbial load of the milk. When aseptically drawn, milk is sterile; however, it is contaminated during and after secretion and during the normal processes of production and processing. Infection of the mammary gland, udder and teat surfaces, milking equipment and storage tanks all have the potential to contaminate milk. Milk is a nutritious medium that can support the growth of a large selection of bacterial contaminants. Bacteria are capable of utilising the proteins, fats, carbohydrates and vitamins in milk for their growth and metabolism. The total bacteria count (TBC) (alternatively known as total viable count [TVC]) measures the amount of bacterial contamination in milk. In general, as long as the TBC of the milk is less than 100,000 colony forming units (cfu)/ml, it is within the regulatory limit.

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However, good quality milk has a TBC of <10,000 cfu/ml. Most of these bacteria are killed by pasteurisation; however, some bacteria can survive pasteurisation and are called thermodurics. Depending on their physiological characteristics, thermodurics are divided into different categories:

*Thermophilic thermodurics:* thermophiles have an optimum growth temperature of 50–55 °C, but can grow in the range of 40–60 °C. If present in milk, they can grow during the pre-heating stages of processing (Murphy *et al.* 1999) and the numbers can be increased after processing. Therefore, initial numbers in milk need to be low.

*Mesophilic thermodurics:* mesophiles have an optimum temperature of about 30 °C although they can grow in the range 5 to 50 °C. Therefore, they can grow during poor refrigeration conditions on-farm or during processing. These sporeforming and non-sporeforming bacteria include, for example, strains of *Bacillus*, *Microbacterium*, *Micrococcus*, *Enterococcus*, *Streptococcus* and *Arthrobacter.* Spore forming bacteria have the ability to form spores when subjected to harsh environments (i.e. high temperatures, extreme pH values or nutrient lacking environments). The original cell makes a copy of its chromosome and surrounds it with a tough wall forming a dormant dehydrated resistant spore. When conditions become favourable the spore can rehydrate and resume metabolism. By these means, spore forming bacteria have the ability to withstand pasteurisation during milk processing. Growth temperature range for thermophilic, mesophilic and psychrophilic bacteria is presented in Table 1.

*Psychrotrophic thermodurics*: psychrophiles can grow at temperatures from freezing to 25 °C. A sub-group within this group are psychrotrophs that can grow at or below 7 °C. Mesophilic bacteria that can grow at refrigeration temperatures are also called psychrotrophs. Most psychrotrophs are not heat resistant, e.g. *Pseudomonas* spp., and are in milk due to post-pasteurisation contamination. Psychrotrophic thermodurics have the ability to withstand pasteurisation and subsequently grow at refrigeration temperatures, in on-farm bulk tank milk or in product. In addition to being spore-forming bacteria, they can produce proteolytic enzymes that degrade protein and fat in milk.

Due to their ability to withstand pasteurisation, thermoduric bacteria can limit the shelf life of pasteurised milk (Te Giffel *et al*. 1997). Late blowing of cheese and 'bitty cream' in milk are issues associated with thermoduric contamination (Stone and Rowlands 1952). Additionally, some thermodurics are food borne pathogens, thus their numbers in dairy products must be minimised. Testing for thermoduric bacteria

**Table 1. Growth temperature ranges for thermophilic, mesophilic and psychrophilic bacteria**

| Bacterial group | Parameter | Growth temperature |
|---|---|---|
| Thermophilic 'heat loving' | Minimum | 40 °C |
| | Maximum | 60 °C |
| | Optimum | 50–55 °C |
| Mesophilic 'medium temperature' | Minimum | 5 °C |
| | Maximum | 50 °C |
| | Optimum | 30–37 °C |
| Psychrophilic 'cold loving' | Minimum | 0 °C or less |
| | Maximum | 25 °C |
| | Optimum | ≤20 °C |

involves pasteurising milk samples in the laboratory by heating for 30 min at 62.8 °C, after which a conventional TBC method such as standard plate count or petrifilm aerobic count is used to enumerate the surviving bacteria (Frank and Yousef 2004).

Major bacterial groups of concern to the dairy industry are the *Bacillus cereus* group, the sulphite-reducing clostridia (SRC) and psychrotrophic thermodurics.

### *Bacillus cereus* group of bacteria

*B. cereus* is a Gram positive, rod-shaped, spore-forming motile bacterium with peritrichous flagella (Christiansson 2011) which can grow in aerobic or faculatively anaerobic conditions and can survive pasteurisation (Granum 2005). It is a member of the *Bacillus cereus* group which, in addition to *B. cereus*, comprises five other very closely related species, *B. thuringiensis, B. mycoides, B. weihenstephanensis, B. pseudomycoides* and *B. anthracis* that are very difficult to distinguish (Table 2; Tallent *et al.* 2012a). *B. cereus* group bacteria can be isolated on selective mannitol egg yolk polymyxin agar (MYP). The colonies are pink (mannitol negative) with a zone of precipitate (lecithinase positive) (Mossel, Koopman and Jongerius 1967). Traditional biochemical tests for the identification of *B. cereus* (*sensu stricto*) are Voges-Proskauer (positive), nitrate reduction (positive), glucose fermentation (positive) and catalase (positive) (Table 2). The optimum temperature for growth of *B. cereus* group is 30–37 °C although they have the ability to grow at temperatures in the range 4.5 to 50 °C (Claus and Berkley 1986).

Spores of the *B. cereus* group are sufficiently heat resistant to survive high temperature short time (HTST) pasteurisation. Instead of destroying the spores, the heat treatment can trigger spores to germinate and subsequently grow in the pasteurised product (Griffiths and Phillips 1990). Species within the *B. cereus* group are foodborne pathogens capable of causing foodborne illness. Diarrhoeal disease occurs when ingested food containing $10^5$-$10^7$ cells of *B. cereus* (*sensu stricto*) produce enterotoxin in the small intestine (Granum 2005). An emetic disease occurs when food containing pre-formed toxin is ingested.

The occurrence of *B. cereus* group species in raw milk and consequently in pasteurised products, particularly dried products, is almost inevitable. Of 92 infant milk formula (IMF) samples examined in a German study, 54% were positive for *B. cereus* group (Becker *et al*. 1994). While there was a high percentage of positive samples the counts were relatively low (0.3–10 cfu/g). Similarly, 59 of 100 IMF samples from Ireland were positive for *B. cereus* group but again counts were low (Haughton, Garvey and Rowan 2010). When stored under unfavourable conditions after reconstitution (>25 °C for 14h), *B. cereus* (*sensu stricto*) became the dominant organism and has antagonistic properties towards other groups of bacteria (Haughton *et al*. 2010). Reports on illness from contaminated IMF are rare. Nevertheless, strict caution to prevent contamination is warranted due to the high susceptibility of the target consumers.

Various agars can be used to isolate *B. cereus* group bacteria, all of which contain egg yolk and rely on the production of lecithinase by *B. cereus* colonies for identification (Tallent *et al.* 2012b). Further identification to species level requires the use of traditional biochemical tests (Table 2) or molecular methods.

### Sulphite-reducing clostridia

Sulphite-reducing clostridia (SRC) are Gram positive anaerobic spore-forming

**Table 2. Differentiating characteristics of the species in the *Bacillus cereus* group (Modified from Tallent *et al.* 2012a)**

| Test | *B. cereus* | *B. thuringiensis* | *B. mycoides* | *B. weihenstephanensis* | *B. anthracis* | *B. megaterium* |
|---|---|---|---|---|---|---|
| Gram reaction | +[a] | + | + | + | + | + |
| Catalase | + | + | + | + | + | + |
| Motility | +/–[b] | +/– | –[c] | + | – | +/– |
| Nitrate reduction | + | + | + | + | + | – |
| Decomposed of tyrosine | + | + | +/– | + | – | +/– |
| Lysozyme-resistant | + | + | + | + | + | – |
| Egg yolk reaction | + | + | + | + | + | – |
| Anaerobic utilisation of glucose | + | + | + | + | + | – |
| Voges-Proskauer reaction | + | + | + | + | + | – |
| Acid produced from mannitol | – | – | – | – | – | + |
| Haemolysis (sheep RBC[d]) | + | + | + | ND | – | – |

[a]90–100% of strains are positive.
[b]50% of strains are positive.
[c]90–100% of strains are negative.
[d]Red blood cells.
ND = Not determined.

bacteria (Donnelly and Busta 1981). Similar to *B. cereus*, SRC describes a group of bacteria consisting of 12–14 different species that are difficult to distinguish to species level. The spores are highly resistant to extreme chemical and physical conditions and are ubiquitous in nature where they can germinate when conditions are favourable (Aureli and Franciosa 2002). Growth temperatures for each species can vary from 3.3 °C to 80 °C (Aureli and Franciosa 2002). The ability of organisms in this group to reduce sulphite to sulphide and form black colonies on selective agar is a fundamental basis for their identification and thus they are referred to as sulphite-reducing clostridia (Angelotti *et al*. 1962; Weenk, Fitzmaurice and Mossel 1991; Aureli and Franciosa 2002). *Clostridium perfringens* is one of the key SRC species and is commonly found in faeces (of both humans and animals) and is widely distributed in soil, dust, vegetation and raw foods. Characteristics of both *C. perfringens* and *B. cereus* are outlined in Table 3. On the farm, silage can be a major source of this and other *Clostridia* since the silage making process provides the bacteria with an anaerobic environment in which they can grow. Spores ingested by cows eating contaminated silage are excreted in the faeces which can contaminate raw milk at milking (Aureli and Franciosa 2002). A heat labile cytotoxic enterotoxin can be produced by *C. perfringens.* The toxin disrupts the membrane of epithelial cells resulting in diarrhoea, cramps, nausea and vomiting which follow shortly after ingestion of numbers in excess of $10^8$ cells (Andersson, Ronner and Granum 1995; Aureli and Franciosa 2002). Because of their potential toxicity to infants, it has been recommended that spore counts in infant foods are minimised (Aureli and Franciosa 2002). The detection of sulphite-reducing *Clostridia,* as the

name suggests rely on *Clostridia* spp. to reduce sulphite in iron sulphite agar base to form ferrous sulphide which results in the formation of black colonies. Polymyxin is added to the agar to select for *Clostridia* spp. The growth of sulphite-reducing *Clostridia* requires anaerobic incubation at 30–37 °C for up to three days (Aureli and Franciosa 2002).

## Regulations

As *B. cereus* in milk can be toxin-producing, the European Union (EC, 1771/2007) has set a threshold for *B. cereus* spores in dried infant formulae intended for infants below 6 months of age. For product to be considered satisfactory and within the threshold levels, five samples are analysed and four samples must be below 50 cfu/g while the remaining sample can be between 50 and 500 cfu/g. In order to achieve this target level, the specifications for *B. cereus* in raw milk are frequently set at <10 cfu/ml. In order to achieve these specifications, it is necessary to understand the sources of these bacteria and the control measures that can facilitate their reduction.

Ideally, thermoduric counts should be below 100 to 200 cfu/ml and counts below 10 cfu/ml indicate excellent equipment hygiene (Reinemann *et al.* 2003). While in the past a limited number of processors in Ireland tested for thermoduric bacteria, more have been obliged to include it as part of the payment schemes in order to comply with customer requirements. Those that conduct this analysis carry out approximately two tests per month and have set a penalty threshold for thermoduric counts in raw milk at 1000 cells/mL.

## Sources of thermoduric bacteria in raw milk

Contamination of bulk tank milk by microorganisms occurs during and after milking (Figure 1). Thermoduric bacteria in milk can arise from soil, bedding, feed, dust, all of which contaminate cow's teats, from which raw milk can be contaminated. Additionally, contamination can occur from deposits on milking equipment.

### *Contamination from teat skin*

Teat skin is considered the major source of thermoduric spores in raw milk and

**Table 3. Characteristics of *Clostridium perfringens* and *Bacillus cereus* group**

| | *Clostridium perfringens* | *Bacillus cereus* group |
|---|---|---|
| Relevance | Foodborne intoxications | Foodborne intoxications, spoil pasteurised dairy products |
| Number of species | 12 to 14 | 6 |
| Growth conditions | Anaerobic | Anaerobic and facultatively anaerobic |
| Growth in milk | Yes | Yes |
| Temperature range for growth | 12–60 °C | 4–55 °C |
| Optimum temperature | 43–47 °C | 30–37 °C |
| Example of industry specification for spore concentrations in bulk tank milk | < 5 spores/mL | < 10 spores/mL |
| Origin of spores in bulk tank milk | Environment – exterior of teats (soil, faeces, silage, milking/ processing equipment) | Environment – exterior of teats (soil, faeces, silage, milking/processing equipment) |

subsequent attachment to surfaces and growth are responsible for the majority of the spore contamination in bulk-tank milk (McKinnon and Pettipher 1983; McKinnon, Rowlands and Bramley 1990; Christiansson, Bertilsson and Svensson 1999). Dirt attached to teats rinses off during milking and spores present in the rinsed off dirt contaminate the milk. The concentration of spores transmitted to milk depends on the amount of dirt on teats and the spore concentration in the dirt. Predictive models have been used to quantitatively identify factors that have the greatest effect on spore concentrations in bulk tank milk. Using such models, Vissers *et al.* (2007) estimated that when teats were contaminated with soil, 33% of tank milk will contain >1000 spores/L, whereas when feed is the main source of contamination, this figure is only 2%. Therefore, cleaning of teats prior to milking can reduce the spore count of milk. Different teat-cleaning methods have been evaluated to determine their effect on the presence of spores in milk. The most effective method (showing a reduction of 96%) was when a moist washable towel followed by drying with a dry paper towel for a total of 20 s was used (Magnusson *et al.* 2006). That study also showed that cleaning was independent of the contamination source (soil, manure, sawdust) or type of spore. Significant reductions in TBC, thermoduric bacteria, enterocci and coliforms were shown with full teat preparation as compared to no teat preparation (Murphy *et al.* 2005). Furthermore, teat preparation involving the use of disinfectant wipes or teat disinfection followed by drying with individual paper towels have also been shown to reduce *Staphylococcus*, *Streptococcus* and coliform numbers on teat skin prior to milking (Gleeson *et al.* 2009). However, teat washing using an iodine solution was shown to be ineffective in reducing bacterial populations of the teat unless followed by thorough drying (Zarkower and Schenchenzuber 1977). This highlights the importance of drying in any teat preparation procedure. A recent survey of over 400 farms in Ireland showed that only 2% of farms wash and dry cow's teats during the summer months with this

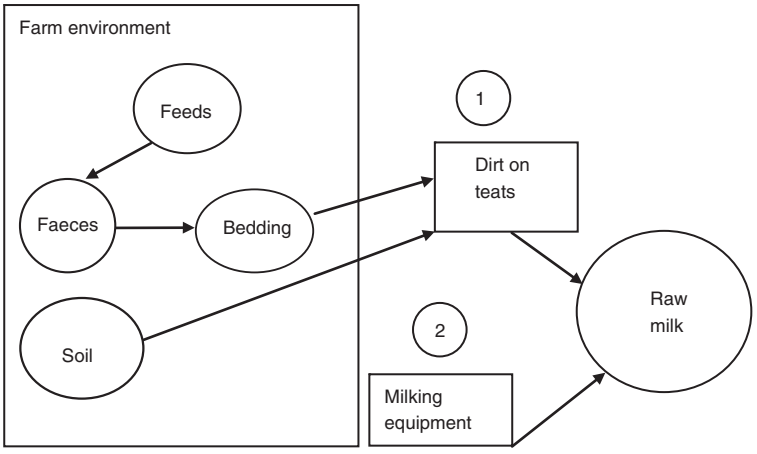


*Figure 1. Sources and route of microbial contamination into milk. Microorganism and spores are transmitted to milk (1) via the exterior of cows' teats, and (2) via surfaces of the milking equipment.*

figure increasing to only 7% during the winter months (Kelly *et al.* 2009). This demonstrates an opportunity for reducing the contamination of Irish milk with spore-forming bacteria.

It has been demonstrated that during the grazing season, the spore concentration in bulk tank milk can be directly related to the contamination of teats with soil (Christiansson *et al.* 1999). High water content of soil and dirty access roadways were considered the most important factors associated with high spore concentrations. It was also suggested that increased soil consumption occurs during wet weather (Herlin and Anderson 1996) due to increased levels of soil present on grazed grass. Spores in the subsequent faeces can contribute to some extent to the spores in milk via contamination of teats from cows lying on pasture. In addition to dirty teats and soil, the concentration of spores in animal feed is a source of contamination during the housing period. Poor quality silage with spore counts of 4.5 $\log_{10}$ cfu/g was considered a likely source of teat contamination (Slaghuis *et al*. 1997). Reductions in spore counts of 60% were feasible indoors by ensuring that the spore concentration of feed was less than 3 $\log_{10}$ cfu/g and the pH of the ration is less than 5.0 (Vissers *et al.* 2007). Cubicle bedding was also considered as a source of milk contamination with *B. cereus* (Davies and Wilkinson 1973). High *B. cereus* counts were shown to be present in the upper layers of sawdust used for bedding cows whilst indoors, which was in direct contact with cow's udders (Magnusson *et al.* 2007). The addition of some cubicle bedding materials such as hydrated lime can lower bacterial counts on cubicle beds (Hogan *et al.* 1989).

In addition, the standard of hygiene of dairy facilities may affect the levels of bacteria found in milk. In Ireland 57% of milking facilities surveyed were considered unsatisfactory in terms of cleanliness (Kelly *et al.* 2009). Improved dairy and parlour hygiene, the fitting of individual feed hopper covers and keeping the bulk milk tank lid closed during the milking process would minimise the possibility of dust getting into raw milk.

*Contamination from milking equipment*

Inadequate cleaning and maintenance of milking equipment can promote biofilm formation on stainless steel surfaces. *B. cereus* biofilms may develop in storage and piping systems that are partially filled or where residual liquid remains after a production cycle (Wijman *et al.* 2007), where they facilitate the growth and multiplication of thermoduric spores which can be released into milk. Biofilms allow greater resistance of bacteria to temperature and sanitation (Frank and Koffi 1990). The inclusion of regular acid cleaning of the milking equipment (Elmoslemany *et al*. 2010) to remove mineral deposits and the inclusion of pre-milking plant disinfection (with sodium hypochlorite or peracetic acid) have both been shown to minimise the levels of thermoduric bacteria in raw milk (Gleeson, O'Brien and Jordan 2013). A number of effective cleaning procedures suitable for milking systems in Ireland have been developed (Gleeson 2013). Sodium hypochlorite has been previously shown to be effective for the cleaning and disinfection of milking equipment (Reinemann *et al.* 2003), but issues with chlorine residues may result from inappropriate use (Ryan *et al*. 2012). Peracetic acid contains environmentally acceptable breakdown products and is effective against bacteria and spores (Watkinson 2008).

The level of water hardness was shown to have an impact on equipment hygiene (Elmoslemany *et al.* 2009). Levels of water hardness expressed as calcium carbonate

($CaCO_3$) can vary from soft (<60 µg/mL) to very hard water (>200 µg/mL) (Watkinson 2008). Hard water has increased magnesium and calcium levels which have a negative effect on disinfectant efficacy and result in mineral deposits on surfaces and the likelihood of biofilm formation. In addition to regular plant descaling, the installation of water softeners provides an effective method for improving water quality and should be considered where water hardness levels are >200 µg/mL (Watkinson 2008).

Automatic cleaning of milk liners between individual cow milkings can also minimise the levels of bacteria (mesophillic *Staphylococcus*, *Streptococcus* and coliform) on liners (Gleeson, O'Callaghan and O'Brien 2010), thus reducing the possibility of bacterial contamination of milk. Only 40% of Irish dairy farmers use heated water, which was shown to have significantly positive effect on total bacterial counts in the milking parlour (Kelly *et al.* 2009). The effectiveness of cleaning agents improves as water temperature was increased (Reinemann *et al.* 2003), and lower wash water temperature can be associated with more variability in farm milk bacterial levels (Bava *et al.* 2009). However, Palmer and O'Shea (1973) concluded that cold circulation cleaning systems compared favourably with conventional circulation cleaning when alkaline powder products (76%) were used with extended surface contact time (rinsing delayed until just before the next milking) and the pH of the working solution was >10. However, hot water provides greater microbial kill than chemical cleaning with cold water. A rise in temperature of 10 °C increased the reaction rates of chemicals by between two and eight times (Watkinson 2008). For effective cleaning an initial cycle water temperature of 70° C and an end of cycle temperature of 50° C is recommended for CIP cleaning with liquid detergent-steriliser solutions (Gleeson 2013).

Furthermore, water quality can also play a role in increasing the spore content of raw milk (Cook and Sandemann 2000), while the use of filter socks is a recommended practice during milking, it is only effective in removing large debris from the milk and is ineffective in removing bacteria from the milk (Chambers 2002).

The rate of bacterial growth in milk is dependent on the initial microbial load and the temperature at which the milk is stored. Ideally, this should be less than 4 °C (Chambers 2002). During the milk cooling period some microorganisms may multiply (Holm *et al.* 2004), especially fast growing psychrotrophic bacteria that reproduce in the temperature range of 4 to 7 °C. The inclusion of a plate cooler has been demonstrated to minimize thermoduric bacteria levels in milk (Elmoslemany *et al*. 2010). Therefore, rapid cooling of milk within the processor regulated temperature (2–4 °C) using efficient plate coolers (Murphy, Upton and O'Mahony 2013) may prevent further psychrotrophic bacteria growth. Ideally the cooling of milk to this required temperature should be completed within a half hour of the completion of each milking session.

**Strategies for control of spores in milk**

The dairy industry depends mainly on pasteurisation to reduce the bacterial count of milk. However, spore forming bacteria can withstand pasteurisation, so the most effective method for reduction of the spore count in milk is increased hygiene at milking.

From the many research studies undertaken, teat preparation is considered a key preventative factor in reducing thermoduric numbers in milk. Bacterial numbers both on teats and in milk are

minimised when teats are washed and dried with individual paper towels or disinfected and dried with individual paper towels prior to milking. Particular attention should be paid to teat preparation whilst cows are indoors, during periods of wet weather and extremely dry weather as soil levels which are high in thermoduric bacteria are more likely to be present on teats. Drying of teats prior to cluster attachment is considered critical. To help maintain a clean udder and prevent teats becoming soiled between milkings, cow's tails should be clipped at least three times a year i.e. at calving, mid summer and at housing. The collecting yard and holding yard should be washed down after each milking and the cow platform and milking cluster should be washed regularly during the milking process. However, in-parlour cleaning should not be conducted whilst cows are still present on the platform or the cluster is still attached to a cow.

Following a recommended plant cleaning procedure is critical to the prevention of an environment that will facilitate milk contamination with thermoduric bacteria. In addition to regular plant descaling, the installation of water softeners provides an effective method for improving water quality. As some thermoduric bacteria will multiply at temperatures above 4 °C, cooling of milk should be completed within a half hour of the completion of milking. Other control factors such as automatic cluster cleaning between individual cow milkings, the addition of peracetic acid as a pre-milking plant rinse and replacing cracked rubberware will also contribute in to minimising thermoduric bacteria levels in milk.

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