MODELING TO CONTROL SPORES IN RAW MILK

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Food producers are responsible for the safety and quality of their products (European Commission, 2000). The quality, safety and economic performance of milk and dairy products depend on the quality of raw milk, processing, distribution and storage conditions. A chain management approach is required to ensure safe and high-quality dairy products. In the dairy chain, dairy farms have an important role. Amongst other factors, the safety and shelf life of dairy products is determined by the contamination of farm tank milk (**FTM**) with microorganisms and their spores.

To produce safe foods the Hazard Analysis Critical Control Points (**HACCP**) approach is implemented throughout the dairy chain. HACCP is a science-based quality-management system developed to ensure the safety of foods (Codex Alimentarius, 2003). Application of HACCP principles to dairy farms is discussed but not yet considered to be generally feasible (Ruegg, 2003). As an alternative to HACCP, codes for good farming practices have been developed (Morgan, 2004). These codes contain qualitative guidelines for all aspects of farm management, aiming to produce milk from healthy animals under generally accepted conditions. However, practice shows that farmers are not able to take measures in all steps of the pathway and tend to focus on specific steps. Furthermore, they do not agree on which measures are most efficient and interpret good hygiene guidelines differently. Therefore, dairy farmers and the dairy industry may benefit from a quantification of the effectiveness of potential control measures. What has to be done first and what is most effective?

An important tool to improve (microbial) quality assurance in production chains is predictive modeling. Predictive models can be used to quantitatively identify the most important steps in the production chain and the most effective control measures. Predictive modeling has been successfully used to optimize production processes and improve the quality and safety of foods including dairy products (Cassin et al., 1998b, De Jong, 1996, De Jong et al., 2002, Van Gerwen, 2000). Predictive models have been developed for some specific parts of the farm environment (Allore and Erb, 1999, Kelly et al., 2000, Ruxton and Gibson, 1993, Zadoks et al., 2002). However, models predicting the microbial contamination of FTM are a missing link in the quantitative approach of the whole production chain. Modeling the microbial contamination of FTM could be used to identify measures that most effectively reduce the concentration of microorganisms and spores in FTM.

To be useful, mathematical models predicting the microbial contamination of FTM should meet some requirements. A model should describe microbial introduction, growth, transmission, inactivation and removal in different parts of the farm environment (e.g. feed, barn and milking parlor) (Figure 1.1). By means of modeling, effects of for example inhibition of microbial growth in feed can be linked and quantitatively compared with the efficiency of cleaning the cow's teats prior to milking. The model should comprise a chain of events that leads to the final microbial contamination level in FTM, as transported to the site of the dairy processor. In order to develop a strategy to minimize microbial concentrations in FTM, variables in the model should relate to aspects a farmer can control. In addition, the model should also account for effects of uncontrollable factors such as seasonal variation of the microbial load of soil. Under specific management conditions, uncontrollable variables determine the highest concentrations in FTM. To develop a model meeting these demands, knowledge and data about possible sources of contamination, contamination routes and effects of specific measures are required.

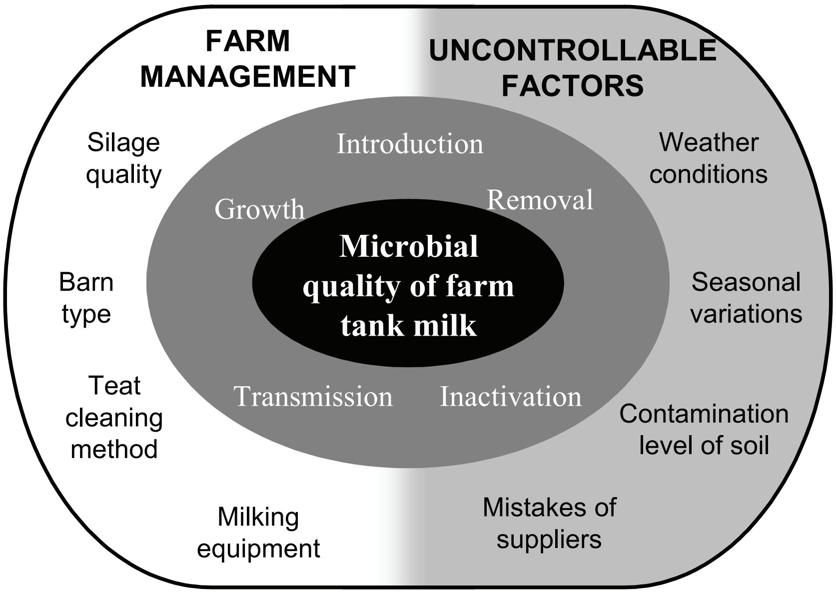

Figure 1.1 Control of microbial quality of farm tank milk (mentioned aspects of farm management and uncontrollable factors are examples and not all inclusive).

Over the years, the dairy industry and research organizations have collected a large amount of data and knowledge about the contamination of FTM by microorganisms and bacterial spores. These existing data and knowledge form the basis for the development of models predicting the concentration of a specific microorganism in FTM. An additional advantage of this approach is that model development will facilitate a structured integration of existing data and knowledge and may reveal relevant aspects that were previously not studied in detail.

Two microorganisms that can spoil dairy products and (partly) originate from the farm environment are butyric acid bacteria (**BAB**) and *Bacillus cereus*. Table 1.1 lists some characteristics of BAB and *B. cereus*. Spores of these microorganisms in raw milk survive pasteurization and, cause the late-blowing defect in semi-hard cheeses (BAB) and limit the shelf life of refrigerated pasteurized dairy products (*B. cereus*), respectively. Because of their relevance, data and knowledge about the contamination of FTM with spores of these microorganisms are available. However, the data are scattered and, in practice, there is disagreement about which measures at the farm level are most effective. Predictive modeling could be used to integrate existing knowledge and data and to identify the most efficient measures to improve control of the contamination of FTM with BAB and *B. cereus* spores. Objective of the dairy industry is to ensure BAB and *B. cereus* spore concentrations in FTM are below 3 $\log_{10}$ spores/L; the maximum spore limit (**MSL**).

Table 1.1 Characteristics of butyric acid bacteria (BAB) and *Bacillus cereus* (Bergère et al., 1968, Griffiths and Phillips, 1990, Klijn et al., 1995, Scientific Panel on Biological Hazards, 2004, Slaghuis et al., 1997, Stadhouders and Jørgensen, 1990, Thylin et al., 1995)

	Butyric acid bacteria	***Bacillus cereus***
Relevance	Cause late-blowing of semi-hard cheeses	Spoil pasteurized dairy products kept at refrigerator temperatures
Growth conditions	Anaerobic	Facultative anaerobic
Growth in milk	No	Yes
Temperature range for growth	10 - 44 °C	4 - 55 °C
pH range for growth	4.4 - 6.8	4.9 - 9.3
Water activity range for growth		0.95 - 1.0
Spore concentrations in FTM	<30 - >10,000 spores/L	<10 – 10,000 spores/L
Origin of spores in farm tank milk	- Exterior of teats (sources: silage, other feed and soil)	- Exterior of teats (sources: silage, other feed and soil) - Milking equipment
Desired concentration or maximum spore limit (MSL)	1,000 spores/L	1,000 spores/L

1.2 SOURCES OF MICROBIAL CONTAMINATION OF FARM TANK MILK

In order to define an effective strategy for controlling the contamination of FTM by microorganisms and bacterial spores it is essential to know of the microbial sources and routes of contamination. Milk is nearly sterile when secreted into the alveoli of the udder (Tolle, 1980). Contamination of FTM by microorganisms occurs during and after milking. Microorganisms and spores are transmitted to milk (1) via the exterior of the cow's teats, (2) via the interior of teats (in the case of mastitis) and (3) via surfaces of the milking equipment (Figure 1.2). After the initial contamination, the concentration in FTM can further increase due to microbial growth (Vissers and Driehuis, 2007). Aerial contamination is insignificant under normal production conditions (Akam et al., 1989, Stadhouders and Jørgensen, 1990, Te Giffel et al., 1995).

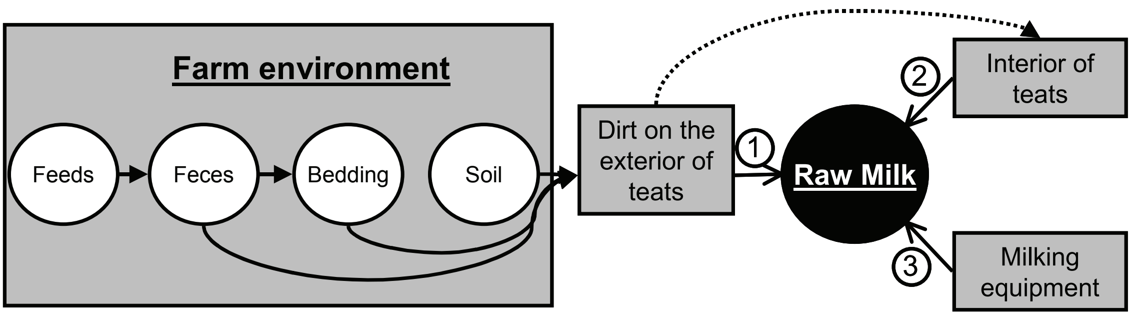

Figure 1.2 Routes for the contamination of raw milk with microorganisms (from Vissers and Driehuis (2007)).

1.2.1 Contamination via the exterior of teats

The most common sources of spores in the farm environment are feed, feces, bedding material and soil. Spores from these sources are transferred to milk in a number of steps. Basically two overlapping routes or contamination pathways for bacterial spores can be distinguished. The first contamination pathway starts with feed. Spores in feed such as silages pass the gastrointestinal tract of cows unharmed and accumulate in feces. Feces and bedding material contaminate the cows' teats. Teat cleaning prior to milking only partly reduces attached dirt and spores (Vissers and Driehuis, 2007). During milking, feces, bedding material and spores on the surface of teats are transmitted to milk (Te Giffel et al., 2002). The second contamination pathway starts with soil. Especially during grazing, soil can contaminate the exterior of teats and, as in the first pathway, spores originating from soil are transmitted to FTM during milking. Elevated *B. cereus* spore concentrations during the summer months have been associated with the transmission of soil to FTM (Christiansson et al., 1999 Slaghuis et al., 1997). In the remainder of this thesis the mixture of feces, bedding material and/or soil attached to the teats is referred to as dirt.

1.2.2 Contamination via the interior of teats

Contamination via the interior of teats is associated with mastitis. After mastitis pathogens have entered the teat canal and infect tissue, the level within the teat may increase significantly. During milking, the mastitis pathogens is transmitted to the milk. For bacterial spores this route is not relevant.

1.2.3 Contamination via surfaces of the milking equipment

Contamination of milk via the milking equipment occurs when microorganisms and milk residues adhered to surfaces in the milking equipment are not removed completely during cleaning (Figure 1.3). In general, bacterial spores are more resistant to cleaning procedures than vegetative cells (Saran, 1995). In the period between two milkings the concentration of adhered microorganisms and spores may increase due to growth. In the Netherlands, the time between two milkings is approximately 10 to 14 h. During milking adhered microorganisms are released into passing milk and increase the microbial contamination of FTM. Especially cracked and decayed rubber parts are sensitive to accumulation of microorganisms (Akam et al., 1989).

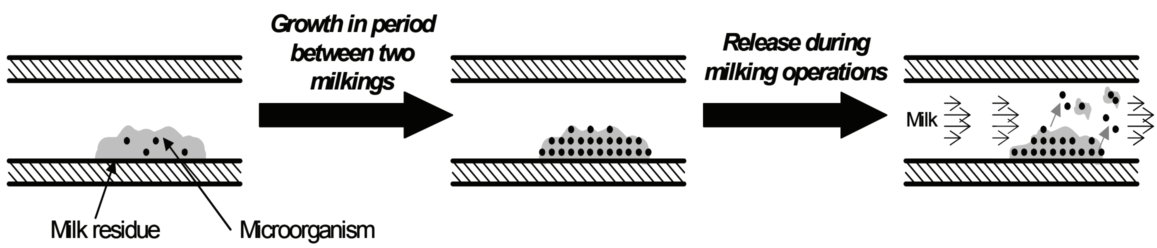

Figure 1.3 Contamination of milk via the surfaces of the milking equipment (from Vissers and Driehuis (2007)).

1.2.4 Growth during storage of FTM

During the period FTM is stored at the farm microbial concentrations may increase due to microbial growth. At dairy farms in the Netherlands, milk is collected in a farm tank before transportation to the dairy processor. Growth in this period, generally 3 days, primarily depends on the temperature of the stored milk. In the Netherlands FTM is cooled to temperatures below 4 °C to prevent growth. Requirements with respect to the cooling rate, farm tank isolation and monitoring of the temperature of the milk in the farm tank are defined in ISO-standard 5708 (ISO, 1983) and in regulations from dairy processors. However, the temperature of FTM in the bulk tank is not constantly at 4 °C. For instance, it takes some time (2-3 h) to cool milk, pumped in an empty tank, to 4 °C. In addition, warm milk is added to the cooled milk every milking, resulting in a temporary increase of the temperature. Under these conditions, growth of psychrotrophic microorganisms, like *B. cereus,* may occur.

1.3 OUTLINE OF THIS THESIS

The objective of the work described in this thesis was to quantify the effectiveness of measures to control the contamination of FTM with spores of BAB and *B. cereus* using model simulations. Results should lead to the definition of a control strategy. For both species models predicting the contamination level of FTM were developed first. Simulation results were validated using a year-long field survey held at 24 Dutch farms. In addition, measurements at specific parts of the contamination pathway (silage, dirt transmission during milking) were performed.

Chapters 2 to 5 deal with the contamination of FTM with BAB spores. The model and simulation results are described in Chapter 2. Chapters 3 and 4 discuss the measurements at two specific parts of the contamination pathway. Chapter 3 focuses on the effects and causes of the heterogeneous distribution of BAB spores in grass- and corn silage. Chapter 4 describes the quantification of the transmission of dirt and spores via the exterior of teats. Chapter 5 describes the validation and refinement of the control strategy derived in Chapter 2 using results from the field survey and results from Chapters 3 and 4.

Chapters 6 and 7 deal with controlling the contamination of FTM with spores of *B. cereus*. Chapter 6 discusses the model developed and simulations. The identified control strategy is validated and refined using data from the field survey (Chapter 7).

In Chapter 8 the effects of implementation of the control strategies identified for the dairy industry are quantitatively assessed.

CHAPTER 2

MODELING THE CONTAMINATION OF FARM TANK MILK WITH BUTYRIC ACID BACTERIA SPORES

Control of the contamination of farm tank milk (FTM) with spores of butyric acid bacteria (BAB) is important to prevent the late blowing defect in semi-hard cheeses. The risk of late blowing can be decreased via control of the contamination level of farm tank milk with BAB spores. A modeling approach was applied to identify an effective control strategy at the farm level. The simulation model developed was based on a translation of the contamination pathway into a chain of unit operations. Using various simulations the effects of factors related to feed quality, feed management, barn hygiene and milking practices on the contamination level of FTM were evaluated. The contamination level of silage was found to be the most important factor. When silage contains on average less than 3 $\log_{10}$ BAB spores/g, a basic pre-treatment of udder teats before milking (approximately 75% removal of attached spores) is sufficient to assure a FTM contamination level below the maximum spore limit (MSL) of 3 $\log_{10}$ BAB spores/L. When silage contains more than 5 $\log_{10}$ BAB spores/g, it should not be fed; it then becomes almost impossible to ensure a FTM contamination level below the MSL. When silage contains on average between 3 and 5 log BAB spores/g, it is most effective not to milk cows that prefer to lie down on dirty patches and to clean teats prior to milking with a more severe and time consuming method (at least 90% removal of attached spores). Measures aimed at improving barn hygiene, decreasing the transmission of spores originating from soil and reducing the contamination level of other feed contribute only marginally to improved control of the contamination of FTM with BAB spores. Application of the modeling methodology could be beneficial for control of the contamination of FTM with other microorganisms such as *Bacillus cereus*.

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Improving farm management by modeling the contamination of farm tank milk with butyric acid bacteria

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2.1 INTRODUCTION

Spores of butyric acid bacteria **(BAB)** are important for the dairy industry with respect to spoilage of semi-hard cheeses. Specifically, *Clostridium tyrobutyricum* is able to convert lactic acid into butyric acid at relatively low pH. These characteristics and the ability to survive pasteurization enable *C. tyrobutyricum* to grow in semi-hard cheeses, such as Gouda. and Emmenthaler. This results in off-flavors and excessive gas formation, so-called late blowing (Bergère and Hermier, 1970, Klijn et al., 1995). Contamination levels of BAB as low as 10 spores/L in pasteurized cheese milk can cause the late-blowing defect in Gouda-type cheeses (Stadhouders, 1990).

To prevent late blowing, the cheese manufacturers have three options: 1) encouraging farmers to reduce the contamination level in farm tank milk **(FTM)**, 2) addition of nitrate and lysozyme to cheese milk to prevent growth of BAB, and 3) removal of 95-99% of the spores during cheese production via bactofugation (Stadhouders, 1990, Waes and Van Heddeghem, 1990). In the Netherlands, farmers are encouraged to control the contamination of FTM with BAB spores via a penalty system. In this system, farmers get a deduction on the milk price when the BAB spore concentration in FTM at delivery results in two BAB spore positive samples of 0.1 mL (Berg et al., 1989). After introduction of this penalty system, the fraction of penalized samples declined from approximately 7% in the 1980's to 2% in the mid 1990's. Since then the fraction of penalized samples stayed more or less constant.

Butyric acid bacteria spores present in FTM originate from the farm environment. The spores are naturally present in soil but during daily practice, silage and other feed such as brewer's grain and hay also act as sources of contamination. Butyric acid bacteria spores are transmitted from these sources to FTM via a number of steps. Feces is considered to be the main carrier in this contamination pathway (Bergère et al., 1968). Along the contamination pathway microbial transmission, growth and inactivation take place. A farmer can reduce the contamination level of FTM via (1) minimization of the introduction of BAB to the farm environment, (2) minimization of transmission, (3) minimization of growth and (4) maximization of inactivation. However, a farmer has to take into account that the concentration also depends on factors the farmer cannot control, e.g. the concentration in soil.

Numerous studies have been performed to identify measures to control the contamination of FTM with BAB (Bergère et al., 1968, Stadhouders and Jørgensen, 1990). Identified measures relate to feed quality, barn hygiene and milking practices. It is suggested to take various measures in all steps of the contamination pathway. However, practice shows that farmers are not able to take measures in all steps and tend to focus on specific steps. In addition, they do not agree on which measures are most efficient, and interpret good hygiene guidelines differently. Consequently, high concentrations of BAB spores in FTM still occur. The Dutch dairy industry wants to ensure a contamination level of FTM below a maximum spore limit (**MSL**) of 3 $\log_{10}$ BAB spores/L. To achieve this, farmers need practical definitions of control measures and quantitative insight into the effectiveness of these measures.

In food safety risk assessment (Cassin et al., 1998b) and industrial process optimization (De Jong et al., 2002) mathematical modeling to quantitatively identify control strategies is frequently applied. This study is a first attempt to apply a similar modeling approach to the contamination of FTM with BAB spores. The objective was to quantitatively identify an effective control strategy in practical and measurable terms.

2.2 MATERIALS AND METHODS

2.2.1 Model development

The contamination pathway

A contamination pathway can be seen as a process flow in industrial processing. A process flow diagram starts with raw materials and consists of the successive unit operations these materials undergo for the production of the end product. Examples of unit operations are mixing, concentration and storage. Because of the similarity with a process flow diagram, a contamination pathway can be translated into a chain of unit operations (Table 2.1).

In this translation, the three sources of contamination (soil, silage and other feed) correspond to raw materials. Silage comprises grass and corn silage; other feed comprise all possible other feeds fed to the cows, such as concentrates, brewer's grain and hay. Silage and the other feed are mixed (unit operation 1) and offered to the cows. The period between the mixing of the feed ration and the actual consumption of the ration by a cow is a storage period (unit operation 2). During storage growth of BAB can occur when conditions such as temperature, pH and availability of nutrients are favorable. In the digestive tract of the cows, the major part of the feed is consumed but BAB spores survive and are excreted with feces. This means that spores are concentrated during transmission from feed to feces (unit operation 3). During excretion and lying down, contaminated feces and soil attach to the surface of the cows' teats. In this paper this mixture of attached soil and feces is referred to as dirt. The contamination of teats with dirt then relates to two unit operations, mixing of soil and feces into dirt (unit operation 4) and cross-contamination (unit operation 5) between dirt and teats. Before milking a farmer can clean teats to remove spores (unit operation 6). The remaining spores are diluted in the raw milk during milking (unit operation 7). Via the milkline, contaminated milk is transferred to the farm tank, where the milk of all cows from a number of milkings is mixed before transportation to the dairy plant (unit operation 8).

The simulation model

For model development, translation of the contamination pathway into a chain of unit operations has the advantage that predictive microbial models are available to describe the effect of unit operations on microbial behavior. All variables in these predictive microbial models can be related to processing parameters or product characteristics. This makes it possible to evaluate different control strategies in practical and measurable terms.

In the Netherlands, FTM transported to the site of the dairy processor is generally a collection of milk yields of individual cows, from six successive milkings over three days. Therefore, the model developed simulates the FTM contamination level after six milkings. Predictive microbial models used to calculate the FTM contamination level (C_{FTM}) are listed in Table 2.1. The simulation model first calculates the number of BAB spores transmitted to the FTM of each cow during each milking ($N_{i,k}$). Then the FTM contamination level after six milkings

(C_{FTM}) is calculated based on $N_{i,k}$ and the milk yield ($V_{milk,i,k}$) of the different cows during the different milkings.

Table 2.1 Predictive microbial models used to calculate the number of butyric acid bacteria (BAB) spores transmitted to farm tank milk (FTM) by each cow of a herd during each separate milking ($N_{i,k}$ in spores) and the resulting contamination level in FTM (C_{FTM} in spores/L).

	Unit-operation	Equation
1	Mixing of silage and other feedtuffs[1]	$C_{ration}(0) = F_{silage} \cdot C_{silage} + (1 - F_{silage}) \cdot C_{otherfeed}$
2	Storage of the feed ration resulting in growth during time t[2]	$\ln(C_{ration}(t)) = \ln(C_{ration}(0)) + \mu \cdot A_N(t) - \ln\left(1 + \left(\left(e^{\mu * A_N(t)} \cdot t\right) / \left(e^{\ln(C_\infty / C_{ration}(0))}\right)\right)\right)$ $A_n(t) = t + \frac{1}{\mu}\ln\left(\frac{e^{-\mu \cdot t} + q_0}{1 + q_0}\right), \quad q_0 = 1/\left(e^{\lambda \cdot \mu} - 1\right),$ $\mu = \gamma(T) \cdot \gamma(pH) \cdot \mu_{opt}, \quad \gamma(T) = \left(\frac{T_{ration} - T_{\min}}{T_{opt} - T_{\min}}\right)^2$ $\gamma(pH) = \left[\frac{(pH_{ration} - pH_{\min}) \cdot (pH_{\max} - pH_{ration})}{(pH_{opt} - pH_{\min}) \cdot (pH_{\max} - pH_{opt})}\right]$
3	Relation between concentration in the feed ration and in feces[3]	$C_{Faeces} = (1/(1 - F_{digested})) \cdot C_{ration}(t)$
4	Mixing of soil and feces resulting in contaminated dirt[1]	$C_{dirt} = F_{soil} \cdot C_{soil} + (1 - F_{soil}) \cdot C_{faeces}$
5	Cross contamination of udder teats[4]	$N_{before\_treatment} = M_{dirt} \cdot C_{dirt}$
6	Removal during teat cleaning[5]	$N_{i,k} = N_{after\_treatment} = TC_{efficiency} \cdot N_{before\_treatment}$
7	Rinsing of teats, dilution of spores in milk and mixing of milk in the farm tank[6]	$C_{FTM} = \sum_{k=1}^{6} \sum_{i=1}^{N_{Herd}} N_{i,k} \Big/ \sum_{k=1}^{6} \sum_{i=1}^{N_{Herd}} V_{i,k}$

[1] The contamination level of a carrier X after mixing ($C_{ration}(0)$, C_{dirt}) was calculated as a function of the contamination level in and fractions of the preceding carriers (C_{silage}, $C_{otherfeed}$, C_{soil}, C_{feces}, F_{silage}, F_{soil}). All contamination levels (noted with C_*) are in spores/g and all fractions (noted with F_*) in %.

[2] The Baranyi growth model (Baranyi and Roberts, 1994) was used to calculated the concentration in the feed ration after growth ($C_{ration}(t)$). Lag time λ (h) equals $1/\mu$. t (h) is the time available for growth. The growth rate μ was estimated using gamma-concept with factors for temperature and pH. (Zwietering et al., 1996).

[3] $F_{digested}$ (%) refers to the fraction of the feed ration digested. F_{digest} was assumed to equal 90% (Hengeveld, 1983)

[4] M_{dirt} (g) refers to the mass of dirt attached to the udder teats that will dilute in the milk without teat cleaning. N_* (spores) refers in all cases to a number of bacterial spores.

[5] Equation is based on Chen et al. (2001). $TC_{efficiency}$ (%) relates to the percentage of spores removed by the applied teat cleaning method

[6] Subscript i refers to the different cows in the herd, subscript k to the different milkings. $V_{i,k}$ is the milk yield for cow i with milking k in L.

The microbial growth in the feed ration is simulated using the Baranyi-growth model (Baranyi and Roberts, 1994). The Baranyi model was applied because it takes a maximum attainable contamination level into account (C_∞), which could be relevant for the growth in the feed laying in the barn. With the gamma-concept of Zwietering et al. (1996), the growth rate μ (1/h) was estimated. This concept was chosen because the effects of temperature and pH can be separated and parameter values (T_{min}, T_{opt}, pH_{min}, pH_{opt}, pH_{max} and μ_{max}) are available in literature. Table 2.2 shows the values of these parameters for *C. tyrobutyricum*.

Table 2.2 Growth characteristics of *Clostridium tyrobutyricum*

Parameter name	**Abbreviation**	**Value**[1]
Minimum temperature required for growth (°C)	T_{min}	9[2]
Optimum temperature required for growth (°C)	T_{opt}	37
Minimum pH required for growth	pH_{min}	4.4
Optimum pH for growth	pH_{opt}	5.6
Maximum pH allowed for growth	pH_{max}	6.8
Growth rate under optimal conditions (h^{-1})	μ_{opt}	0.12

[1] Data from Bergère and Hernier (1970) and Thylin et al. (1995)

[2] T_{min} is the minimum temperature where growth is reported minus 1 °C (Zwietering et al., 1996)

Barn hygiene

Barn hygiene is considered to be an important factor for the contamination of FTM with BAB spores (Stadhouders and Jørgensen, 1990). To account for differences between cows and to have a practical measure of barn hygiene, the herd was divided into three groups. The groups were (1) slightly contaminated cows with no visible dirt attached to the teats, (2) moderately contaminated cows with visible dirt attached to the teats and (3) highly contaminated cows with excessive amounts on the teats. The proportions of slightly and moderately contaminated cows are an indicator of barn hygiene. Highly contaminated cows prefer to lie down on dirty patches. This preference is generally a cow characteristic. Therefore the proportion of highly contaminated cows is not related to barn hygiene per se. In the simulation model, each cow group corresponds to a different amount of dirt attached to the teats. In the calculation procedure each cow was first assigned to a cow group based on the proportions of highly and moderately contaminated cows. Then the number of BAB spores transmitted to the FTM via the teats of this cow was calculated ($N_{i,k}$).

Model variables

The model distinguishes between controllable and uncontrollable variables. A variable was considered controllable when a farmer can directly influence the value via management or can measure the variable easily and respond to the results of this measurement. Worst, optimal and average values currently applicable to common farm practices in the Netherlands were retrieved from published data and from expert opinions (Table 2.3). In this respect, worst and optimal refer to the direction of the effect of the variable on the contamination level in FTM (worst settings result in higher and optimal settings in lower contamination levels).

All other variables selected for the final simulation model were considered to be uncontrollable. All uncontrollable variables were modeled with statistical distributions. Distributions were applied because extreme values of uncontrollable variables affect the occurrence of extremely high values of the FTM contamination level. Because the dairy industry aims to ensure a contamination level below the MSL these extreme values are particularly important. The selected uncontrollable variables and their distributions are listed in Table 2.4. The distributions were also retrieved from published data and expert opinions. All variable values and distributions in Tables 2.3 and 2.4 refer to cows housed in the barn (winter period); for cows in pasture (summer period) different values and distributions will apply.

Table 2.3 Worst, average and optimal values of controllable variables as encountered in the Netherlands. Terms worst and optimal relate to the effect on the contamination level in FTM.

Variable	Abbreviation	Worst value	Average value	Optimal value	Ref.
Herd size	*N-herd*	30	65	125	a
Mean contamination level silage ($\log_{10}$ CFU/g)	*C-silage*	7	4	2	b
Mean contamination other feed ($\log_{10}$ CFU/g)	*C-other feed*	3	1	0	c
Fraction silage in ration (%)	*F-silage*	90	65	50	a
pH of feed ration	*pH-ration*	5.6	5.0	<4.4	d
Temperature of feed in the barn (°C)	*Temp-ration*	37	20	10	a
Time between two feed ration refreshments (h)	*Time-Ration*	144	24	6	a
Fraction of soil in dirt (%)	*F-soil*	0.0	2.5	20.0	a
Proportion moderately contaminated cows (%)	*MC-cows*	25.0	12.5	0.0	a
Proportion highly contaminated cows (%)	*HC-cows*	5.0	2.5	0.0	a
Teat cleaning strategy (pre-treated: 0=no cows; 1=highly contaminated cows; 2=highly + moderately contaminated cows; 3=all cows)	*TC-strategy*	0	2	3	a
Mean teat cleaning efficiency (%)	*TC-efficiency*	30	75	90	a+e

a. Expert opinion; b. Pahlow, et al. (2003); c. Dasgupta and Hull (1989);
d. Unpublished data; e. Stadhouders and Jørgensen, (1990);

2.2.2 Model simulations

The simulation model was programmed in Microsoft Excel (Microsoft-Corporation, 2002). The Excel plug-in @Risk (Palisade-Corporation, 2002) was used to implement the distributions of the uncontrollable variables. Simulations were performed using Latin Hypercube sampling. Each simulation comprised 2,500 iterations.

The following assumptions apply to the programmed model and performed simulations:

- The composition of the feed ration is constant during a period of 6 milkings. In practice feed rations do not change daily, so this assumption does not conflict with practice. A constant feed ration implies that the transit time of the BAB spores in the gastro-intestinal tract can be neglected,
- The lag time for growth in the feed ration is assumed to equal $1/\mu$, an estimate normally applied in food safety risk assessment (Zwietering et al., 1996),
- The maximum attainable contamination level in the feed ration (C_∞) is assumed to be 8 $\log_{10}$ spores/g,
- The same teat cleaning method is applied to cows with the same amount of dirt attached to the udder teats for all milkings,
- All BAB present on the udder teats surface before milking are rinsed off and diluted in the raw milk. This assumption was necessary since data are available only about the amount of dirt transmitted to milk,
- Dutch farmers generally milk twice a day and milkings were assumed to take place with regular intervals of 12 h.

Table 2.4 Distribution applied to uncontrollable variables of the simulation model.

Variable	Abbrevation	Distribution	Ref.
Contamination level of soil (log 10 CFG/g)	*C-soil*	Pert (2; 3; 4)	a
Natural variation around mean contamination level of silage ($\log_{10}$ CFU/g)	*NV-C-silage*	Pert (Mean-1; Mean; Mean+1)	b
Natural variation around mean contamination level of other feed ($\log_{10}$ CFU/g)	*NV-C-other feed*	Pert(Mean-0.5; Mean; Mean+0.5)	c
Dirt mass rinsed off teats of slightly contaminated cows (g)	*DM-SC_cows*	10^Pert(-2.5;-1.5; -0.5)	d+e
Dirt mass rinsed off teats of moderately contaminated cows (g)	*DM-MC-cows*	10^Pert(- 0.75;0;0.75)	d+e
Dirt mass rinsed off teats of highly contaminated cows (g)	*DM-HC-cows*	10^Pert(0.5;1;1.5)	d+e
Milk yield per individual cow per milking(L)	*V-milk*	Pert(10;13;15)	e
Natural variation around mean teat cleaning efficiency of applied method (%)	*NV-TC-efficiency*	Pert (10%*mean; mean; 110%*mean)	d+e

a. Unpublished data; b. Spoelstra (1990); c. Dasgupta and Hull (1989);
d. Stadhouders and Jørgensen (1990); e. expert opinion;

2.3 RESULTS

2.3.1 Model validation

Validation of the developed model was difficult since only data concerning contamination levels in different carriers were available, without information about the related management conditions. Therefore expert opinion was used together with experimental data to validate the model. Predicted contamination levels corresponded with experimental data of Bergère et al. (1968) and Kalzendorf (1996).

To validate observed trends, the simulation results were discussed with field experts. In qualitative terms observed trends corresponded with their experience and did not conflict with practice. The quantitative validation of the predicted contamination levels and qualitative validation of observed trends showed the reliability of the developed simulation model.

2.3.2 Identification of important controllable variables

To identify the most important controllable variables, first the mean contamination level in FTM was simulated with the value of all controllable variables fixed on their average values (Table 2.3). Then for each controllable variable two additional simulations were performed. In the first simulation the mean FTM contamination level was calculated with the value of the specific controllable variable fixed on its worst value, and the other controllable variables fixed on their average value. In the second simulation the same procedure was repeated but with the value of the controllable variable fixed on its optimal value.

Figure 2.1 shows all the calculated mean FTM contamination levels for all controllable variables. Controllable variables are represented in order of decreasing impact on the contamination level of FTM for the worst-case scenario. For the effect of the variation of the mean silage contamination level, a separate *y*-scale was used because the impact of this controllable factor exceeded, by far, the impact of the other factors.

A large difference between calculated mean contamination levels at worst and optimal settings implies that the specific variable has a large impact on the contamination level. This implies that the average contamination level in silage (*C-silage*) is by far the most important determinant for the achieved contamination level in FTM. Other relevant factors are the teat cleaning strategy (*TC-strategy*), efficiency of the applied teat cleaning method (*TC-efficiency*) and the proportion of highly contaminated cows (*HC-cows*). Variables directly related to farm hygiene (*MC-cows, F-soil*) only have a marginal impact, as does the contamination level in the other feed (*C-otherfeed*).

For the teat cleaning strategy the calculated contamination level in FTM for the average and optimal scenario are almost equal. This implies that little can be gained from cleaning teats slightly contaminated cows before milking. Compared to the average situation, it is most effective to remove highly contaminated cows from the herd and to apply a more severe teat cleaning method (more than 90% removal).

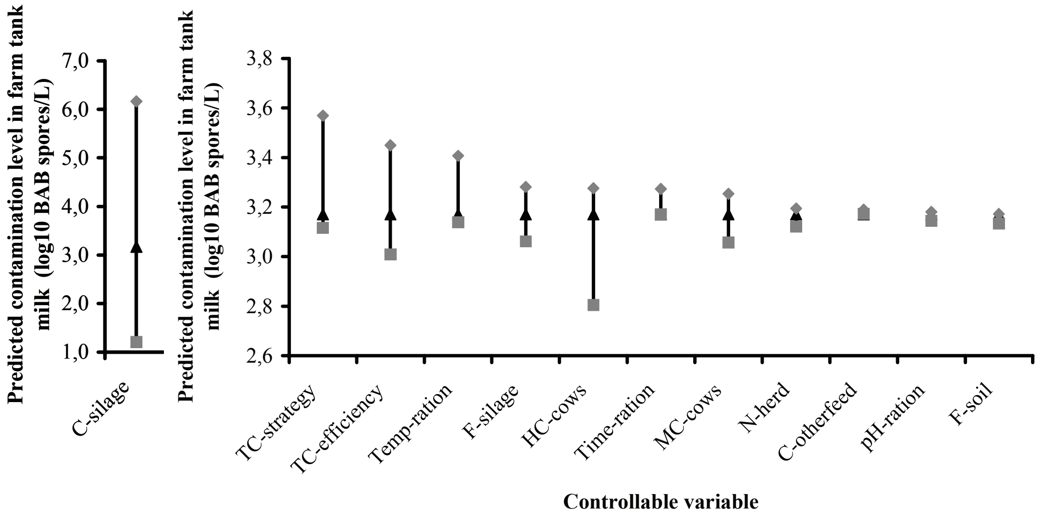

Figure 2.1 Simulated impact of the different controllable variables on the mean bulk tank milk contamination level; *x*-axis labels refer to Table 2.3. (▲All controllable variables fixed on average value, ♦ Specific variable fixed on worst value and all other variables on average value, ■ Specific variable fixed on optimal value and all other variables on average value)

2.3.3 Identification of important uncontrollable variables

To determine the most important uncontrollable variables, the values of all controllable variables were fixed at their average value and the values of all uncontrollable variables, except the one under investigation, were fixed on their most likely value. The mean, and 5% and 95% percentile values of the FTM contamination level were then calculated. For comparison an additional simulation was performed with all uncontrollable variables varying according to their distribution.

Figure 2.2 shows the results, with error bars representing the difference between the mean and the percentile values. The variables are presented in order of decreasing difference between the 5% and 95% percentile value. The larger this difference, the more important the specific uncontrollable variable is for the predicted FTM contamination level. Figure 2.2 shows the natural variation of the silage contamination level (*NV-C-Silage*) to be, by far, the most important uncontrollable variable.

2.3.4 Relation between silage and FTM contamination level

Silage was shown to be the most important factor. To establish the relation between contamination levels in silage and FTM, additional simulations were performed. In these calculations the average silage contamination level was fixed at 2, 3, 4, 5, 6 and 7 $\log_{10}$ BAB spores/g and for each of these contamination levels in silage three simulations were performed: one with all controllable variable values fixed at their worst value, one with all these variable values fixed at their average value and one with all these variable values fixed at their optimal value.

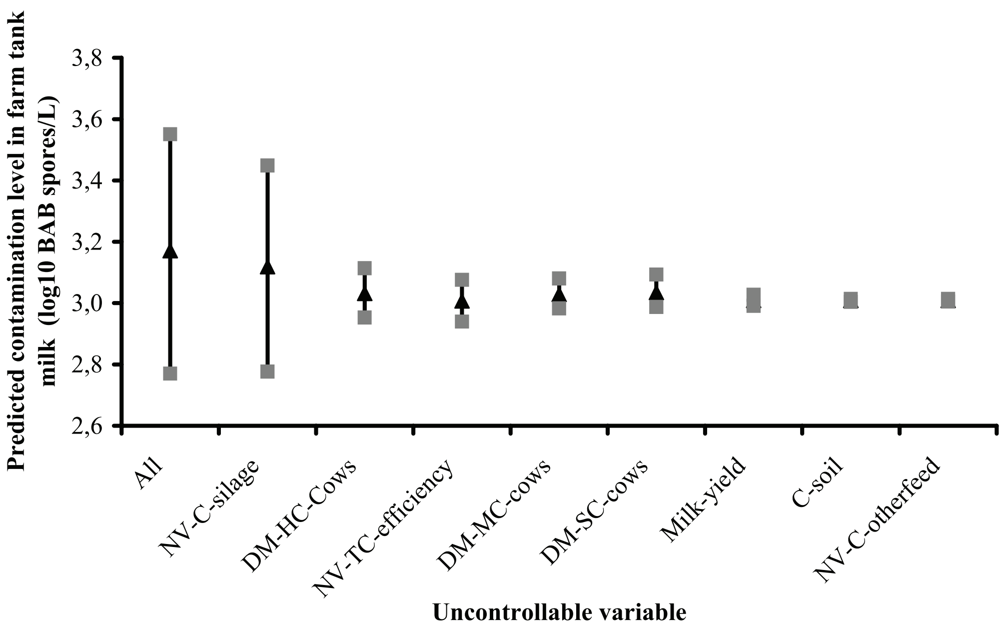

Figure 2.2 Simulated impact of uncontrollable variables on the variation of bulk tank milk contamination with average values for the controllable variables; *x*-axis labels refer to Table 2.4. (▲ Mean of the bulk tank milk contamination level, ♦ 95% percentile value of the simulated farm tank milk contamination level, ■ 5% percentile value of the simulated farm tank milk contamination level.)

The results were plotted in a graph with the average silage contamination on the *x*-axis and the FTM contamination on the *y*-axis; error bars represent the 5% and 95% percentile values (Figure 2.3). This graph also shows the MSL (3 $\log_{10}$ spores/L). The 95% percentile value is important since the industry wants to assure that the FTM contamination is below the desired level at all times. Figure 2.3 shows that feeding silage with an average contamination level above 5 $\log_{10}$ BAB spores/g should be avoided. Average management is sufficient when the mean silage contamination is 3 $\log_{10}$ BAB spores/g or lower. Between 3 and 5 $\log_{10}$ BAB spores/g additional measures compared to average management are needed, e.g. removal of highly contaminated cows or another teat cleaning method (Figure 2.1).

2.4 DISCUSSION

In this study an approach to model the microbial contamination of FTM is applied to the case of the contamination of raw milk with BAB spores. In this approach, the complex interactions between microorganisms, farm management and uncontrollable factors are simplified in a mathematical model based on the contamination pathway. The objective was to illustrate the usefulness of the approach and to identify an effective strategy to control the contamination of FTM with BAB spores.

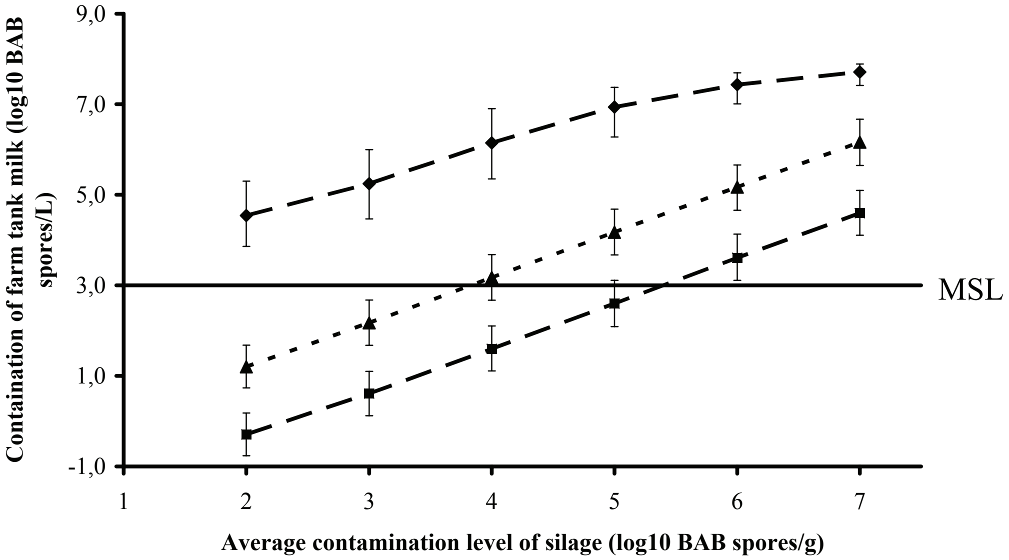

Figure 2.3 Simulated relation between the simulated contamination level of FTM with BAB spores and average contamination level of silage. (♦ All controllable variables fixed at the worst value, ▲All controllable variables fixed at the average value, ■ All controllable variables fixed at the optimal value. Error bars indicate 90% confidence interval. Average, worst and optimal values correspond to Table 2.3.)

For a number of variables in the developed model, expert opinion was needed to obtain variable values. Inclusion of expert opinion often increases uncertainty of model predictions. Figure 2.1 and 2.2 show that, although large differences for worst and optimal value were applied, of these variables only the teat cleaning strategy, teat cleaning efficiency and fraction highly contaminated cows had a moderate effect on the predicted FTM contamination level. But, the impact of these two variables (approximately 0.5 $\log_{10}$ difference between highest and lowest value) is far less than the impact of the concentration in silage (approximately 5 $\log_{10}$ difference between highest and lowest value).

Empirical results of Bergère et al. (1968) showed that feeding silage can result in an increased contamination of FTM with BAB spores. They concluded that feeding high quality silage is most important in controlling the contamination level. But that measures at other steps in the contamination pathway are also necessary. Dasgupta and Hull (1989) confirmed the importance of silage experimentally. The simulation results correspond with these finding; the added value of the simulation model to these publications is the quantification of the importance of silage quality compared to other, presumed, relevant factors.

The simulation confirmed that the first objective of a farmer should be to control the contamination level of the mixed grass/corn silage fed to cows. Additional control measures are ineffective when the mixed silage contains on average more than 5 $\log_{10}$ BAB spores/g. Dry matter content **(DM)** and pH of the silage during storage have been identified as important factors to control of the contamination of silage with BAB spores (Pahlow et al., 2003). Grass silage with a high DM can be achieved via wilting of the grass before ensiling (Pauly, 1999). A quick decrease of the pH can for example be achieved via addition of formic acid, early in the fermentation process (Beaudouin, 1985).

Figure 2.2 indicates the importance of natural variation of the contamination level in silage to the final contamination level in FTM. Variation of the silage contamination level is related to the heterogeneous nature of silage (Pauly, 1999, Spoelstra, 1990). Harvesting and ensiling practices influence the heterogeneity of the silage and therefore offer a possibility to further control the contamination of the FTM with BAB spores. In practice, other risk factors mentioned with respect to high levels of BAB spores in silage are the initial contamination of silage with soil and the deterioration of silage during the feed-out phase. Currently, further research is being performed to identify which of these factors (pH, DM, heterogeneity, initial contamination and deterioration) impose the highest risk for the contamination of mixed silage, and which factors or combinations of factors are the most practical indicators for the contamination level in silage.

Bergère et al. (1968) and Stadhouders and Jørgensen (1990) emphasized the importance of hygienic milking practice to control the contamination of FTM with spores of BAB. Hygienic milking practice relates to the contamination of teats with dirt and the removal of the dirt during teat cleaning. In this respect, cows should be prevented to lie down on dirty patches (this leads to highly contaminated teats before milking) and a teat cleaning method with an average efficiency of at least 75% should be applied. Moderately contaminated cows (a small amount of visible dirt) have far less impact on the contamination level of FTM. Herlin and Christiansson (1993) found no relation between the housing system and the contamination of FTM with anaerobic spores. Therefore, it can be expected that the proportions heavily and moderately contaminated cows are independent of the housing system. However, all these measures have far less impact on the concentration in FTM than silage quality (Figure 2.1).

Contrary to common beliefs in practice, soil and feed other than silage are unimportant sources of BAB spores. The maximum contamination levels in these sources (3 $\log_{10}$ BAB spores/g and 4 $\log_{10}$ BAB spores/g respectively) are generally negligible compared to the levels in silage (up to 7 $\log_{10}$ BAB spores/g). Even when only highly contaminated feed are fed in the absence of silage feeding, average management will be sufficient to achieve the desired FTM quality.

Summarizing, using data presented in Figures 2.1 and 2.3, a general strategy to control the contamination of the FTM below the MSL can be defined. When the average contamination level of silage is below 3 $\log_{10}$ BAB spores/g, it is sufficient to clean the teats with a method with an average efficiency of 75%. At average silage contamination levels between 3 and 5 $\log_{10}$ BAB spores/g additional measures are necessary. The most efficient additional measures are removing highly contaminated cows from the herd and improving the efficiency of the applied teat cleaning method, although Stadhouders and Jørgensen (1990) consider the most efficient teat cleaning method too time consuming. Silage with on average more than 5 $\log_{10}$ BAB spores/g should not be fed because at these contamination levels even optimal control of all other factors will not be sufficient to assure a contamination level below the MSL.

2.5 CONCLUSIONS

A mathematical model based on the contamination pathway was shown to be a useful tool for the identification of a strategy to control the FTM contamination below 3 log_{10} BAB spores/L. The interpretability of the model variables will facilitate the implementation of the research results into practice. The contamination level of silage was shown to be the most important factor and silage containing on average more than 5 log_{10} BAB/g should not be fed to cows. Further research is needed to identify the factors that impose the highest risk of an insufficient microbial quality of the silage. Other identified control measures are application of an teat cleaning method with an efficiency of at least 75% and removal from the herd of cows with a preference for lying down on dirty patches.

There are other spore-forming microorganisms originating from the farm, such as *Bacillus cereus*, that impose problems for the dairy industry besides BAB (Te Giffel et al., 1995). When the contamination pathway for these microorganisms is known, the described approach could also be applied to improve and decrease the contamination level of FTM with these microorganisms.

CHAPTER 3

BUTYRIC ACID BACTERIA SPORES IN GRASS AND CORN SILAGE

Germination and growth of spores of butyric acid bacteria (BAB) may cause severe defects in semi-hard cheeses. Silage is the main source of BAB spores in cheese milk. The objectives of the study were to determine the significance of grass and corn silages as sources of BAB spores and to investigate relationships between high concentrations of BAB spores in corn silage and aerobic deterioration. In the first survey, samples were taken at 21 farms from various locations in silos containing grass and corn silages and from mixed grass/corn silage fed to cows in the barn. It was demonstrated that the quantity of BAB spores consumed by cows was determined by a small fraction of silage with a high concentration (above 5 $\log_{10}$ BAB spores/g). High concentrations were found most often in corn silage within areas with visible molds (69% of the samples). Areas with visible molds in grass silage and surface layers of corn silage contained respectively 21% and 19% of the cases a concentration above 5 $\log_{10}$ BAB spores/g. Based on these results it was concluded that currently in the Netherlands, corn silage is a more important source of BAB spores than grass silage. In a second survey, eight corn silages were divided into 16 sections and each section was studied in detail. High concentrations of BAB spores were only found in the top 50 cm. Elevated concentrations of BAB spores were associated with different signs of aerobic deterioration. In 13% of the sections in corn silage with more than 5 $\log_{10}$ yeasts and molds/g, more than 5 $\log_{10}$ BAB spores/g were found. Sections with a temperature of more than 5 °C above ambient temperature contained in 21% of the cases more than 5 $\log_{10}$ BAB spores/g. Concentrations above 5 $\log_{10}$ BAB spores/g were measured in 50% of the sections with a pH above 4.4. All sections with a pH above 4.4 also showed increased temperatures and concentrations of yeasts and molds. Based on these results it is postulated that high concentrations of BAB spores in corn silage are the result of oxygen penetration into the silage, this results in aerobic deterioration and the formation of anaerobic niches with an increased pH just below the surface. Growth of BAB in these anaerobic niches with an increased pH causes locally high concentrations of BAB spores in (corn) silage.

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Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration

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3.1 INTRODUCTION

In semi-hard cheeses, such as Gouda and Emmental, growth of spore-forming bacteria of the genus *Clostridium* can lead to off-flavors and excessive gas formation. This defect is called late-blowing. These bacteria, butyric acid bacteria **(BAB)**, are able to convert lactic acid into butyric acid, hydrogen and carbon dioxide at relatively low pH. In particular *Clostridium tyrobutyricum* is associated with this problem (Klijn et al., 1995). Without preventive measures, late-blowing of Gouda type cheeses can occur at a concentration above 10 BAB spores/L of pasteurized cheese milk (Stadhouders, 1990).

Spores of BAB in milk originate from the farm environment. Reducing the concentration of BAB spores in farm tank milk **(FTM)** is an important option to prevent late-blowing of cheeses (Stadhouders and Spoelstra, 1990). Additional measures are removal of spores from milk by bactofugation, addition of inhibitors, such as nitrate and lysozyme, and the use of nisin-producing cheese starter cultures (Stadhouders, 1990, Waes and Van Heddeghem, 1990, Delves-Broughton et al., 1996). To encourage farmers to produce milk with low concentrations of BAB spores, Dutch dairy processors have FTM analyzed monthly for BAB spores. The milk payment to farmers is partly dependent on the result of these tests. The objective of dairy processors is to always receive FTM with less than 3 $\log_{10}$ BAB spores/L.

Silage is the most important source of BAB spores. The spores survive passage through the alimentary tract of the cow and are excreted with the feces. Transmission to milk occurs via fecal contamination of the cow's teats (Bergère et al., 1968). Control of the concentration of BAB spores in silage is essential to control the concentration in FTM (Vissers et al., 2006; Chapter 2). The preservation of forage by ensiling is achieved via the attainment of a low pH by lactic acid fermentation and the maintenance of anaerobic conditions within the silage silo. A rapid and sufficient decline in pH after ensiling decreases the chance of clostridial growth in silage. The rate and extent of pH decline are influenced by many factors, e.g. dry matter content **(DM)**, concentration of fermentable sugars and buffer capacity of the crop, and activity of the lactic acid bacteria (Weissbach, 1996).

Creation and maintenance of anaerobic conditions in silage is important to prevent growth of aerobic microorganisms. However, in practice it is unavoidable that silage is exposed to air. During storage small amounts of air will penetrate the silage, for instance, because silage covers (usually plastic sheets) are not completely airtight. Calculations showed that during storage, oxygen can penetrate up to a depth of 0.2 m from the top of the silage (McGechan and Williams, 1994). After opening for feeding, usually air will penetrate via the silage face. Parsons (1991) calculated that after opening, oxygen can penetrate silage via the feed-out face up to distances of 4 m from the feed-out face. During storage and after opening of the silage, surface layers are most sensitive to the penetration of air. The main factors influencing the extent of air penetration are the porosity and density of the silage and the rate of silage feeding (Honig, 1991). As a result of air infiltration, acid-tolerant (facultative) aerobic microorganisms start to proliferate. This aerobic deterioration process is usually initiated by yeasts, which use residual sugars and lactic acid as substrates. As this process proceeds, silage pH rises and other, less acid-tolerant micro-organisms, such as molds and *Bacillus* species start to proliferate (Pahlow et al., 2003).

In the past, high concentrations of BAB spores in FTM were associated with anaerobically unstable silages, made of crops with high buffer capacity such as grass and alfalfa (Stadhouders and Spoelstra, 1990, Weissbach, 1996). Anaerobically, unstable silages are generally characterized by a high pH and high levels of butyric aid and ammonia. Traditionally, corn silages were considered a less significant source of BAB spores. Due to a combination of DM, fermentable sugars and buffer capacity, silages made of corn generally have a fast pH decline and a low final pH (<4.0). These conditions do not allow significant growth of clostridia. Furthermore, low concentration of BAB spores were found in unopened corn silage silos (Stadhouders and Spoelstra, 1990). But, Driehuis and Te Giffel (2005) recently identified corn silage as the main source of BAB spores at five dairy farms that produced FTM with elevated BAB spore concentrations. A relationship between occurrence of high concentrations of BAB spores in the corn silages and aerobic deterioration was suggested because concentrations above 5 $\log_{10}$ spores/g were detected in surface layers.
The objectives of this study were to determine the importance of grass silages and corn silages as a source of BAB spores in the mixed silage fed to the cows in the barn at farms in The Netherlands (survey 1) and to further investigate relationships between high concentrations of BAB spores in corn silage and aerobic deterioration (survey 2).

3.2 MATERIALS AND METHODS

3.2.1 BAB spores in grass and corn silage (survey 1)

In the first survey, 21 farms located in different regions in the Netherlands were visited. Farms were selected based on the detection of BAB spores in FTM delivered to the dairy processor in 2004 using data of the Netherlands Milk Control Station (MCS, Zutphen, Netherlands). Bad, moderate and well performing farms were selected to ensure that not only high quality silages were surveyed. At these farms, all silages were ensiled in bunker or clamp silos and covered with plastic sheeting. The width of the silages sampled ranged from 6 to 15 m; the height ranged from 1.5 to 4 m. At three farms inoculants of unknown brand were added to the grass and corn silages during ensiling. At each farm, samples were taken from the grass silage and the corn silage and from the mixture of these silages that was offered to cows in the barn (mixed silage). Per grass and corn silage, three samples were taken: a sample from the core, the surface layer (outermost 0.2 m of the silage, Figure 3.1A) and of areas with visible molds. From both the surface layer and the core, ten sub samples were taken at different positions and mixed to provide composite samples. Areas with visible molds (molded spots) and the 15 cm surrounding these areas were excluded from the core and surface layer samples. Sub samples from a maximum of 10 molded spots per silo were taken and mixed to provide a composite sample for molded spots. All samples were stored at 4 °C until determination of the pH and microbial analysis within 24 h.
Silage pH was determined with a Metrohm 532 pH-meter (Metrohm, Herisau, Switzerland) in extracts prepared by adding 180 ml of demineralized water to 20 g of silage sample and homogenizing for 2 min in a laboratory blender (Stomacher 400 Circulator; Seward, London, UK)

3.2.2 Relationships between aerobic deterioration and BAB spores in corn silage (survey 2)

The objective of the second survey was to further investigate the relationship between aerobic deterioration and elevated concentrations of BAB spores in corn silage. In this survey eight corn silages at randomly selected farms in the middle and eastern part of the Netherlands were sampled. The silages were stored in bunker or clamp silos and were fed to dairy cows. The width of the silages sampled ranged from 7 to 11 m; the height ranged from 1.6 to 2.5 m. At none of the farms an inocculant had been added to the corn silages during ensiling. The feed-out face was divided into three layers with widths of 0.2, 0.3 and 0.4 m. The different layers are referred to as surface, second and third layer. Each layer was divided into five sections of approximately the same length. Including the core, this resulted into 16 sections per silage (Figure 3.1B). In each section the temperature and the penetration resistance (explained below) were measured in duplicate. The temperature was measured at a depth of 0.1 m using a Pt-100 probe (Ebro, Ingolstadt, Germany). In addition, the ambient temperature was measured in °C. The area characteristic dT was defined as the difference between the temperature measured in the silage section and ambient temperature. Sampling for pH measurement and microbiological analyses was conducted as described for survey 1.

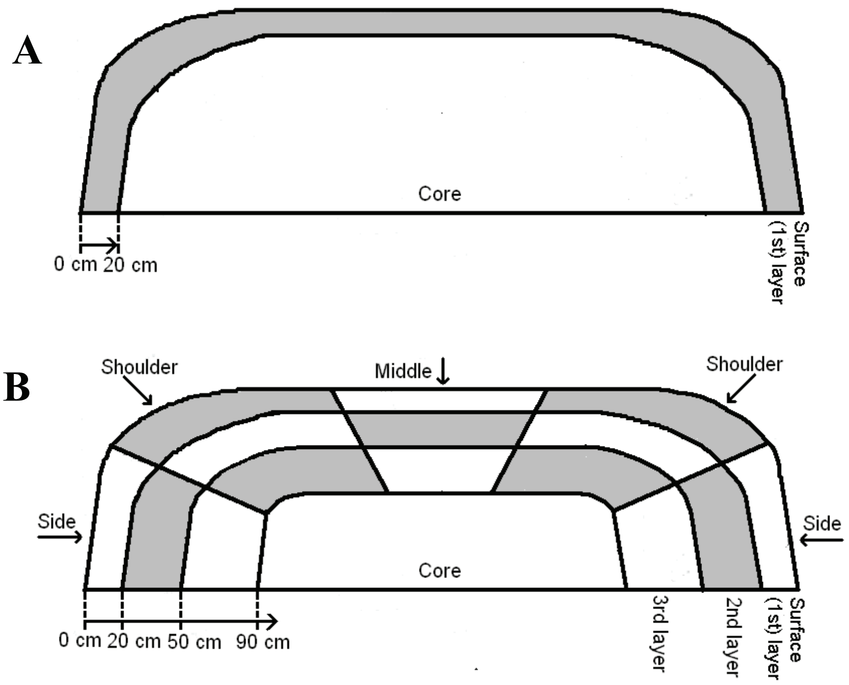

Figure 3.1 Partitioning of the feed-out face of the silages sampled in survey 1 (A) and survey 2 (B)

Penetration resistance was used as an indicator of the density of the silage silo; a higher resistance indicates a higher degree of compaction and a higher density. Penetration resistance was measured using a manual penetrometer (Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands), consisting of a manometer, a probing rod 1 m in length and a cone of 5 cm^2

(Figure 3.2). The device was pushed horizontally into the feed-out face with a constant speed of approximately 2 cm/s until the probing rod had penetrated the silage for 0.9 m. The maximum resistance during the measurement was read out from the manometer and corrected for the surface of the cone to obtain a value in N/cm^2.

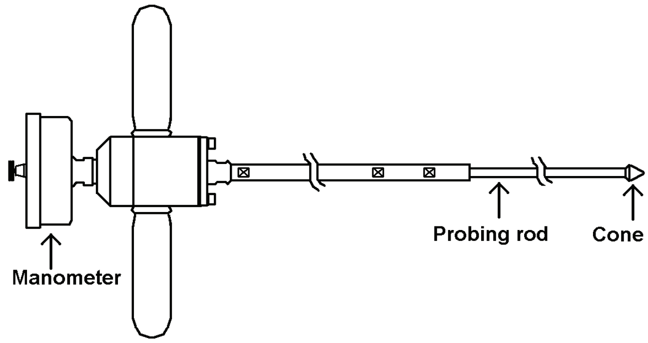

Figure 3.2 Schematic representation of hand penetrometer (Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands)

3.2.3 Microbial analyses

Silage extracts were prepared by adding 90 mL of peptone physiological salt solution (**PPS**) (1 g of neutralized bacteriological peptone and 8.5 g sodium chloride per liter) to 10 g of silage sample and homogenizing for 2 minutes in a laboratory blender (Stomacher, Sward Medical London, UK). Microbial counts were determined using decimal dilution series of silage extracts in PPS.

Yeasts and molds (**Y&M**) were enumerated on oxytetracycline glucose yeast extract agar (Oxoid, Haarlem, The Netherlands) and plates were counted after incubation for 4 days at 25 °C.

The concentration of BAB spores was determined by a most probable number (**MPN**) method according to Dutch Standard (NEN-ISO-6877, 1994). A volume of 0.1 mL of diluted extract was added to tubes containing 10 mL of sterilized milk, supplemented with glucose and lactic acid. The tubes were heated for 5 minutes at 80 °C to inactivate vegetative cells and to trigger the germination of spores. The tubes were sealed with paraffin and incubated for 4 days at 37 °C. A tube scored positive if after incubation gas formation was visible.

3.2.4 Statistical analyses

All microbial counts were $\log_{10}$ transformed to obtain log-normal distributed data. To calculate averages, the values below the detection level (detection levels: 30 BAB spores/g and 100 Y&M/g) were assigned a value corresponding to half of the detection level. (i.e. 15 BAB spores/g and 50 Y&M/g). The *Student t*-test was used to detect significant differences between samples (Snedecor and Cochran, 1989).

3.3 RESULTS

3.3.1 BAB spores in grass and corn silage (survey 1)

Table 3.1 lists the concentrations of BAB spores and Y&M and the pH measured in the samples obtained in the first survey. The average concentration of BAB spores in the samples from the core of both, grass and corn silage was 3 $\log_{10}$ spores/g. The concentrations in the core of grass and corn silage were in 52% and 62% of the silages, respectively, below 3 $\log_{10}$ spores/g; the initial contamination level of silage (Pahlow et al, 2003). Based on a mathematical model of the contamination of milk with BAB spores, Vissers et al. (2006; Chapter 2) determined that concentrations of BAB above 5 $\log_{10}$ spores/g in silage gives a probability of more than 5% that the concentration in farm tank milk will exceed 3 $\log_{10}$ BAB spores/L. Based on this criterion, all silage cores, except for one corn silage, were considered to be of sufficient quality. The average concentrations of BAB spores in surface layers of grass and corn silage were not significantly different (P>0.10). However, concentrations above 5 $\log_{10}$/g were detected more frequently in the surface layers of corn silage (19%) than in the core of corn silages (5%), surface layer of grass silages (5%) and core of grass silages (0%) (Figure 3.3).

Table 3.1 Concentration of butyric acid bacteria (BAB) spores and yeast & molds (Y&M) and pH of samples from core, surface layer and molded spots of grass and corn silages and from mixed silage (survey 1).

Sample	Concentration BAB spores ($\log_{10}$/g)[1]		Concentration Y&M ($\log_{10}$ cfu/g)		pH	
	Mean[2]	Range	Mean	Range	Mean	Range
Grass – core (N=21)	3.0[3,a]	<1.5 - 4.6	3.2[a]	<2.0 - 4.9	4.7[a]	4.0 - 5.6
Grass – surface layer (N=21)	3.1[a]	1.6 - 5.4	5.1[d]	2.3 - 8.3	5.3[d]	3.6 - 7.8
Grass – molded spots (N=14)	3.9[b,c]	2.0 - 5.4	6.8[d]	4.5 - 8.5	7.1[e]	4.6 - 8.5
Corn – core (n=21)	3.0[a]	<1.5 - 5.4	5.8[b,c]	2.2 - 8.3	3.9[b]	3.5 - 4.5
Corn – surface layer (N=21)	3.6[a,b]	1.6 - 6.2	6.9[d]	2.5 - 8.0	4.2[c]	3.5 - 5.8
Corn – molded spots (N=16)	5.5[d]	2.8 - 7.2	7.8[e]	5.2 - 8.5	6.6[e]	4.5 - 8.1
Mixed silage (N=21)	4.2[b,c]	2.9 - 5.4	6.4[c,d]	3.7 - 7.7	4.7[a]	4.0 - 6.3

[1] Concentration of BAB spores was determined using Most Probable Number technique for enumeration

[2] Within columns, value with different superscript (a,b,c etc) are significantly different (P<0.05)

In both grass silage and corn silage, the concentrations of BAB spores were higher in molded spots than in the surface layer and core. The highest concentrations were present in molded spot samples from corn silage (average of 5.5 log_{10} spores/g). In approximately 70% of the cases, these samples contained more than 5 log_{10} spores/g. The average concentration of BAB spores in molded spots present in grass silage was 3.9 log_{10} spores/g, which was significantly lower than the concentration in molded spots present in corn silage ($P<0.001$). In both silage types, visible molds were most often present in the shoulders of the silage or as blue colored lumps at depths up to approximately 1 m from the surface. In general the molded spots were roughly 5 to 10 times larger in corn silages than in grass silages. Most farmers did not remove these spots from the mixed silage fed to the cows in the barn.

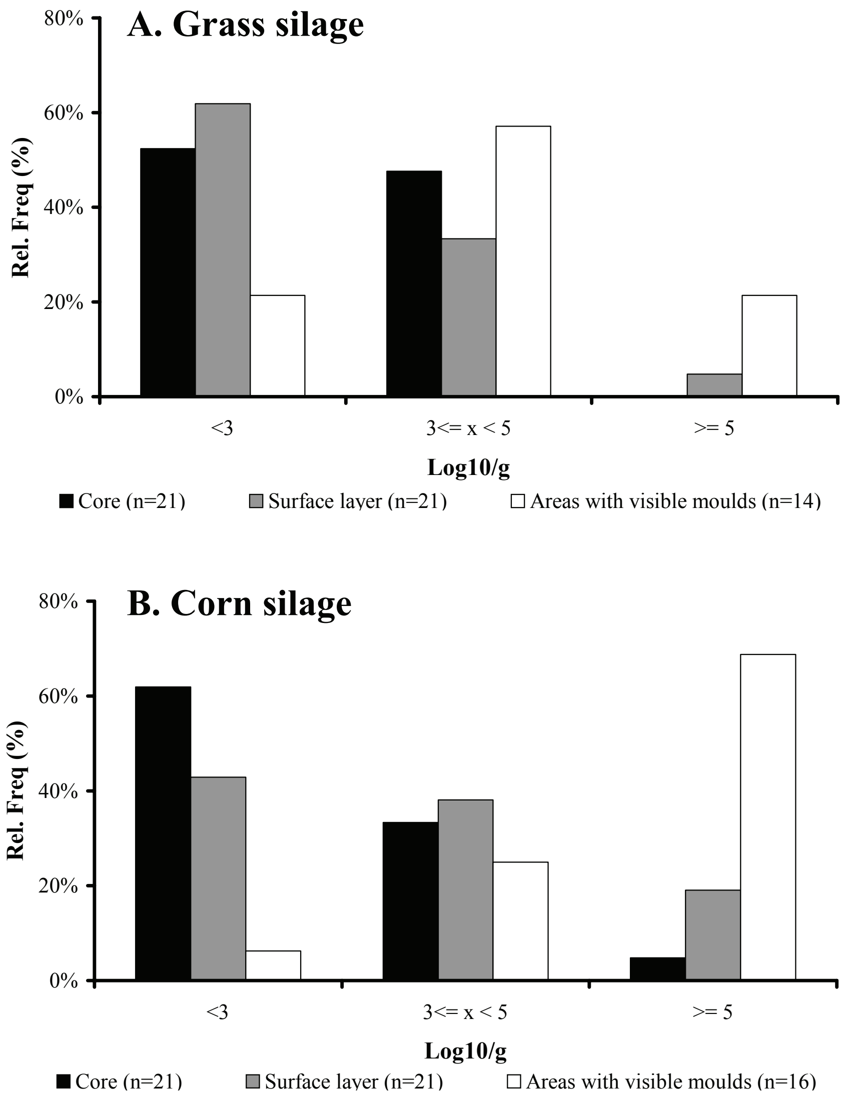

Figure 3.3 Distribution of the concentration of BAB spores measured in grass (A) and corn (B) silage in survey 1

3.3.2 Relationships between aerobic deterioration and BAB spores in corn silage (survey 2)

Table 3.2 shows the concentration of BAB spores, Y&M, penetration resistance, temperature difference between silage and ambient temperature (dT) and pH measured in the 16 sections of the 8 corn silages sampled in the second survey. Significant differences were observed between the layers (surface, 2nd and 3rd (Figure 3.1)) but not between the different segments (side, shoulder and middle). Therefore, the results are summarized per layer.

In total, 16 (out of 128) samples contained more than 5 $\log_{10}$ BAB spores/g. These contamination levels were most often (63%) found in the surface layer and never in the third layer. In samples from the core, BAB spore concentrations were always below 3 $\log_{10}$ spores/g, implying that after ensiling no growth of BAB had occurred within the cores. All corn silages included in this survey had at least one section with more than 5 $\log_{10}$ spores/g. This indicates that in all silages growth of BAB had occurred in at least one section.

Penetration resistance was measured as an indicator for density. The penetration resistance increased by approximately 25 N/cm^2 per layer going from the surface layer (average 25 N/cm^2) to the core (average 96 N/cm^2). The differences observed between the layers were significant ($P<0.001$). Within layers, no significant differences were observed, although the resistance in the shoulders of the outer layer tended to be lower than the resistance in the sides ($P=0.07$) and middle ($P=0.08$). The results indicate that the lowest penetration resistances were measured in the layers where the highest concentrations of BAB spores were observed.

Similar to the concentration of BAB spores, the concentration of Y&M, dT and pH were highest in the surface layer and lowest in the core. For dT and pH the range of values measured was largest in the surface layer and lowest in the core. However, for Y&M the largest range of concentrations was measured in the core and the smallest range in the surface layers. All sections in the surfaces layer contained more than 5 $\log_{10}$ Y&M/g.

The results demonstrate that high concentrations of BAB spores and indicators of aerobic deterioration (high Y&M counts, increased dT and pH) occur in the same layer. To confirm the relationship between high BAB spore concentrations and aerobic deterioration, the results of survey 2 were analyzed using three criteria for aerobic deterioration:

1. Concentration of Y&M higher than 5 $\log_{10}$ cfu/g
2. dT larger than 5 °C
3. pH of the section higher than 4.4 (minimal pH required for growth of *Clostridium tyrobutyricum* (Thylin et al., 1995).

First, the number of sections that met each criterion or none of the criteria was counted. Secondly, for the sections meeting a criterion, the distribution of the concentration of BAB spores was determined. Results are presented in Figure 3.4.

The percentage of sections that met criteria 1, 2 and 3 were respectively 88%, 38% and 13%, respectively. All but one of the sections with a dT larger than 5 °C contained more than 5 $\log_{10}$ Y&M/g. All sections with a pH higher than 4.4 contained more than 5 $\log_{10}$ Y&M/g and showed a dT of more than 5 °C. These observations correspond with a process of aerobic deterioration that starts with growth of Y&M, followed by an increase of the temperature due to microbial activity, finally resulting in an increase of the pH.

Table 3.2 Concentration of Butyric acid bacteria (BAB) spores (log_{10} spores/g) and yeasts & molds (Y&M) (log_{10} cfu/g), temperature (°C) difference between silage section and ambient temperature (dT), pH and penetration resistance (N/cm^2) of samples from core and three layers of corn silage (survey 2)

Layer	**Concentration BAB spores**[1]		**Log_{10} concentration Y&M**		**$dT_{section-ambient}$**		**pH**		**Penetration resistance**	
	Mean[2]	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Surface layer (n=40)	3.7^c	<1.5-7.0	7.5^b	5.5 - 8.7	10.9^c	0.4- 32.4	4.4^c	3.7 - 6.4	25^d	10 - 60
Second layer (n=40)	3.0^b	<1.5-7.4	6.8^a	4.3 - 8.6	5.8^b	-0.1 -25.7	4.0^b	3.7 - 5.1	50^c	26 - 76
Third layer (n=40)	2.5^a	<1.5-4.0	6.4^a	<4.0 - 8.7	3.4^b	-0.7 -15.3	3.8^a	3.6 - 5.2	76^b	54 - 94
Core (n=8)	2.3^a	<1.5-2.9	5.4^a	<4.0 - 9.0	1.4^a	-0.4 - 3.3	3.8^a	3.6 - 4.1	96^a	80 - 108

[1] Concentration of BAB spores was determined using Most probable number technique for enumeration

[2] Within columns, value with different superscript (a,b,c etc) are significantly different ($p<0.05$).

The relative frequency of concentrations of more than 5 log_{10} BAB spores/g increases with increasing signs of aerobic deterioration. The distribution of the concentration of BAB spores in the sections with a concentration of Y&M above 5 log_{10}/g was similar to the distribution observed in all sections sampled; approximately 13% of the samples contained more than 5 log_{10} BAB spores/g and 19% between 3 and 5 log_{10} BAB spores/g. The relative frequency of a concentration of BAB spores above 5 log_{10}/g increased to 21% when dT was more than 5 °C and to 50% when the pH of the section was above 4.4. Concentrations below 3 log_{10} BAB spores/g were never observed in sections with an increased pH, whereas sections that met none of the criteria always contained less than 5 log_{10} BAB spores/g.

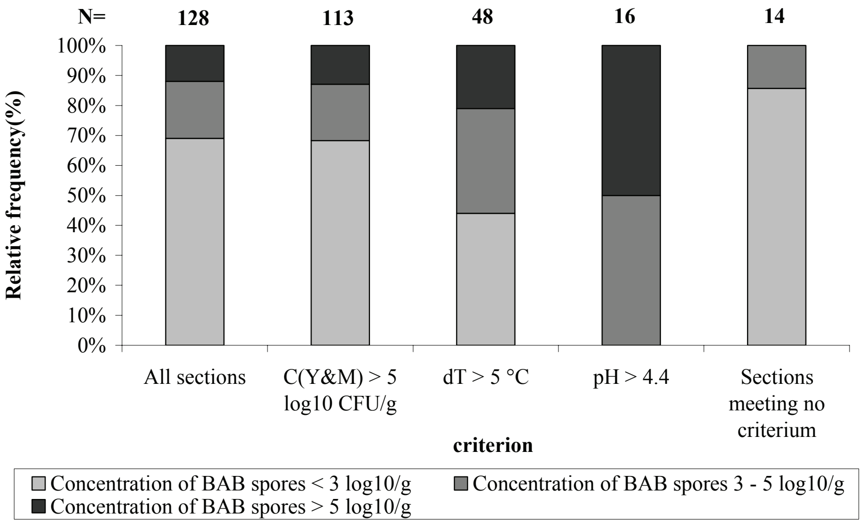

Figure 3.4 Distribution of the concentration of BAB spores measured in the sections meeting the criteria defined on the x-axis (survey 2)

3.4 DISCUSSION

Traditionally, grass silages were considered a more important source of BAB spores than corn silages because in grass silages much higher BAB spore concentrations were measured than in corn silages (Stadhouders and Spoelstra, 1990). The results of this study indicate that nowadays at dairy farms in The Netherlands, BAB spore concentrations in grass and corn silages are comparable. Consequently, corn silage contributes significantly to the concentration of BAB spores in the ration and in FTM. Based on the distribution of BAB spores in grass and corn silages (Figure 3.3), the contribution of corn silage appeared more significant than that of grass silage at the farms sampled.

3.4.1 Aerobic deterioration of corn silage and growth of BAB

A generally accepted view is that high BAB spore concentrations in silage are associated with anaerobic instability of silage due to insufficient pH decline during the primary fermentation phase (Pahlow et al., 2003). The results of the present study, however, show that increased BAB spore concentrations in both grass and corn silage were related to aerobic instability problems rather than to anaerobic instability. High BAB spore concentrations were detected particularly in corn silage samples showing signs of aerobic deterioration, such as high Y&M concentration, high dT and elevated pH (Table 3.1 and 3.2). These samples were almost exclusively located in surface layers (top 50 cm) and in sections with a low density, i.e. in parts which are easily infiltrated by oxygen. Obvious signs of aerobic deterioration (e.g. visibility of molds, increased pH) were always accompanied by increased (above 3 $\log_{10}$ spores/g) or high concentrations of BAB spores (above 5 $\log_{10}$ spores/g) (Figure 3.3 and 3.4).

Growth of strictly anaerobic BAB in aerobically deteriorated parts of silage may seem contradictory. However, microbial ecosystems with aerobic and anaerobic zones are found in many en vironments, e.g. in sediments, biofilms and intestines (Brune et al., 1995, Stoodley et al., 2002, Fourcans et al., 2004). The underlying mechanism of growth of BAB in air-exposed parts of silage is presumably related to the succession of steps occurring in the process of aerobic deterioration (Pahlow et al., 2003). Oxygen penetrating the silage initiates growth of aerobic, acid-tolerant yeasts and acetic acid bacteria. These bacteria oxidize residual sugars and organic acids, leading to an increase of pH. Since the initial concentration of aerobic microorganisms is low, oxygen penetrates initially relatively deep into the silage. But, as the concentration of the aerobic microorganisms increases, the consumption of oxygen increases. As a result, oxygen penetrates less, and deeper parts of the silage return to anaerobic conditions (Muck and Pitt, 1994). Consequently, just below the surface, anaerobic niches with an increased pH may develop. We hypothesize that the high concentration of BAB spores detected in the top 50 cm of the corn silage and in molded spots were due to frequent occurrence of anaerobic niches as a result of the mechanism described above. Growth of BAB associated with aerobic deterioration has been found previously in grass silage (Jonsson, 1991).

Oxygen can penetrate the surface layers of corn silage during the storage period and after opening of the silo (McGechan et al., 1994, Parsons, 1991). Additional research should be undertaken to establish whether high concentrations of BAB spores already occur during the storage period or whether growth of BAB is only initiated after opening of the silage. The process where the penetration of oxygen into silage ultimately leads to growth of BAB requires sufficient amounts of oxygen penetration and requires time. The growth rate of BAB is relatively low, 0.12/h at the optimal temperature of 37 °C and pH of 5.6 (Thylin et al., 1995). In silage growth conditions are not optimal, resulting in a growth rate (μ) less than 0.12/h. During the storage period, the amount of oxygen that penetrates the silage per unit of time will be relatively low, but the time available to go through the entire process described above is several months. After opening of the silage presumably larger amounts of oxygen can penetrate the silage, speeding up the process of aerobic deterioration, but the time available for the formation of anaerobic niches with an increased pH and growth of BAB is less, since the silage face is removed regularly (in the Netherlands a feed-out speed of 1.5 m per week is advised). Control measures that have to be implemented to minimize the occurrence of locally elevated concentration in corn silage depend on which of the two process of oxygen penetration described above is dominant.

In the current study silage samples were not analyzed for organic acids. Therefore, it is unknown whether the corn silage samples with high BAB spore concentrations contained significant amounts of butyric acid, as is usually the case in for instance grass silage with a high BAB spore concentration caused by anaerobic instability. This subject needs further investigation in future studies.

3.4.2 Impact on BAB spores in the ration

The results of this study demonstrate that the concentration of BAB spores within the same corn silage varies strongly (from <1.5 $\log_{10}$ spores/g to >7.0 $\log_{10}$ spores/g). Extremely high concentrations (>5 $\log_{10}$ spores/g) were only found in parts that constitute a relatively small fraction of the total mass (1-10%). However, these small fractions are the main source of BAB spores in the mixed silage fed to cows in the barn. If for example 10% of the silage fed contains 5 $\log_{10}$ spores/g, the silages fed to the cows will contain at least 4 $\log_{10}$ spores/g. This explains why in the first survey the average concentration found in the mixed silage was significantly higher than the average concentrations measured in the core and surface layers of grass and corn silage, which represent the bulk of the material fed (Table 3.1).

One could argue that the elevated concentration of BAB spores in mixed silage in the barn may be due to growth of BAB in the period that the mixed silage is in the barn (generally less than 1 day). However, calculations using the theoretical growth rate of *C. tyrobutyricum* (Vissers et al., 2006), the most important BAB, as well as additional experiments investigating the change in the concentration of BAB spores in mixed silage over time (results not shown) indicate that growth of BAB does not occur at this stage.

3.4.3 Control measures

To control the concentration of BAB spores in mixed silage, it is most important to prevent local growth of BAB in grass and corn silage. Growth can probably be prevented by limiting the penetration of oxygen or inhibiting the detrimental effect of oxygen penetration, for instance by addition of an inhibitor of aerobic deterioration by yeasts and molds, such as propionic, sorbic and benzoic acid and *Lactobacillus buchneri* (Driehuis et al., 1999, Kung et al., 2003). Achieving a high density of the silage is the most important way to limit the penetration of oxygen. Muck and Holmes (2000) performed an extensive study to identify factors that affect the density of corn and alfalfa silages. They found that using heavier packaging tractors, lower initial layer thicknesses and longer packing times affects silage density. A lower initial layer thickness means that each delivered load of freshly harvested corn is spread over a large area allowing, for a higher compaction of the freshly added corn before a new load is delivered. Over recent years the capacity of corn harvesting machines has increased, increasing the mass of harvest silage delivered to the silo per unit of time. However, ensiling practices have not been adjusted accordingly resulting in decreased packing times per delivery of harvested corn. This could explain why high concentrations of BAB spores have only recently been detected in corn silages. After opening of the silage a high feed removal rate may be important because it limits the time available for aerobic deterioration, formation of anaerobic niches with an increased pH and growth of BAB. However, in practice, complete prevention of penetration of oxygen and growth of Y&M is be impossible. The highest concentration of BAB spores were found in visibly molded and deteriorated parts of the silage. During feed-out, removal of these areas is probably an effective measure to reduce the overall concentration in the mixed silage.

3.5 CONCLUSIONS

At farms in the Netherlands, corn silage was shown to be a more important source of BAB spores in FTM than grass silage. The concentration of BAB spores consumed by cows is determined mainly by a small fraction of silage with a high concentration (above 5 $\log_{10}$ spores/g). High concentrations, above 5 $\log_{10}$ spores/g, in corn silage were associated with signs of aerobic deterioration, namely a concentration of Y&M above 5 $\log_{10}$ /g, a temperature difference between silage and ambient temperature of more than 5 °C, a pH higher than 4.4 and visibility of molds. The higher the extent of aerobic deterioration, the higher the observed concentration of BAB spores.

CHAPTER 4

QUANTIFICATION OF THE TRANSMISSION OF SPORES VIA THE EXTERIOR OF TEATS

Pathogens and spoilage microorganisms can be transmitted to milk via dirt (e.g. feces, bedding material and/or soil) attached to the exterior of the cows' teats. To determine the relevance of this pathway and to perform quantitative microbial risk analysis of the microbial contamination of farm tank milk (FTM), it is important to know the amount of dirt transmitted to milk via the exterior of teats. In this study at 11 Dutch farms the amount of transmitted dirt was determined using spores of mesophilic aerobic bacteria (MAB) as a marker. The amount of transmitted dirt to FTM varied among farms from approximately 3 to 300 mg/L, with an average of 59 mg/L. The usefulness of the data for microbial risk analyses is briefly illustrated using the contamination of FTM with spores of butyric acid bacteria (BAB) as a case study. In a similar way the data can be used to quantitatively identify measures to control the contamination of FTM with other microorganisms or chemical residues.

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Quantification of the transmission of microorganisms to milk via dirt attached to the exterior of teats

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4.1 INTRODUCTION

The dairy industry assures the microbial quality and safety of dairy products via a chain management approach. Therefore from "grass to glass", quality assurance systems such as good farming practices, good hygienic practices, and Hazard Analysis and Critical Control Points (**HACCP**) are implemented (Morgan, 2004, Te Giffel, 2003). In addition, qualitative risk analyses can be used to quantitatively assess risks throughout the food chain and to identify effective measures to improve food safety (Cassin et al., 1998a, Notermans et al., 1997). A similar quantitative approach can be followed to identify measures to control the microbial quality of farm tank milk (**FTM**) (Vissers et al., 2006; Chapter 2).

At the farm level many microorganisms, e.g. butyric acid bacteria spores (**BAB**), *Bacillus cereus* (spores) and *Listeria monocytogenes* are transmitted from the environment to FTM via the exterior of teats (Bergère et al., 1968, Sanaa et al., 1993, Slaghuis et al., 1997). Contamination of milk via the exterior of the cows' teats occurs when teats are contaminated with dirt material consisting of e.g. feces, bedding material and/or soil. Dirt attached to exterior of teats rinses off during milking. Subsequently, milk is contaminated with microorganisms originating from the dirt. The concentration of microorganisms transmitted depends on the amount of transmitted dirt and the concentration in transmitted dirt.

This implies that for quantitative risk analysis of the microbial quality of FTM data are required with respect to the amount of transmitted dirt and microbial concentrations in transmitted dirt. Data on the microbial concentrations in dirt or constituents of dirt (e.g. feces, bedding material and soil) are generally available. However, only Stadhouders and Jørgensen (1990) give insight into the amount of transmitted dirt. They estimated that FTM contains on average 40 mg dirt/L milk. The objective of this study was to obtain more, up-to-date and robust data about the amount of dirt transmitted to FTM via the exterior of teats.

4.2 MATERIALS AND MEHTODS

4.2.1 General methodology

Most likely due to the heterogeneous composition of dirt and its low concentrations in comparison to the gross components of milk, a reliable method to directly determine the amount of dirt in milk is not available. Stadhouders and Jørgensen (1990) applied an indirect method based on the transmission route as an alternative. If a specific dirt component is transmitted from the farm environment to milk exclusively via the dirt attached to the exterior of teats, this specific component can be used as a marker for transmitted dirt: when the marker concentrations are measured in samples of milk and dirt, the amount of transmitted dirt can be calculated using equation 4.1.

$$M_{dirt} = C_{marker}(milk)/C_{marker}(dirt) \qquad [4.1]$$

with M_{dirt} Amount of dirt transmitted (g/L)

$C_{marker}(milk)$ Concentration of marker in sample of milk (unit/L)

$C_{marker}(dirt)$ Concentration of marker in sample of dirt (unit/g)

Stadhouders and Jørgensen (1990) used spores of BAB as a microbial marker for transmitted dirt. In order to obtain more robust data in our study, spores of mesophilic aerobic bacteria **(MAB)** were used as a microbial marker. Like for BAB spores, there is ample evidence that MAB spores are transmitted from the farm environment to milk via the exterior of teats (Cooke and Sandeman, 2000, Slaghuis et al., 1997). Major advantage of using MAB spores in stead of BAB spores as microbial marker, is that the MAB spore concentration is generally above the detection limit (Slaghuis et al., 1997). Stadhouders and Jørgensen (1990) were not able to obtain a value for the concentration of BAB spores in 36% of their milk samples. Consequently it was not possible to calculate the amount of transmitted dirt for these farms. An additional advantage of using MAB spores as microbial marker was that a plate-count method could be used for enumeration. Stadhouders and Jørgensen (1990) had to use a most-probable-number method to determine the concentration of BAB spores in dirt and milk.

To determine the amount of transmitted dirt, milk samples were taken from milk recording jars to minimize the possibility that milk is contaminated with spores of MAB from surfaces of the milk equipment. Milk of a single cow is collected in these jars before further transportation through the milklines to the farm tank.

Dirt samples were collected from the surface of the udder. Dirt attached to the udder was assumed to have the same history and composition as dirt attached to teats. It was not possible to take dirt samples from the teats because this would affect the amount of transmitted dirt.

Using a milk sample from the recording jar and a dirt sample from the udder of the same cow, the amount of transmitted dirt for an individual cow can be calculated. Via sampling of multiple cows a farm average can be obtained.

4.2.2 Preliminary measurements

To obtain accurate data it is important that the dirt sample is representative of dirt rinsed off teats. Therefore, first the representativeness of dirt samples taken from the udder for dirt attached to teats was determined at an experimental farm (Animal Sciences Group, Wageningen UR, Lelystad). At this experimental farm cows were housed 24 h of the day. Dirt samples were collected from udder and teats of six cows that had visible dirt attached to the teats. MAB spore concentrations in dirt samples originating from teats and udder were statistically compared (*Student t*-test) to establish if dirt samples from the udder are representative for dirt attached to the teats.

4.2.3 Main study

In the main study the general methodology described was used to determine the amount of dirt transmitted to milk at 11 farms, selected at random, in different regions of the Netherlands. The farms milked between 40 and 125 cows and cows were housed 24 h a day during the experimental period. Per farm dirt and milk samples were collected from 10 cows.

4.2.4 Microbial analyses

The MAB spore concentrations in the samples were determined via dilution of the collected dirt in physiological salt solution (**PPS**) followed by pasteurization of 5 mL of diluted dirt and milk samples for 10 minutes at 80 °C. Pasteurized samples were cooled in melted ice prior to serial dilution in PPS. Appropriate dilutions were plated on Plate Count Milk Agar. Spore counts were determined after two days incubation at 30 °C.

4.3 RESULTS AND DISCUSSION

4.3.1 Preliminary study

The average MAB spore concentration in dirt attached to the udder was 6.9 $\log_{10}$ spores/g (standard deviation (sd) of 0.4 $\log_{10}$ spores/g) and in dirt attached to the teats 6.6 $\log_{10}$ spores/g (sd of 0.3 $\log_{10}$ spores/g). The difference was not significant (P=0.14). These results implies that a dirt sample from the udder can be considered to be representative for dirt rinsed off teats during milking.

4.3.2 Main study

Table 4.1 shows the MAB spore concentrations measured in dirt and milk plus the calculated amount of transmitted dirt per farm. Spore concentrations measured in milk and dirt corresponded to the concentration in milk, feces, bedding and soil previously observed in the Netherlands (Slaghuis et al., 1997). MAB spore concentrations in dirt and milk were highly correlated (correlation coefficient = 0.79; P=0.02). This implies that MAB spores in milk most likely did originate from dirt attached to the teats and that no relevant contamination of the milk via the milk cups and tubing to the milk recording jar had occurred.
The calculated average amount of transmitted dirt ranged from approximately 3 to 300 mg/L, with an average across farms of 59 mg/L. Significant differences between farms existed. This suggest that farm management could affect the amount of transmitted dirt. However, the obtained dataset was too small to detect the existence of relations between for example type of bedding used, application of fore stripping or the applied teat cleaning method and the amount of transmitted dirt.
The minimum (3 mg/L) and average (59 mg/L) values found correspond with previous estimations, i.e. a minimum of 2 mg/L and average of 40 mg/L (Stadhouders and Jørgensen, 1990). The similarity of the average values suggest that since the end of the 1980's implementation and improvement of quality control systems and hygienic measures taken at the farm level have not reduced the amount of transmitted dirt. But, it should be kept in mind that Stadhouder and Jørgensen (1990) had to estimate the average value because they could not measure the concentration of their marker for transmitted dirt, spores of BAB, in 36% of the milk samples. Consequently, our estimates are more robust.
The usefulness of the data obtained can be illustrated using the contamination of FTM with spores of BAB as a case study. Minimization of the contamination of FTM with spores of BAB is important for the production of semi-hard cheeses (Klijn et al., 1995). FTM is contaminated with spores of BAB when feces is transmitted to milk via the exterior of teats (Bergère et al., 1968). BAB spore concentrations in feces vary by more than a factor 10,000 (from 2 to >6 log spores/g) (Kalzendorf, 1996). The amount of transmitted dirt varies across farms by a factor of 100 only (Table 4.1). This implies that BAB spore concentrations in FTM

depend more on the spore concentration in feces than on the amount of transmitted dirt. Consequently, it is more important to minimize BAB spore concentration in feces than to minimize the contamination of teats with feces or to maximize the efficiency of teat cleaning, as has been shown by Vissers et al. (2006; Chapter 2). However, the role of teat hygiene should not be neglected since a factor 100 difference between the amount of dirt transmitted to milk at the best and worst farms is substantial. In addition, for the identification of control measures at the farm level, the data acquired in the presented study will also facilitate the extension of "farm to fork" risk analyses to "grass to glass" analyses. In this way for instance the importance of animal feed for the safety of dairy products could be assessed more accurately.

Table 4.1 Concentrations of spores of mesohilic aerobic bacteria (MAB) in milk of individual cows (log_{10} spores/L) and in dirt attached to the udder (log_{10} spores/g) and the calculated amount of transferred dirt (mg/L) for the 11 farms visited. Per farm, milk and dirt attached to the udder of 10 cows were sampled.

	Concentration in milk of individual cows		**Concentration in dirt attached to udder**		**Calculated amount of dirt in milk of individual cows**	
	Farm average[1]	Range	Farm average	Range	Farm average[2]	Range
Farm 1	4.4^{c}	4.1-4.9	7.2^{e}	6.7-7.8	3^{a}	1 - 10
Farm 2	3.2^{a}	3.0-4.0	$5.7^{a,b}$	5.1-6.0	$6^{a,b}$	1 - 17
Farm 3	4.8^{d}	4.1-5.2	7.2^{e}	6.7-7.6	6^{a}	1 - 20
Farm 4	$4.7^{c,d}$	4.1-5.2	6.7^{d}	6.4-7.1	15^{b}	3 - 39
Farm 5	4.0^{b}	3.0-4.4	$6.4^{c,d}$	4.9-7.0	$17^{a,b}$	1 - 129
Farm 6	4.9^{a}	4.3-5.1	$6.8^{d,e}$	5.8-7.4	$20^{b,c}$	5 - 16
Farm 7	4.8^{d}	4.1-5.2	6.4^{c}	6.0-6.7	$32^{c,d}$	5 - 69
Farm 8	5.3^{e}	5.0-5.5	$6.8^{d,e}$	6.0-6.8	$55^{c,d}$	2 - 163
Farm 9	3.9^{b}	3.2-4.7	5.3^{a}	4.5-6.2	69^{d}	2 - 258
Farm 10	4.9^{d}	4.2-5.4	$6.3^{c,d}$	5.3-7.0	129^{d}	8 - 779
Farm 11	$4.9^{c,d,e}$	4.1-5.5	$6.0^{c,d}$	5.0-6.9	299^{d}	2 - 1160

[1] Within columns values with a different superscript (a,b,c etc.) are significantly different ($P<0.05$)
[2] Statistics performed on log_{10} transformed data

4.4 CONCLUSIONS

Spores of MAB can be used as a microbial marker for the amount of dirt transmitted to FTM via the exterior of teats. To determine this amount, dirt samples from the surface of the udder and milk samples from the milk recording jar can be used. In the Netherlands during housing of cows per farm, approximately between 3 to 300 mg of dirt is transmitted to 1 L of milk, with an average of 59 mg/L.

CHAPTER 5

MONITORING THE CONTAMINATION OF FARM TANK MILK WITH BUTYRIC ACID BACTERIA SPORES

A year-long survey of 24 dairy farms was conducted to determine effects of farm management on the concentrations of butyric acid bacteria (BAB) spores in farm tank milk (FTM). The results were used to validate a control strategy derived from model simulations. BAB spore concentrations were measured in samples of FTM, feces, bedding material, mixed corn/grass silage fed to cows in the barn, and soil. Furthermore, a questionnaire was used to gather farm management information such as bedding material used and teat cleaning method applied. The average BAB spore concentration in FTM was 2.7 $\log_{10}$ spores/L and 33% of the FTM samples exceeded a maximum spore limit (MSL) of 3 $\log_{10}$ spores/L. The average spore concentration in mixed silage was the only aspect of farm management that was significantly related to the concentration of BAB spores in FTM. Farms that fed mixed silage with the lowest average BAB spore concentrations (3.4 $\log_{10}$ spores/g) produced FTM with the lowest average concentration (2.1 $\log_{10}$ spores/L). The efficiency of farm management in controlling BAB spore concentration in FTM depended to a large extent on the ability of farmers to prevent incidents with elevated BAB spore concentrations in mixed silage (>5 $\log_{10}$ spores/g) and not specifically on the average BAB spore concentration in mixed silage across the year. The survey showed that farmers should aim for a concentration in mixed silage below 3 $\log_{10}$ spores/g and prevent the concentration from exceeding 5 $\log_{10}$ spores/g in order to assure a concentration in FTM below the MSL. These results correspond with the previously reported model simulations.

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Minimizing the level of butyric acid bacteria spores in farm tank milk

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5.1 INTRODUCTION

In semi-hard cheeses such as Gouda and Emmental growth of spore-forming anaerobic bacteria, butyric acid bacteria **(BAB)**, can lead to off-flavors and excessive gas formation, a defect known as late-blowing (Klijn et al., 1995). BAB are able to convert lactic acid into butyric acid, hydrogen, and carbon dioxide at relatively low pH. In particular *Clostridium tyrobutyricum* is associated with the late-blowing defect. Without preventive measures, concentrations in pasteurized cheese milk above 10 BAB spores/L can already lead to late-blowing of Gouda-type cheeses (Stadhouders, 1990).

To prevent late-blowing, cheese manufacturers have three main options: 1) minimization of BAB spore concentrations in raw milk; 2) removal of BAB spores from raw milk via bactofugation; and 3) preventing growth of BAB in cheese by adding inhibitory agents, such as nitrate and lysozym, to cheese milk (Stadhouders, 1990, Waes and Van Heddeghem, 1990).

In the Netherlands farmers are encouraged to produce farm tank milk **(FTM)** with low concentrations of BAB spores via the milk quality payment system. Two tubes of 0.1 mL FTM are tested monthly for the presence of BAB spores. If both tubes test positive the farmer receives a deduction on the milk price. The Dutch dairy industry wants farmers to produce FTM with a maximum spore limit (**MSL**) of 3 $\log_{10}$ spores/L in order to obtain after bactofugation fewer than 1 $\log_{10}$ spores/L in pasteurized cheese milk.

BAB spores in FTM originate from the farm environment. Grass and corn silage are the most important source of BAB spores. Grass and corn silage are fed to cows together (referred to as mixed silage). Because, spores pass the gastrointestinal tract of cows unharmed, the BAB spores in the mixed silage accumulate in feces. Via the exterior of the cow's teats, feces and BAB spores are transmitted to milk during milking (Bergère et al., 1968).

Using model simulations a strategy to achieve BAB spore concentration in FTM below the MSL was defined. Control of the spore concentration in the mixed silage was shown to be the most important control strategy (Vissers et al., 2006; Chapter 2). The model showed that BAB spore concentration in mixed silage should ideally be below 3 $\log_{10}$ spores/g and not exceed 5 $\log_{10}$ spores/g. If mixed silage contain more than 5 $\log_{10}$ spores/g it is not possible to ensure FTM concentration below the MSL.

The aim of this study of Dutch farms was to determine the effect of different aspects of farm management on BAB spore concentrations in FTM via a year-long survey. In addition, the results of the survey were used to validate the control strategy derived from model simulations.

5.2 MATERIALS AND METHODS

5.2.1 Selection of farms

In total 24 farms spread across the Netherlands were selected for the survey. The farms were selected using historical data from The Netherlands Milk Control Station **(MCS)** (Zutphen, The Netherlands). MCS performs the monthly tests for presence of BAB spores in FTM. To select the 24 survey farms, 270 farms spread across the Netherlands were divided into three groups. The first group (Group A) consisted of farms where none of the 12 measurements, performed in the framework of the milk quality payment system in 2004, resulted in a double-positive test result for presence of BAB spores in FTM (i.e. two tubes of 0.1 mL of FTM tested positive for spores of BAB). The second group (Group B) consisted of farms with one or two double-positive test results in 2004. The third group (Group C) consisted of farms with more than 2 double positive test results in 2004. Out of groups A, B and C, respectively, 10, 9 and 5 farms were selected randomly for the survey. In the total population of Dutch farms, 92.1% of the farms belonged to group A, 7.5% of the farms belong to Group B and 0.3% of the farms belongs to Group C.

5.2.2 Sample collection

Samples were collected between January and December 2005. Each farm was visited 6 times at intervals of 8 weeks. At each visit, besides sample collection a questionnaire was answered to collect actual farm management data. The questionnaire contained questions related to the feed ration fed to the cows, housing conditions and milking practices.

The following sample types were collected: FTM (every visit), feces from the barn (when cows were housed half or all day), feces from the pasture (when cows were grazing all day), bedding material from the barn (when cows were housed half or all day), soil (when cows were at pasture half or all day), and mixed silage in the barn (every visit when appropriate). No samples of individual grass and corn silage were taken because earlier studies showed that a sample of the mixed silage is a better indicator of the microbial quality of the silages fed (Vissers et al., 2007a; Chapter 3). All but the FTM sample, were composite samples. Each composite sample consisted of 10 randomly collected sub samples that were mixed on location. Samples were stored at 4-6 °C until microbial analysis within 24 h after sampling.

5.2.3 Microbial analyses

The concentration of BAB spores in FTM, feces, bedding material, mixed silage and soil was determined by a most probable number (**MPN**) method according to the Dutch Standard (NEN-ISO-6877, 1994). Feces, bedding, soil, silage, and feed extracts were prepared by adding 90 mL of peptone physiological salt solution (**PPS**) (1 g of neutralized bacteriological peptone and 8.5 g sodium chloride per liter) to 10 g of sample and homogenizing for 2 minutes in a laboratory blender (Stomacher, Seward Medical, London, UK). Decimal dilution series of extracts were prepared in PPS. A volume of 0.1 mL of diluted extract was added to tubes containing 10 mL of sterilized milk, supplemented with glucose and lactic acid. The tubes were heated for 5 minutes at 80 °C to inactivate vegetative cells and to trigger the germination of spores, then sealed with paraffin and incubated for 4 days at 37 °C. A tube scored positive if after incubation gas formation was visible. The detection limit was 30 spores/L for FTM and 300 spores/g for feces, bedding, mixed silage, and soil.

5.2.4 Statistical Analyses

Statistical analyses were performed using Statistica® and Microsoft Excel©. In order to perform statistical analysis, samples containing BAB spore concentrations below the detection limit were assigned a concentration equaling half of the detection limit. All statistical analyses were performed on $\log_{10}$ transformed data.

Student t-tests were used to determine significant differences between farms and spore concentrations in different carriers (i.e. feces, bedding material, mixed silage, and soil) and to detect possible effects of farm management practices on BAB spore concentrations in FTM. The *Fisher-Exact* test was used to determine whether high concentrations occurred more frequently in a specific carrier or group.

5.3 RESULTS

5.3.1 BAB Spore concentration in FTM and the farm environment

Table 5.1 gives an overview of BAB spore concentration measured in FTM and carriers of BAB spores in the farm environment (environmental carriers) at all 24 farms during the survey. The average BAB spore concentration in FTM was close to the MSL (3 $\log_{10}$ spores/L). In 33% of the FTM samples the MSL was exceeded. Also in feces, bedding material, and mixed silage high concentrations (above 5 $\log_{10}$ spores/g) were frequently detected. Spore concentrations in feces and bedding material did not differ statistically ($P>0.2$). Spore concentrations in feces were significantly higher than spore concentrations in the mixed silage ($P<0.01$), most likely due to a concentration effect (Te Giffel, 2002). BAB spore concentrations in soil varied much less than the concentration in the other environmental carriers and never exceeded 5 $\log_{10}$ spores/g. No relation between soil type (e.g. clay, sand and peat) and the BAB spore concentration was found ($P>0.2$).

Table 5.1 Overview of butyric acid bacteria (BAB) spore concentrations measured in farm tank milk (FTM, $\log_{10}$ spores/L) and in environmental carriers ($\log_{10}$ spores/g).

	Number of samples	**BAB spores[1]** Mean	Standard error of mean (SEM)	Range	**Fraction of samples with critically high concentration**
Farm tank milk	142	2.7	0.06	<1.5 – 4.2	33%[2]
Feces	141	4.7	0.07	<1.5 – 7.0	33%[3]
Bedding material	113	4.5	0.07	<1.5 – 6.0	20%[3]
Mixed silage	122	4.2	0.09	<1.5 – 6.0	18%[3]
Soil	59	4.2	0.06	3.2 – 4.9	0%[3]

[1] $\log_{10}$ spores/L for FTM, $\log_{10}$ spores/g for feces, bedding material, mixed silage and soil

[2] Fraction of samples with concentration above 3 $\log_{10}$ spores/L

[3] Fraction of samples with a concentration above 5 $\log_{10}$ spores/g

5.3.2 Comparison of farms

The results were evaluated in two ways. Firstly, the farms were grouped based on the results of the BAB test in the milk payment system of 2004 (Table 5.2A). Secondly, farms were grouped based on the average BAB spore concentration detected in FTM per farm in the survey (Table 5.2B). Based on the historical grouping group A farms had lower spore concentrations in FTM than Group B farms and Group B farms had lower spore concentrations than Group C farms. In Table 5.2B the Low group represents the six farms with the lowest average BAB spore concentration in FTM, the High group the six farms with the highest average BAB spore concentration in FTM, and the Medium group the remaining 12 farms. Average BAB spore concentrations in FTM per farm ranged from 1.8 to 3.4 log_{10} spores/L.

There was no distinct relationship between the historical grouping and the grouping based on survey data. Of the six farms with the lowest average BAB spore concentration in FTM in the survey, five farms originated from group A and one from group C of the historical grouping. The farms with the highest average FTM concentration in the survey originated from groups A (one farm), B (three farms) and C (two farms) of the historical grouping.

Clear relationships were found after grouping based on the survey data (Table 5.2B). The six farms that fed mixed silage with the lowest average spore concentration (3.4 log_{10} spores/g) also produced FTM with the lowest average concentrations (2.1 log_{10} spores/g). Conversely, the six farms with the highest FTM concentration (3.2 log_{10} spores/g) also fed silage with the highest average concentration (4.7 log_{10} spores/g). These concentrations were around the critical levels defined for FTM and silage by Vissers et al. (2006; Chapter 2). Table 5.2B also indicates that the relationship between average concentrations in mixed silage, feces, and FTM is almost linear since the absolute differences observed at the Low, Medium and High farms were similar for these three carriers (i.e. approximately 1.1 log_{10} difference between Low and High and 0.7 log_{10} difference between Low and Medium). Historical grouping (Table 5.2A) showed less clear relationships than grouping based on the survey data. However, the farms that performed best based on the historical data (Group A) also had the lowest average BAB spore concentration in FTM.

On all farms in the survey, substantial fluctuations of the BAB spore concentration in FTM and environmental carriers were observed. Only at four farms (three from the low group and one from the medium group) the BAB spore concentration in FTM was consistently below the MSL. Also in feces, bedding material and mixed silage critically high concentrations (>5 log_{10} spores/g) were found occasionally on farms with the lowest spore concentration in FTM. For example, the highest BAB spore concentration in feces (7 log_{10} spores/g) was observed at the farm with the third lowest average BAB spore concentration in FTM. At the visit to this farm, the feces sample contained 7 log_{10} spores/g and the corresponding BAB spore concentration in FTM was 4.1 log_{10} spores/L. However, critically high BAB spore concentrations in feces, bedding material and mixed silage were more frequently observed at the farms with the highest average concentration in FTM. But except for one farm, all farms were able to meet the MSL at least at two of the visits. At one farm, at all visit the BAB spore concentration in FTM was above the MSL.

Table 5.2 Overview of butyric acid bacteria (BAB) spore concentrations measured in farm tank milk (log_{10} spores/L) and environmental carriers (FTM, log_{10} spores/g) after grouping based on historical data (2A) and BAB spore concentration observed in farm tank milk during the survey (2B).

	Farm tank milk		**Feces**		**Bedding**		**Mixed silage**		**Soil**	
	Mean[1] (SEM)	Fraction >3 log_{10}	Mean (SEM)	Fraction >5 log_{10}	Mean (SEM)	Fraction >5 log_{10}	Mean (SEM)	Fraction >5 log_{10}	Mean (SEM)	Fraction >5 log_{10}
2A. Historical grouping (2004: group A performed better than Group B and Group B better than Group C)										
Group A (N=10)	2.4 (0.10)^{a}	20%^{a}	4.6 (0.11)^{a}	31%^{a}	4.1 (0.11)^{a}	7%^{a}	4.0 (0.12)^{a}	10%^{a}	4.2 (0.08)^{a}	0%^{a}
Group B (N=9)	2.9 (0.08)^{b}	43%^{b}	4.8 (0.10)^{a}	40%^{a}	4.7 (0.09)^{b}	30%^{b}	4.5 (0.14)^{b}	31%^{b}	4.2 (0.10)^{a}	0%^{a}
Group C (N=5)	2.9 (0.15)^{b}	40%^{a,b}	4.5 (0.21)^{a}	27%^{a}	4.7 (0.13)^{b}	30%^{b}	3.9 (0.19)^{a}	11%^{a,b}	4.5 (0.14)^{a}	0%^{a}
2B. Grouping based on survey results (2005: Low performed better than Medium and Medium performed better than High)										
Low (N=6)	2.1 (0.13)^{a}	8%^{a}	4.1^{a} (0.19)	17%^{a}	3.8 (0.15)^{a}	0%^{a}	3.4 (0.20)^{a}	4%^{a}	4.1 (0.13)^{a}	0%^{a}
Medium (N=12)	2.8 (0.07)^{a,b}	32%^{b}	4.7 (0.08)^{a,b}	33%^{a,b}	4.6 (0.08)^{b}	26%^{b}	4.2 (0.10)^{b}	16%^{a,b}	4.4 (0.08)^{a}	0%^{a}
High (N=6)	3.2 (0.08)^{b}	61%^{b}	5.2^{b} (0.10)	50%^{b}	4.7 (0.12)^{b}	27%^{b}	4.7 (0.16)^{b}	33%^{b}	4.2 (0.12)^{a}	0%^{a}

[1] Within columns values with a different superscript (a,b,c etc.) are significantly different ($P<0.05$)

[2] SEM = standard error of mean

5.3.3 Effects of farm management

In addition to the determination of BAB spore concentrations, data about farm management practices at the farms that participated in the survey were collected via a questionnaire. Table 5.3 gives an overview of farm management practices related to housing of cows, milking hygiene and type of silage fed. Statistically significant relations with the BAB spore concentration in FTM were only found for the location of the cows during the summer period and for the presence in the herd of cows preferring to lie down on dirty patches. Therefore these aspects were analyzed in more detail.

Table 5.3 Overview of farm management practices at the farms in the survey

Aspect of farm management	**Different behaviors observed**	**Number of farms with this behavior**
Type of housing system	Loose-housing system	21
	Tied-housing system	3
Type of silage fed	Grass and corn silage	22
	Grass silage only	2
Type of bedding material in the boxes[1]	Sawdust	15
	Straw	6
Presence of cows preferring to lie down on dirty patches[2]	Present at each visit	8
	Never present	8
	Present at some of the visits	8
Fore-stripping prior to milking to detect abnormal milk	No	11
	Yes	13
Teat cleaning method	Dry towel	20
	Wet towel	4
Location of cows during the summer period (May to October)	Housed during nighttime, at pasture during daytime	9
	Housed all day	5
	At pasture all day	1
	A combination of the above	9

[1] No bedding material used at farms with tied-housing system

[2] Teats of cows that lie down on dirty patches are generally more heavily contaminated than teats of other cows

Table 5.4 shows the BAB spore concentration measured in the winter period and for the different housing situations observed during the summer period. During the winter period cows were housed 24 h of the day at 96% of the farm visits. Table 5.4 shows that keeping cows 24 h a day at pasture leads to a reduction of the BAB spore concentration in FTM. The observed lower BAB spore concentration in FTM for this situation seems related to the lower concentration in feces. BAB spore concentrations in mixed silage did not statistically differ.

Table 5.4 Overview of the concentration of BAB spores in farm tank milk (log_{10} spores/L) and environmental carriers (log_{10} spores/g) during summer period (May to October) for different housing conditions and the winter period (November to April)

Housing situation	**Farm tank milk**		**Feces**		**Bedding**		**Mixed silage**		**Soil**	
	Average[1] (SEM[2])	Fraction $>3\ log_{10}$	Average (SEM)	Fraction $>5\ log_{10}$	Average (SEM)	Fraction $>5\ log_{10}$	Average (SEM)	Fraction $>5\ log_{10}$	Average (SEM)	Fraction $>5\ log_{10}$
Barn all day (winter) (N=66)	2.9 $(0.10)^{b}$	$41\%^{b}$	4.8 $(0.11)^{b}$	$32\%^{a}$	4.5 $(0.10)^{a}$	$17\%^{a}$	4.2 $(0.12)^{a}$	$15\%^{a}$	Soil not sampled	
Barn all day (summer)(N=19)	2.9 $(0.14)^{b}$	$44\%^{b}$	4.6 $(0.17)^{a,b}$	$32\%^{a}$	4.8 $(0.18)^{a}$	$47\%^{b}$	4.5 $(0.22)^{a}$	$32\%^{a}$	Soil not sampled	
Barn at night (summer)(N=38)	2.7 $(0.10)^{b}$	$30\%^{a.b}$	4.7 $(0.11)^{b}$	$33\%^{a}$	4.4 $(0.11)^{a}$	$10\%^{a}$	4.0 $(0.20)^{a}$	$13\%^{a}$	4.3 $(0.08)^{a}$	$0\%^{a}$
Pasture all day (summer)(N=14)	2.1 $(0.21)^{a}$	$7\%^{a}$	4.0 $(0.39)^{a}$	$14\%^{a}$	Bedding not sampled		4.0 $(0.56)^{a}$	$14\%^{a,3}$	4.2 $(0.08)^{a}$	$0\%^{a}$

[1] Within columns values with a different superscript (a,b,c etc.) are significantly different ($P<0.05$)

[2] SEM=standard error of mean

[3] Mixed silage was only fed in 64% of the cases

However, further analysis suggested that it was probably not the location of the cows that was important but whether the cows were fed silage or not. The questionnaire revealed that in 64% of the cases that cows were grazing all day the diet of fresh grass was supplemented with silage. In these cases cows were offered silage in the barn before milking. When the diet of grazing cows was not supplemented with silage, the BAB spore concentration in FTM never exceeded 1.6 $\log_{10}$ spores/L (5 samples), whereas when silage was fed to cows grazing all day, BAB spore concentrations in FTM ranged from <1.5 to 3.7 $\log_{10}$ spores/L (9 samples).

Another farm management factor that was studied in detail was the presence in the herd of cows that prefer to lie down on dirty patches. Teats of these cows are generally more heavily contaminated with feces and likely more spores are transmitted to milk. Indeed, statistical analysis of the data indicated that when cows preferring to lie down on dirty patches were absent in the herd, BAB spore concentrations were significantly lower (0.5 log10 spores/L). However, further analysis of the data demonstrated that the observed difference was more likely due to differences in spore concentrations in the mixed silage and not to the absence or presence of cows preferring to lie down in dirty patches in the herd. When such cows were absent, the average spore concentration in mixed silage and feces was 0.5 log10 spores/g lower than when these cows were present.

5.3.4 Critical concentrations in feces, bedding and mixed silage

Figures 5.1 and 5.2 show the fraction of FTM samples with a BAB spore concentration below and above the MSL in relation to BAB spore concentrations in feces and mixed silage. Figure 5.1 shows that BAB spore concentration in feces above 5 $\log_{10}$ spores/g are critical with respect to the production of FTM with less spores than the MSL. When feces contained more than 5 $\log_{10}$ spores/g, almost 60% of the FTM exceeded the MSL. On the other hand when feces contained less than 4 $\log_{10}$ spores/g, none of the FTM samples exceeded the criterion. These results suggest that farmers should try to take measures that result in BAB spore concentration in feces below 4 $\log_{10}$ spores/g.

The BAB spore concentration in feces relates to the BAB spore concentration in mixed silage. Figure 5.2 shows that also in mixed silage a critical concentration can be distinguished. As in feces, a BAB spore concentration above 5 $\log_{10}$ spores/g in mixed silage should be prevented. When this contamination level in silage was observed almost 70% of the FTM samples contained more spores than the MSL. Conversely, when the silage contained less than 3 $\log_{10}$ spores/g, only about 13% of the FTM contained more spores than the MSL of 3 $\log_{10}$ spores/L.

5.4 DISCUSSION

5.4.1 Control strategy

The data presented in this paper confirm conclusions drawn in a modeling study concerning control of BAB spore concentrations in FTM (Vissers et al., 2006; Chapter 2). In this study a model was developed that described transmission of BAB spores from mixed silage, other feed and soil, via feces to FTM as a function of farm management factors. In the modeling study, it was concluded that to control the FTM concentration, by far, the most important to control the concentration of BAB spores in the silage fed to cows. Other control measures were considered to have only a marginal impact.

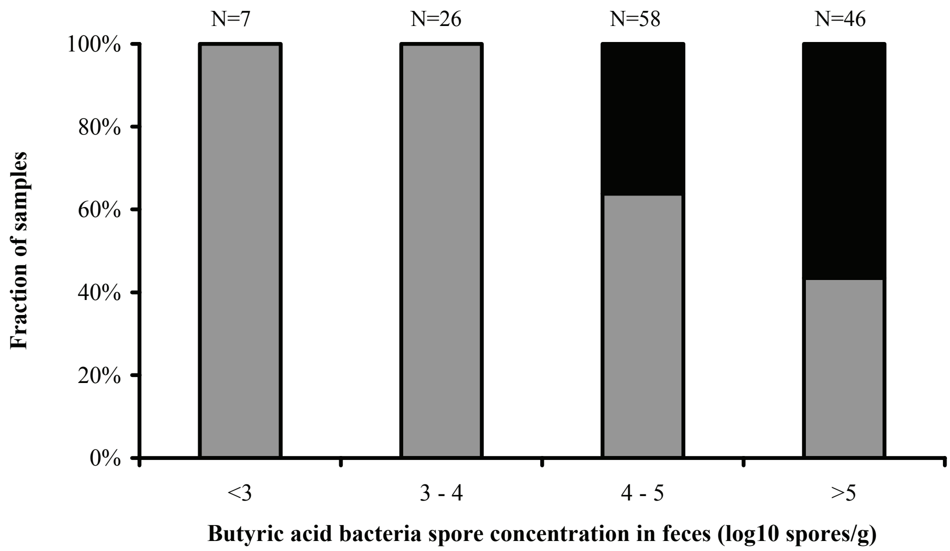

Figure 5.1 Fraction of farm tank milk (FTM) samples with concentration above (black bars) and below (rey bars) the maximum spore limit of 3 $\log_{10}$ spores/L as a function of the butyric acid bacteria (BAB) spore concentration in feces.

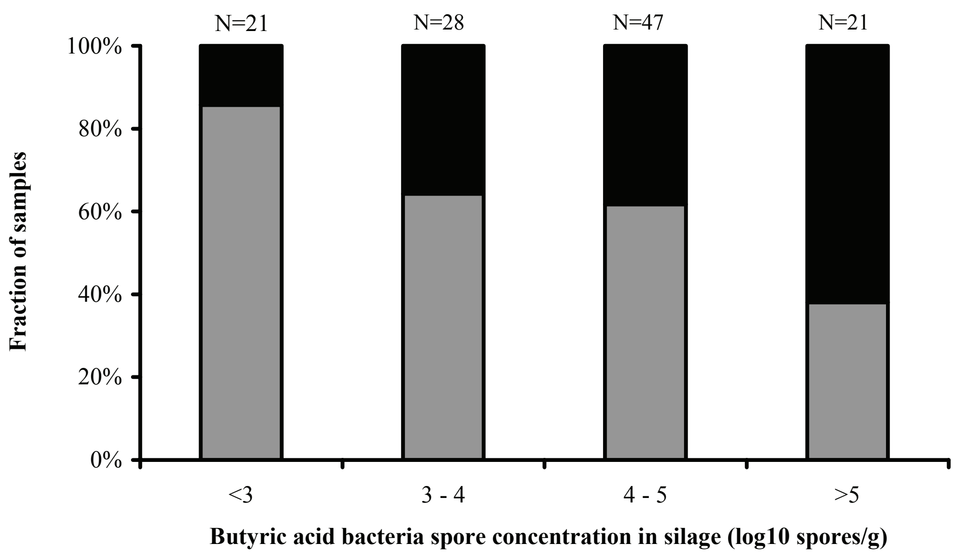

Figure 5.2 Fraction of farm tank milk (FTM) samples with concentration above (black bars) and below (grey bars) the maximum spore limit of 3 $\log_{10}$ spores/L as a function of the butyric acid bacteria (BAB) spore concentration in mixed silage.

The survey confirmed the importance of silage for control of the BAB spore concentration in FTM. In line with the model of Vissers et al. (2006; Chapter 2) a more or less linear relation between BAB spore concentrations in mixed silage offered to the cows, feces and FTM was found (Table 5.2). On the rare occasions (5 of the 144 visits) that no grass- or corn silage was fed to the cows, the spore concentration in FTM never exceeded 1.6 log_{10} spores/L.

The most efficient way to control the BAB spore concentration in FTM was shown to be control of the BAB spore concentration in mixed silage. Farmers with the lowest average BAB spore concentration in FTM (2.1 log_{10} spores/L) also fed mixed silage to the cows with the lowest average concentration (3.4 log_{10} spores/g). Conversely, farmers with the highest average BAB spore concentration in FTM (3.2 log_{10} spores/L) fed mixed silage with the highest average concentration (4.7 log_{10} spores/g). Apart from silage feed and silage quality, no relevant relations with other aspects of farm management were detected.

In the modeling study by Vissers et al. (2006; Chapter 2) it was postulated that in order to consistently produce FTM meeting the MSL, farmers should aim for a concentration of BAB spores in the silage fed below 3 log_{10} spores/g (in combination with a moderate level of teat hygiene) and should prevent concentrations above 5 log_{10} spores/g. Figures 5.1 and 5.2 confirm that these concentrations are critical for control. When mixed silage contained more than 5 log_{10} spores/g, the majority of FTM samples exceeded the MSL. On the other hand when the silage fed contained less than 3 log_{10} spores/g, FTM only in a minority of the samples (13%) exceeded the MSL. At concentrations in mixed silage between 3 and 5 log_{10} spores/g, the fractions with a BAB spore concentration above and below MSL was similar. At these concentrations in mixed silage presumably the level of fecal contamination of the exterior of teats plays a role, as also stated in the modeling study (Chapter 2). The agreement between the survey results and results obtained in a modeling study emphasize the usefulness of the modeling approach for the identification of measures to control the microbial contamination of FTM.

5.4.2 Incident management

The heterogeneous distribution of BAB spores in grass and corn silage, is a difficulty in achieving and maintaining the BAB spore concentration in silage fed to cows below 3 log_{10} spores/g. A number of studies have shown that BAB spore concentrations in grass and corn silage can be highly elevated locally (up to >7 log_{10} spores/g), while the concentration in the bulk of the silage is low (less than 3 log_{10} spores/g) (Driehuis and Te Giffel, 2005, Jonsson, 1991, Vissers et al., 2007a; Chapter 3). When such local spots with high BAB spore concentration are mixed with the bulk of the silage and other silage, the average BAB spore concentration in the mixed silage fed to cows increases significantly, as was confirmed in a recent study (Vissers et al., 2007a; Chapter 3). The heterogeneous distribution of BAB spores in silage can be an explanation why even farms with a low average BAB spore concentrations in FTM, occasionally produce FTM with a BAB spore concentration above the MSL (Table 5.2). As such, efficient farm management with respect to control of the contamination of FTM with BAB spores probably depends on the ability of farmers to minimize the incidents of elevated BAB spore concentration (> 5 log_{10} spores/g) in mixed silage.

5.4.3 Control of BAB spores in mixed silage

To secure that mixed silage fed contain less than 3 $\log_{10}$ spores/g, it is important to limit the initial contamination of silage with BAB spores and to prevent growth of BAB during ensilage, storage and feed-out. Silage is initially contaminated with BAB spores via enclosure of soil during harvesting (Pahlow et al., 2003). The BAB spore concentrations measured in soil (Table 5.1) indicate that an initial contamination level below 3 spores/g silage can be secured when less than 1% soil is enclosed. To prevent growth of BAB, it is first of all important to produce an anaerobically stable silage by assuring a fast and sufficiently deep decline of the pH by lactic acid fermentation (Pahlow et al., 2003, Weissbach, 1996). Secondly, penetration of oxygen into the silage during storage and feed-out should be prevented. Penetration of oxygen is the most likely cause of locally increased BAB spore concentrations in silage (Jonsson, 1991, Vissers et al., 2007a; Chapter 3). Presence of oxygen in silage enables yeasts to grow and consume the organic acids. As a consequence, pH rises locally and BAB may start to proliferate, resulting in small pockets with a very high BAB spore concentration. Adequate sealing of silage and a high silage density limits the penetration of oxygen (McGechan et al., 1994, Parsons, 1991).

5.4.4 Late-blowing in summer

Traditionally, late-blowing of cheeses was exclusively associated with milk produced in the winter period. Therefore, in the Dutch milk quality payment system, FTM was initially tested for BAB spores during the winter period only. However, in recent years late-blowing occurred more often during the summer, suggesting that also during the summer period FTM contained significant concentrations of BAB spores. As a reaction, since 2004 FTM is tested for BAB spores all year round. The results shown in Table 5.4 about BAB spore concentrations in FTM in summer and winter confirm that this change in the milk payment system was justified. The survey presented in this paper clearly demonstrates that the increased late-blowing frequency during summer is most likely due to changed feeding and housing patterns in the summer. In the past, in the Netherlands cows were fed silage and housed all day only during the winter period. In summer time cows were grazing all day and no silages were fed. Nowadays, in the summer period cows are generally in the barn for at least half of the day (Table 5.3), but more importantly silages are fed all year round, even when cows graze all day.

5.5 CONCLUSIONS

To ensure BAB spore concentrations in FTM below the MSL farmers should aim for an average BAB spore concentration in the mixed silage fed below 3 $\log_{10}$ spores/g and prevent concentrations above 5 $\log_{10}$ spores/g. Other aspects of farm management did not affect the observed spore concentrations in FTM. Efficient control of the BAB spore concentration in FTM below 3 $\log_{10}$ spores/L mainly depends on the ability of farmers to prevent incidents with elevated concentrations in mixed silage fed (more than 5 $\log_{10}$ spores/g). The results of the survey were agreement with a previously performed modeling study of the contamination of FTM by BAB spores (Chapter 2). This emphasizes the usefulness of a modeling approach for the identification of measures to control the microbial contamination of FTM.

CHAPTER 6

MODELING THE CONTAMINATION OF FARM TANK MILK WITH *BACILLUS CEREUS* SPORES

The shelf life of pasteurized dairy products depends partly on the concentration of *Bacillus cereus* spores in raw milk. Based on a translation of contamination pathways into chains of unit-operations, two simulation models were developed to quantitatively identify major factors that have the greatest impact on the spore concentration in farm tank milk (FTM). In addition, the models were used to determine the reduction in concentration that could be achieved via measures at the farm level. One model predicts the concentration when soil is the only source of spores, most relevant during grazing of cows. The other model predicts the concentration when feed is the main source of spores, most relevant during housing of cows. It was estimated that when teats are contaminated with soil, 33% of the FTM contains more than the maximum allowed spore limit (MSL) of 3 $\log_{10}$ spores/L. When feed is the main source this is only 2%. Based on the predicted spore concentrations in FTM it was calculated that the average spore concentration in raw milk stored at the dairy processor during the grazing period is 3.5 $\log_{10}$ spores/L of milk and during the housing period is 2.1 $\log_{10}$ spores/L. It was estimated that during the grazing period a 99% reduction can be achieved, if all farms minimize the contamination of teats with soil and teat cleaning is optimized. During housing, a reduction of the concentration by 60% should be feasible by assuring spore concentrations in feed below 3 $\log_{10}$ spores/g and a pH of the silage-based ration fed to the cows below 5. Implementation of these measures at the farm level assures that the concentration of *B. cereus* spores in raw milk rarely exceeds the MSL.

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6.1 INTRODUCTION

Growth of *Bacillus cereus* often limits the shelf life of pasteurized dairy products kept at refrigeration temperatures. Dairy products spoiled by *B. cereus* show defects like off-flavors, sweet curdling and bitty cream (Overcast and Atmaram, 1971, Stone and Rowlands, 1952). *B. cereus* in dairy products originates, at least partly, from the farm environment. The spore-forming properties of *B. cereus* enable the organism to survive pasteurization processes. In addition, recontamination during milk processing via (improperly cleaned) pasteurization equipment and during filling of the product can occur (Lin et al., 1998, Svensson et al., 2000, Te Giffel et al., 1996).

To prevent spoilage of pasteurized dairy products, *B. cereus* should be controlled by a chain management approach. It is important to reduce the *B. cereus* spore concentration in milk via measures at the farm level or by bactofugation and to prevent recontamination and growth during processing. Of these aspects, least is known about measures that are needed to achieve a significant reduction at the farm level and quantitative effects of potential control measures on the contamination of FTM.

Raw milk in the silo tank at the site of dairy processors is a collection of a relatively large number of farm tank milk **(FTM)** deliveries (up to 50 in the Netherlands). The concentration of *B. cereus* spores in the raw milk is the weighted average of the spore concentrations in the different FTM deliveries. The highest concentrations occur at the end of summer and in early autumn (Slaghuis et al., 1997, Te Giffel et al., 1995). A maximum allowed spore limited **(MSL)** of 3 $\log_{10}$ spores/L has been defined based on the required shelf life of pasteurized milk (Walstra et al., 2005).

Farm tank milk is contaminated with spores of *B. cereus* via the exterior of the cow's teats and via improperly cleaned milking equipment (Griffiths and Phillips, 1990, Saran, 1995). A further increase of the concentration could occur due to growth and sporulation of *B. cereus* during storage of the milk in the farm tank. Contamination via the exterior of teats occurs when teats are contaminated with dirt. During the grazing period, dirt attached to the teats mainly consists of soil. During housing attached dirt predominantly consists of feces and bedding material (Christiansson et al., 1999). Dirt attached to the exterior of teats rinses off during milking. Subsequently spores present in rinsed-off dirt contaminate the milk. The concentration of *B. cereus* spores transmitted to milk depends on the amount of dirt rinsed off the teats and the spore concentration in attached dirt.

Various measures, ranging from providing feed of high (microbial) quality to thorough cleaning of teats prior to milking, potentially reduce the concentration of *B. cereus* spores in FTM. For efficient farm management, it is important to identify the most effective measures to reduce the concentration of *B. cereus* spores in FTM. Based on the contamination pathway, Vissers et al. (2006; Chapter 2) developed a simulation model to identify a strategy to control the concentration of butyric acid bacteria spores in FTM. The model contained interpretable and measurable variables and accounted for effects of uncontrollable variables. A control strategy was defined using Monte-Carlo simulations.

In this study, a similar approach was applied to the contamination of FTM with *B. cereus* spores. The objectives of this study were to identify, quantitatively, factors that affect the concentration of *B. cereus* spores in FTM and to determine the reduction of the concentration that can be achieved via measures at the farm level. Based on this information a control strategy can be defined.

6.2 MATERIALS AND METHODS

6.2.1 Model development

Model assumptions

Based on the two contamination pathways (Figure 6.1), two simulation models were developed following the approach of Vissers et al. (2006; Chapter 2). In the first model, soil is the only source of *B. cereus* and teats are only contaminated with soil (the soil-based model). During the grazing period teats are predominantly contaminated with soil (Christiansson et al., 1999). Therefore the soil-based model most likely corresponds to the contamination process during the grazing period. In the second model feed is the only source and teats are solemnly contaminated with (feces and) bedding (feed-based model). The feed-based model reflects the housing period.

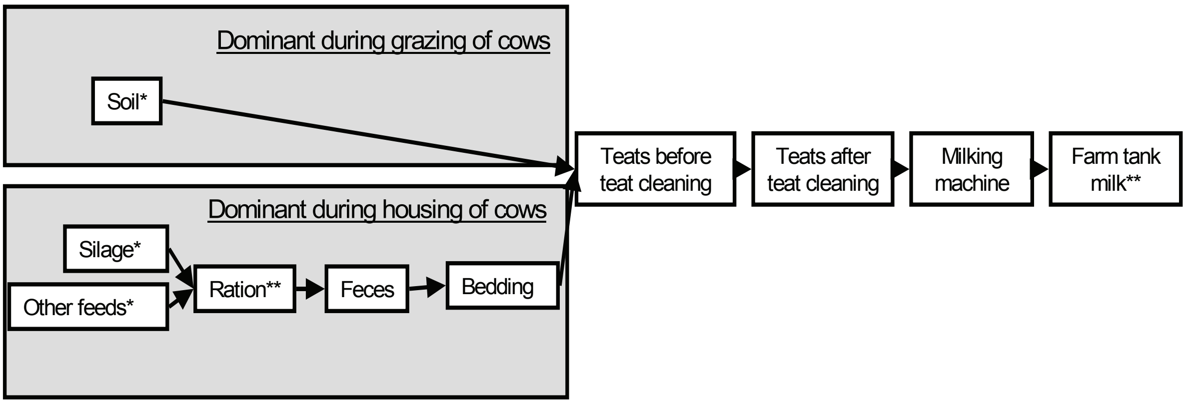

Figure 6.1 Contamination pathways for *Bacillus cereus* spores in farm tank milk (* source of contamination, ** growth can occur within this carrier)

The following assumptions apply to the models developed:

- Growth of *B. cereus* is possible in the mixed grass/corn silage provided to cows in the barn and in milk stored in the farm tank. Based on data of Te Giffel et al. (1995) and Slaghuis et al. (1997) no growth in bedding material was assumed;
- The spore concentration increases at the same rate as the vegetative cells when *B. cereus* can grow in the mixed silage and in FTM;
- Lag time is assumed to be equal to 1 divided by the growth rate (μ). This is a common assumption in predictive microbiology (Zwietering et al., 1996);

- The maximum attainable spore concentration in the feed ration ($C_{ration,\infty}$, Table 6.1) is assumed to be 5 $\log_{10}$ spores/g. This value is slightly above the maximum concentration of *B. cereus* spores measured in animal feed (Christiansson et al., 1999, Slaghuis et al., 1997, Te Giffel et al., 1995);
- The composition of the silage-based ration provided to cows is constant for the period of six milkings and is refreshed at constant intervals. Residence time in the gastro-intestinal tract can be neglected when the composition of the ration is constant;
- The cows are milked twice a day at a constant interval of 12 h;
- The milking equipment is cleaned properly and no contamination of the milk via the milking equipment occurs;
- The concentration of *B. cereus* spores in the milk entering the farm tank ($C_{milking}$ (spores/L)) is equal for all milkings collected in the farm tank;
- FTM is collected after six milkings. This is common practice in the Netherlands.

Model Structure

To have a measure of the hygiene status of pasture, cattle housing and milking parlor the herd is divided into three cow groups (slightly, moderately and highly contaminated cows). The proportions of slightly, moderately and highly contaminated cows represent the hygiene status. First, the concentration of *B. cereus* spores in the milk of each cow group ($C_{cowgroup\_y}$ *(spores/L)*) is calculated using the equations in Table 6.1. Then, the concentration of spores in the milk entering the farm tank each milking ($C_{milking}$ *(spores/L)*) is calculated as the weighted average of $C_{cowgroup}$ calculated for each cowgroup (based on the proportion of each cowgroup within the herd). The effect of growth of *B. cereus* in FTM is calculated using $C_{milking}$ and the equations in Table 6.2. The equations in Table 6.2 simulate the temperature of the FTM over time and the effects of this temperature profile and regular addition of $C_{milking}$ to the concentration of *B. cereus* spores in FTM (C_{FTM} *(spores/L)*).

Model Parameters and Variables.

The model distinguishes model parameters, controllable and uncontrollable variables. Variables are considered controllable when a farmer can directly influence the value of the variable via his/her management or when a farmer can measure the value of the variable and respond accordingly. Typical controllable variables are the temperature of the silage-based ration (T_{ration}*(°C)*) and teat cleaning method (TC_{mtd}). Uncontrollable variables relate to natural variability. A farmer cannot influence their value nor can he/she adjust his/her management based on the values of the variables. Uncontrollable variables determine the range of FTM concentrations occurring under a specific combination of controllable variables. A typical uncontrollable variable is the concentration of *B. cereus* spores in soil.

Table 6.1 Equations used to calculate the concentration of *Bacillus cereus* spores in the milk of each cow group ($C_{cowgroup\_y}$ in spores/L)

Intermediate concentration	**Equation**
Concentration in mixed feed components ($C_{ration}(0)$ in *spores/g*)[1]	$C_{ration}(0) = F_{silage} \cdot C_{silage} + (1 - F_{silage}) \cdot C_{otherfeed}$
Concentration in the mixed feed components at consumption, after growth ($C_{ration}(t)$ in *spores/g*)[2]	$\ln(C_{ration}(t)) = \ln(C_{ration}(0)) + \mu \cdot A_N(t) - \ln\left(1 + \frac{e^{\mu \cdot A_N(t)} \cdot t}{e^{\ln(C_\infty / C_{ration}(0))}}\right)$
	$A_n(t) = t + \frac{1}{\mu}\ln\left(\left(e^{-\mu \cdot t} + q_0\right)/\left(1 + q_0\right)\right)$ with $q_0 = 2/\left(e^{\mu \cdot \lambda} - 1\right)$
	$\mu = \gamma(T) \cdot \gamma(pH) \cdot \gamma(a_w) \cdot \mu_{opt}$ with $\gamma(T) = \left(\frac{T_{ration} - T_{\min}}{T_{opt} - T_{\min}}\right)^2$,
	$\gamma(pH) = \left[\frac{(pH_{ration} - pH_{\min}) \cdot (pH_{\max} - pH_{ration})}{(pH_{opt} - pH_{\min}) \cdot (pH_{\max} - pH_{opt})}\right]$ and $\gamma(a_w) = \frac{a_w - a_{w,\min}}{1 - a_{w,\min}}$
Concentration in feces (C_{feces}) and bedding ($C_{bedding}$ both in *spores/g*)[3]	$C_{bedding} = C_{faeces} = \left(1/\left(1 - F_{digested}\right)\right) \cdot C_{ration}$
Number attached to teats before teat cleaning (N_{before} in *spores*)[4]	$N_{before} = DM_y \cdot C_{soil}$ or $N_{before} = DM_y \cdot C_{bedding}$
Number attached to teats after teat cleaning (N_{after} in *spores*)[5]	$N_{after} = (1 - TC_{eff}) \cdot N_{before}$
Concentration in milk of each cow group per milking ($C_{cowgroup}$ in *spores/L*)[6]	$C_{cowgroup} = N_{after} / Y_{cow}$

[1] F_{Silage} (%) is the fraction of silage in the ration, C_{silage} and C_{other_feed} are the concentration of *Bacillus cereus* in fed silage and other feed;

[2] The Baranyi growth model (Baranyi and Roberts, 1994) is used to simulate growth in the ration. Lag time λ (in h) equals $1/\mu$ and t(h) is the time available for growth. Growth rate is estimated with the gamma concept of Zwietering et al. (1996);

[3] $F_{digested}$ (%) refers to the fraction of the feed ration that is digested;

[4] DM_y (g) is the mass of dirt attached to the teats that will dilute in the milk without teat cleaning of the teats of cow group y. During the grazing period soil with concentration C_{soil} (spores/g) attaches to the teats; during the housing bedding with concentration $C_{bedding}$ attaches to the teats;

[5] This equation is based on Chen et al. (2001). TC_{eff} (%) is the percentage of spores removed by the teat cleaning method applied;

[6] Y_{cow} is the average milk yield in L

Table 6.2 Equations used to calculate the concentration of *Bacillus cereus* spores in farm tank milk (FTM, C_{FTM} in *spores/L*)

Intermediate concentration	Equation
1. Maximum capacity of farm tank (I_T in m^3)[1]	$I_T = 6 \cdot Y_{cow} \cdot N_{herd} \cdot 110\%$
2. Temperature of farm tank milk (T_{FTM} in °C)[2]	$T_{FTM} = Q_{FTM} / (V_{FTM} \cdot \rho_m \cdot Cp_m) + 273$
3. Milk flow into the farm tank (ϕ_{FTM} in m^3/h)[3]	$\phi_{FTM} = Y_{cow} \cdot N_{herd} / t_{milking}$
4. Binary indicator if farmer is milking at time t (B_m)	If Milking B_m=1 else B_m=0
5. Binary indicator if cooling system of farm tank is switched on (B_c)[4]	If ($V_{FTM} / I_T \geq FTC_{start}$ and T_{FTM}>4 °C then B_c=1 else B_C=0
6. Iterative calculation of heat exchanging surface (A_{heat} in m^2)[4]	$\frac{V_{FTM}}{I_T} = \frac{1}{2\pi} \cdot \left(\frac{A_{heat} \cdot \pi \cdot D_T}{8 \cdot I_T} - \sin\left(\frac{A_{heat} \cdot \pi \cdot D_T}{8 \cdot I_T} \right) \right)$
7. Change of the energy of FTM over time (dQ_{FTM}/dt in J/h)[5]	$\frac{dQ_{FTM}}{dt} = B_m \cdot \phi_m \cdot \rho_m \cdot Cp_m \cdot (T_{milkline} + 273) - 3600 \cdot$ $(B_c \cdot k_c \cdot A_{heat} \cdot (T_{FTM} - T_{med}) - (1 - B_c) \cdot k_{env} \cdot A_{heat} \cdot (T_{FTM} - T_{env}))$
8. Change of the number of *B. cereus* in FTM over time (dN_{FTM}/dt in cfu/h)[6]	$dN_{FTM} / dt = \mu \cdot N_{FTM} + B_m \cdot \phi_{FTM} \cdot C_{milking}$
9. Change of milk volume in the FTM over time (dV_{FTM}/dt in m^3/h)[7]	$dV_{FTM} / dt = B_m \cdot \phi_{FTM}$
10. Concentration of *B. cereus* spores in FTM (C_{FTM} in *spores/L*)	$C_{FTM} = N_{FTM} / V_{FTM} \cdot 1{,}000$

[1] Y_{cow} (m^3) is the milk yield per cow per milking, N_{herd} is the number of lactating cows in the herd. Factor 110% is derived from ISO-5708 (ISO, 1983)

[2] Q_{FTM} is described with equation 7, V_{FTM} (m^3) is the volume of milk in the farm tank, ρ_m (kg/m^3) is the density and Cp_m ($J/kg/K$) is the heat capacity of milk

[3] Variables Y_{cow} and N_{herd} are explained under χ and $t_{milking}$ (*h*) is the duration of one milking

[4] FTC_{start} (%) is the filling degree of the farm tank at the moment the cooling system of the farm tank starts. Other variables explained under equations 1 and 2.

[5] D_T (m) is the diameter of the farm tank and obtained from www.meko.nl using the maximum capacity of the farm tank (I_T (m^3), equation 1)

[6] ρ_m and Cp_m ($J/kg/K$) are described under 2, $T_{milkline}$ (°C) is the temperature of the milk at the end of the milk line, k_c ($W/m^2/K$) expresses the cooling capacity of the farm tank, T_{med} (0 °C) is the temperature of the cooling medium, k_{env} ($W/m^2/K$) is the heat resistance of the isolation of the farm tank and T_{env} (°C) is the temperature in the milking parlor. All other variables are explained in equations 2, 4, 5 and 6.

[7] μ ($1/h$) is the growth rate of *B. cereus* at the applicable FTM temperature (T_{FTM} in °C) and is calculated with the equations described in Table 2 (point 2), t is time in s and $C_{milking}$ (*spores/L*) in the concentration of *B. cereus* in the milk entering the farm tank. All other variables are explained in equations 3 and 4.

Values for the model parameters were retrieved from published and unpublished data, from a survey involving 288 Dutch farmers or from consultation of experts in the Dutch dairy industry. In the survey, farmers were sent a short questionnaire (20 questions) to collect basic farm management data, such as the size of the herd and teat cleaning methods. The data used to retrieve characteristics of the ration (e.g. pH and water activity) consisted of 241 records with various characteristics of grass and corn-silages (Figure 6.2) and was collected by Blgg (Oosterbeek, the Netherlands). Blgg is a Dutch laboratory that determines characteristics (e.g. nutritional quality, composition and pH) of amongst others grass and corn silage.

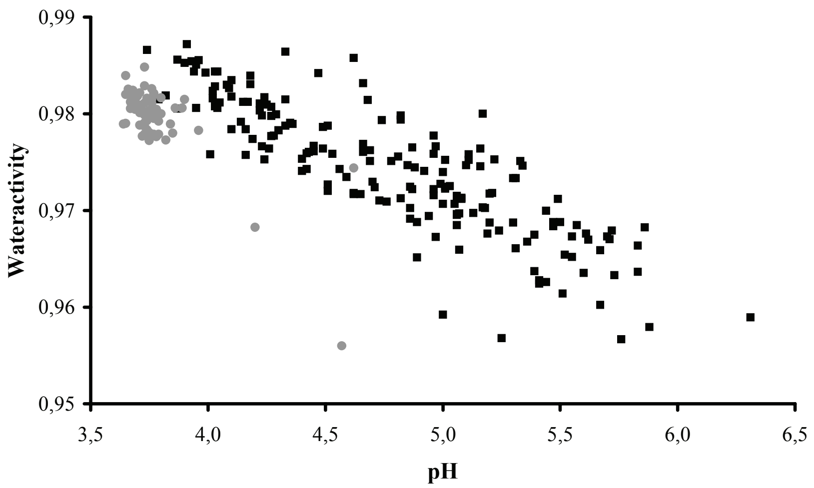

Figure 6.2 pH and water activity of grass (■) and corn (•) silages (Blgg, Oosterbeek, the Netherlands)

Table 6.3 lists the controllable variables and the worst, average and best values as encountered in the Netherlands. Worst and best refer to the direction of the effect of the variable on the *B. cereus* spore concentration in FTM, i.e. worst settings increase and best settings decrease the spore concentration in FTM. The average values correspond to the average values encountered in the Netherlands.For model simulations, values of uncontrollable variables were preferably randomly sampled from an available dataset in order to stay as close as possible to the actual variation occurring in practice. If no dataset was available PERT distributions were used. PERT distributions are defined by minimum, most likely and maximum values, values that can be obtained relative easily from literature or experts. If the difference between the minimum and most likely value and the difference between the most-likely and maximum value are equal, the shape of a PERT distribution is roughly similar to a normal distribution. Table 6.4 lists the uncontrollable variables and applicable datasets and statistical distributions. Table 6.5 lists the model parameters and their values.

Table 6.3 Controllable variables in the simulation models with worst, average and best values as encountered in the Netherlands. The terms worst and best relate to the effect on the concentration of *Bacillus cereus* spores in farm tank milk.

Notation	Description	Worst	Ave.	Best	Ref.
	Initial feed quality				
C_{silage_a}	Average concentration in silage ($\log_{10}$ spores/g)	4.5	2.0	0.0	a
$C_{other\,feed\_a}$	Average concentration in all other feed besides silage ($\log_{10}$ spores/g)	3.5	2.0	0.0	a
F_{silage}	Fraction silage in ration (%)	90	65	50	b
	Feed management				
$a_{w,ration}$	Water activity of silage-based ration[1]	0.99	0.98	0.95	c
pH_{ration}	pH silage-based ration[1]	6.5	4.6	<4.5	c
T_{ration}	Temperature of silage-based ration (°C)	37	20	10	d
$t_{refresh}$	Time between two ration refreshments (h)	120	24	6	b
	Hygiene				
P_{HCcows}	Proportion highly contaminated cows (%)	10.0	2.0	0.0	b
P_{MCcows}	Proportion moderately contaminated cows (%)	25.0	12.5	0.0	d
	Teat cleaning				
TC_{mtd}	Teat cleaning method (1=dry paper towel; 2=moist paper towel; 3= first with moist paper towel followed by dry paper towel)	1	1	3	b
TC_{strat}	Teat cleaning strategy (0=no teat cleaning; 1= only highly contaminated cows; 2=highly + moderately contaminated cows; 3=all cows)	0	3	3	b
	Milking equipment				
FTC_{start}	Filling degree of tank at start cooling (%)	15	15	4	b
k_c	Cooling capacity of farm tank (W/m^2/K)	250	400	850	b+e
$t_{milking}$	Duration of one milking (h)	2.0	1.5	1.0	b
T_{env}	Temperature in the milking parlor (°C)	25	15	5	f
$T_{milkline}$	Temperature milk at end of milk line (°C)	35	33	15	f

[1] pH and water activity of the ration are correlated variables (correlation factor= -0.83) (Figure 8.2)

a. Slaghuis et al. (1997);
b. Survey of 288 Dutch farmers;
c. Data Blgg (Oosterbeek, The Netherlands, 2003);
d. Expert Opinion (2006)
e. NEN-ISO 5708 (ISO, 1983)
f. Expert opinion

Table 6.4 Distributions applied to the uncontrollable variables in the simulation models. .

Notation	Variable	Distribution	Ref.
C_{soil}	Concentration in soil (spores/g)	Sampled from data *(min.=10, avg=*10^4 and *max.=*10^6 *spores/g)*	a+b
C_{silage}	Silage concentration taking into account heterogeneity (spores/g)	10^pert(C_{Silage_a}-0.5; C_{Silage_a}; C_{Silage_a}+0.5)[1]	a+c
$C_{other\ feed}$	Concentration in other feed with variation (spores/g)	10^pert($C_{other\_feed\_a}$-0.5; $C_{other\_feed\_a}$; $C_{other\_feed\_a}$+0.5)	a+c
DM_{SC_cow}	Dirt mass on uncleaned teats of slightly contaminated cows (g)	10^Pert(-2.5;-1.5;-0.5)	d
$DM_{MC\text{-}cow}$	Dirt mass on uncleaned teats of moderately contaminated cows	10^Pert(-0.75;0;0.75)	d
$DM_{HC\text{-}cow}$	Dirt mass on uncleaned teats of highly contaminated cows (g)	10^Pert(0.5;1;1.5)	d
$NV_{TC\text{-}eff}$	Efficiency of the three teat cleaning methods (% removed); for methods see Table 6.3.	Sampled from data	e
Y_{milk}	Milk yield per individual cow per milking (L)	Pert(0.010;0.013;0.015)	d

[1] Pert (minimum, most likely, maximum) specifies PERT distribution with minimum and maximum values as shown. The shape of the distribution is calculated from the most likely value. 10^Pert means 10 to the power pert.

a. Slaghuis et al (1997); b. Data NIZO food research, 2003; c. Expert opinion, d. Expert Opinion (Vissers et al., 2006); e. Magnusson et al. (2006)

Table 6.5 Model constants

Notation	Description	Value	Ref.
$a_{w,min}$	Minimal water activity required for growth	0.95	a
Cp_m	Heat capacity of milk (J/kg/K)	4180	b
C_∞	Log_{10} of maximum concentration of *B. cereus* spores in silage-based ration (cfu/g)	5	c
k_{env}	Heat resistance of isolation of the farm tank (W/m^2/K)	5	d
N_{herd}	Herd size (average in the Netherlands)	70	e
pH_{min}	Minimum pH required for growth	4.9	a
pH_{max}	Maximum pH allowing growth	8.1	a
pH_{opt}	Optimum pH for growth	6.5	a
T_{med}	Temperature of cooling medium in farm tank (°C)	0	d
T_{min}	Minimum growth temperature (°C)	0	a
T_{opt}	Optimum growth temperature (°C)	37	a
μ_{opt}	Growth rate under optimal conditions (h^{-1})	2.0	a
ρ_m	Density of milk (kg/m^3)	1030	b

a. Zwietering et al. (1996), b. Walstra et al. (2005) c. Slaghuis et al. (1997) d. NEN-ISO 5708 (ISO, 1983) e. Survey of 288 Dutch farmers

6.2.2 Model simulations

Both simulation models were programmed in Microsoft Excel (2002 version, Microsoft Corp. Redmond, WA). @Risk (2002 version, Palisade Corp, Newfield, NY) was used to perform Monte-Carlo simulations using Latin Hypercube sampling. Each simulation comprised 2,500 iterations.

Model validation

The simulation models predicts the concentration of *B. cereus* spores in FTM based on management information as inputs. Each calculation results in the prediction of a value for the concentration of *B. cereus* spores in FTM. However in published surveys, farm management information is minimal. Furthermore concentrations of *B. cereus* spores in FTM were often below the detection limit and results are generally reported as the fraction of samples with a concentration below the detection limit, e.g. 97% of the FTM during housing contained less than 2.3 $\log_{10}$ spores/L (Slaghuis et al., 1997). In other words no quantitative data are available. This complicated validation of the simulation models developed. Therefore, predicted cumulative probability distributions (**CPD**) were used to determine the validity of the models developed. The derived CPD's describe the probability (between 0% and 100%) that the concentration of *B. cereus* spores in FTM is below a concentration x. For example, when a concentration of 2 $\log_{10}$ spores/L corresponds to a predicted cumulative probability of 45%, the simulation model predicted in 45% of the cases a concentration below 2 $\log_{10}$ spores/L. In addition, the derived CPD's give an indication of the range of concentrations occurring in practice and can be used to determine spore concentrations raw milk stored at the dairy processor and in pasteurized milk.

For each model a CPD was determined using Monte-Carlo simulations. For each simulation, values for controllable variables were sampled from an available dataset (C_{silage_a}, $C_{other\_feed\_a}$, $a_{w,ration}$, pH_{ration}, $t_{refresh}$, P_{HCcows}, TC_{mtd} and TC_{strat}, FTC_{start} and $t_{milking}$, see Table 6.3) or from PERT distributions with the worst, average and optimal values of Table 6.3 as distribution parameters (F_{silage}, T_{ration}, P_{MCcows}, k_c, T_{env} and $T_{milkline}$). Rationale for using a dataset or PERT distribution was similar to the rationale for using a dataset or PERT distributions for the uncontrollable variables. Values for uncontrollable variables were sampled from the datasets and distributions listed in Table 6.4. The derived CPDs were compared with data of Te Giffel et al. (1995) and Slaghuis et al. (1997). In both these surveys FTM concentrations were measured at Dutch experimental farms for a period of 1 year.

Calculation of concentration in raw milk stored at the dairy processor

At the dairy processor, FTM of approximately 50 farms is mixed in a silo tank before further processing (Lankveld, 2002). The concentration in pasteurized milk depends on the concentration in raw milk in the silo tank. Therefore, the spore concentrations in raw milk stored in the silo tank at the dairy processor during the grazing period (using data soil-based model) and housing period (using data feed-based model) were simulated using data derived for model validations.

The concentrations in the silo tank were simulated using Monte Carlo simulations. It was assumed that milk of 50 farms is stored in one silo tank and that each farm produces the same volume of milk. For each iteration, 50 concentrations were randomly sampled from the data obtained in the validation study. The silo concentration is the average of the 50 sampled points. For both grazing and housing period, 2500 iterations were performed.

Sensitivity analysis

Worst- and best-case sensitivity analyses can be used to determine quantitatively the most important aspects affecting microbial contamination levels (Zwietering and van Gerwen, 2000). The impact of five major aspects of farm management on the concentrations of *B. cereus* spores in FTM was evaluated using worst- and best-case sensitivity analyses. The five aspects of farm management considered were: the initial feed quality, feed management (related to growth of *B. cereus* in the silage-based ration fed), hygiene, teat cleaning and milking equipment functioning. These aspects apply to different parts of the contamination pathways of Figure 6.1 and comprise different controllable variables. In Table 6.3 all controllable variables are grouped following the evaluated five aspects of farm management.

For each aspect the worst-case sensitivity for aspect x (WCS_x) was calculated with equation 6.1. In a similar way the best-case sensitivity was calculated. The worst- and best-case sensitivities were calculated for the 95% percentile value of 2,500 simulated concentrations in order to identify aspects of farm management that affect the highest concentrations occurring.

$$WCS_x = \log_{10}\left(\frac{C_x(worst)}{C(base)}\right) \qquad [6.1]$$

With:

C_x (worst)	95% percentile value of the FTM concentration when values of the controllable variables belonging to category x are fixed at their worst value, while all other controllable variables are fixed at their average value;
C (base)	95% percentile value of the FTM concentrations when the values of all controllable variables are fixed at their average value.

For the soil-based model worst- and best-case sensitivities were determined for the aspects hygiene, teat cleaning and milking equipment. In addition, the sensitivity for the uncontrollable concentration of *B. cereus* spores in soil was determined in order to evaluate the importance of soil as a source of *B. cereus* spores. The worst and best values used for the concentration of *B. cereus* spores in soil were 6 $\log_{10}$ spores/g and 1 $\log_{10}$ spores/g. For the feed-based model, worst- and best-case sensitivities were determined for all five aspects of farm management.

6.3 RESULTS

6.3.1 Model validation

To interpret model simulation results, it is important to determine the validity of the models. This was done by determination of CPDs of the concentration of *B. cereus* spores in FTM (Figure 6.3). Besides the CPDs derived with the soil- and feed-based model, survey data collected by Slaghuis et al. (1997) during the grazing and housing period are shown in Figure 6.3. Higher concentrations can be expected when teats are contaminated with soil (solid line in Figure 6.3) than when teats are contaminated with feces and bedding (dashed line in Figure 6.3). When soil is the source of *B. cereus* spores, simulated spore concentrations in FTM were in 50% of the iterations below 2.1 $\log_{10}$ spores/L and in 95% below 4.1 $\log_{10}$ spores/L. Of the predicted concentrations with the soil-based model 33% were above the MSL. When feed act as the source of spores and spores are transferred to milk via feces and bedding, 50% of the simulated spore concentration in FTM were below 1.3 $\log_{10}$ spores/L and 95% of the simulated concentrations below 2.7 $\log_{10}$ spores/L. Only 2% of the spore concentrations were above the MSL. This implies that when the milking equipment is cleaned properly, concentrations above the MSL are most likely due to the contamination of teats with soil.

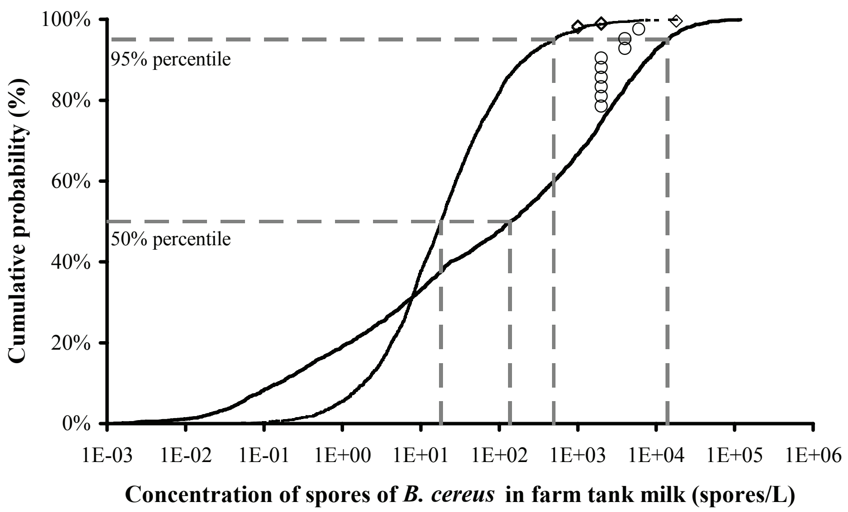

Figure 6.3 Simulated cumulative probability distributions of the concentration of *B. cereus* in FTM (data collected during housing (◊) and grazing (○) period (Slaghuis et al., 1997))

The CPD simulated with the soil-based model can be best compared with data acquired during grazing of cows, and the CPD derived with the feed-based model with data acquired when cows are housed. The soil-based model predicts higher concentrations of *B. cereus* spores in FTM than Slaghuis et al. (1997) measured during the grazing period (solid line vs circles). The CPD derived with the feed-based model is in agreement with the data Slaghuis et al. (1997) obtained at farms where cows were housed (dashed line vs diamonds). In the survey of Te Giffel (1995) 36% of the 250 mL bulk tank milk samples tested positive for spores of *B.*

cereus, but exact concentrations were not reported. The soil-based and feed-based model predicted higher concentrations. Taking into account the lack of management information presented in these surveys, the limited number of farms sampled and simplifications implicit in a model approach, it can be concluded that the model predictions are in line with concentrations observed in practice.

6.3.2 Concentrations at the dairy processor

The derived CPDs of the concentration of *B. cereus* spores in FTM were used to calculate concentrations of *B. cereus* spores in raw milk stored in collection tanks at the site of the dairy processors. This concentration is an important predictor for the spore concentration in pasteurized milk. The calculated average concentration of *B. cereus* spores in the silo tank at the dairy processor during the grazing period was 3.5 log_{10} spores/L. This implies that the concentration in raw milk during the grazing period is likely to be above the MSL.

The simulated average concentration for the housing period was 2.1 log_{10} spores/L and the concentration was below 3 log_{10} spores/L in 99% of the iterations. Thus, during the housing period, the defined criterion of the dairy industry is generally met.

The calculated average concentrations in raw milk stored at the dairy processors are higher than the median concentration of the FTM in both periods (2.1 log_{10} spores/L for the grazing period and 1.3 log_{10} spores/L for the housing period, Figure 6.3). This is due to the fact that generally less than 15% of the FTM deliveries stored in one silo tank determine the final concentration of *B. cereus* spores in raw milk processed. In other words a limited number of FTM deliveries (with the highest spore concentrations) determine the microbial quality of raw milk.

6.3.3 Sensitivity analysis

Sensitivity analyses were performed at the 95% percentile values of the simulated concentrations during the grazing and housing period. The 95% percentile value simulated with the soil-based model under average settings of the controllable variables (base case) was 3.0 log_{10} *B. cereus* spores/L. This implies that at an average farm during the grazing period the FTM is generally below the MSL.

During the grazing period, the spore concentration in FTM mostly depends on the (uncontrollable) concentration of *B. cereus* spores in soil (Figure 6.4A). Decreasing the contamination of teats with soil (improved hygiene) reduces the spore concentration in FTM by approximately 1 log_{10} spores/L compared to the base level; cleaning teats of all cows with a moist and a dry towel instead of cleaning with only a dry towel, results in a similar reduction. Faster or slower cooling of the milk affects growth of *B. cereus* in FTM only marginally and is not expected to affect the FTM spore concentration.

The predicted 95% of the FTM spore concentration during the housing of the cows and with average settings of the controllable variables was 2.1 log_{10} spores/L; 0.9 log_{10} spores/L lower than during the grazing period. The predicted concentration is most sensitive to the initial spore concentrations in the feed (i.e. initial feed quality) and to feed management (Figure 6.4B). Feed management relates to growth of *B. cereus* in the silage-based ration fed to cows.

At an average farm the pH of the ration (4.6) prevents an increase of the concentration of *B. cereus* in the ration (best-case sensitivity = 0 $\log_{10}$ spores/L). However, when the pH of the ration increases above 5.0 and when the periods between two feed ration refreshments are long (>24 h) *B. cereus* could grow and the spore concentration in the ration could increase as indicated by the worst-case sensitivity for feed management (approximately 3 $\log_{10}$ spores/L). Significant growth of *B. cereus* could occur when the pH of the ration is above 5.2, temperature is above 20 °C and feed is not refreshed daily.

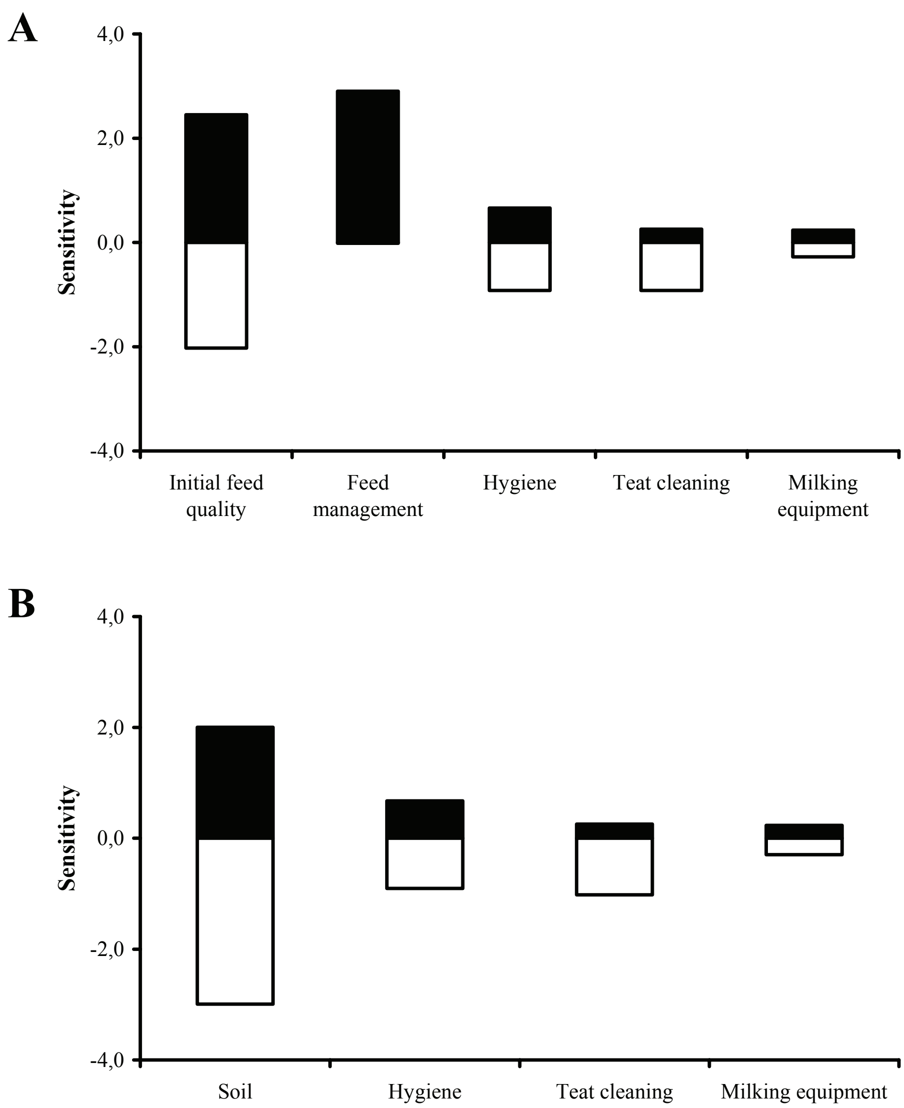

Figure 6.4 Worst- (black bar) and best- (white bar) case sensitivities of the 95% percentile of the concentration of *Bacillus cereus* spores in farm tank milk for different aspects of farm management plus the soil concentration for feed-based (A) and soil-based (B) model.

A comparison of Figures 6.4A and 6.4B showed that the concentrations in the sources of contamination (soil and feed) are most important for the final spore concentration in FTM. The most important difference is that the spore concentration in soil is uncontrollable, whereas a farmer can influence the concentration in the feed, for example by making good silage. This is problematic because, compared to the base level, a reduced concentration of *B. cereus* spores in soil (best-case sensitivity of approximately 4 log_{10} spores/L) affects the FTM concentration more than improvements of the initial feed quality (best-case sensitivity of approximately 2 log_{10} spores/L). In addition, a high initial quality of the feed could be completely negated if growth of *B. cereus* in the ration is possible. In contrast, bad hygiene and inefficient teat cleaning do not diminish the positive effect of a low concentration of *B. cereus* spores in soil.

6.3.4 Identification of control strategies

From Figure 6.3 it can be concluded that the highest concentrations of spores occur when teats are contaminated with soil and that 15% of the FTM deliveries with the highest concentration determine the concentration of *B. cereus* spores in the raw milk in the collection tank at the dairy processor. Figure 6.4A shows that during grazing, when teats are contaminated with soil, the highest concentration can be reduced by optimizing (pasture and milking parlor) hygiene and optimal teat cleaning practices (i.e. cleaning with moist and dry towels). The solid line in Figure 6.5 shows the CPD of the FTM concentration when all farmers implement measures that minimize the contamination of teats with soil (approximately 0.3 g dirt attached to the teats of all cows prior to teat cleaning) and apply the most efficient teat cleaning methods (average reduction of 95% of the spores; see Tables 6.3 and 6.4). After implementation of these measures at all farms during the grazing period, the concentration of *B. cereus* spores in FTM will nearly always be below 3 log_{10} spores/L (Figure 6.5). It can be calculated that after implementation of the suggested measures the expected concentration in raw milk stored at the dairy processor will be 1.5 log_{10} spores/L, a reduction of 99% compared to the base situation.

To obtain similar concentrations when teats are contaminated with feces and bedding, farmers should ensure that the feed initially contain less than 3 log_{10} spores/g and the pH of the ration offered to the cows is below 5. The dotted line in Figure 6.5 shows the predicted CPD when the requirements with respect to the initial feed quality and feed management are met. The predicted average concentration of *B. cereus* spores in raw milk stored at the site of the processors during the housing period is then 1.7 (σ=0.2) log_{10} spores/L, a reduction of 60% compared to the base situation.

6.4 DISCUSSION

Predictive models were developed to simulate the concentration of *B. cereus* spores in FTM. The aim was to evaluate different scenarios in order to determine the factors that have the most significant impact on the *B. cereus* spore concentration in FTM and to evaluate how FTM concentrations could be reduced. In light of these objectives, the models developed were useful. Firstly, the sensitivity analyses revealed the most important factors affecting the spore

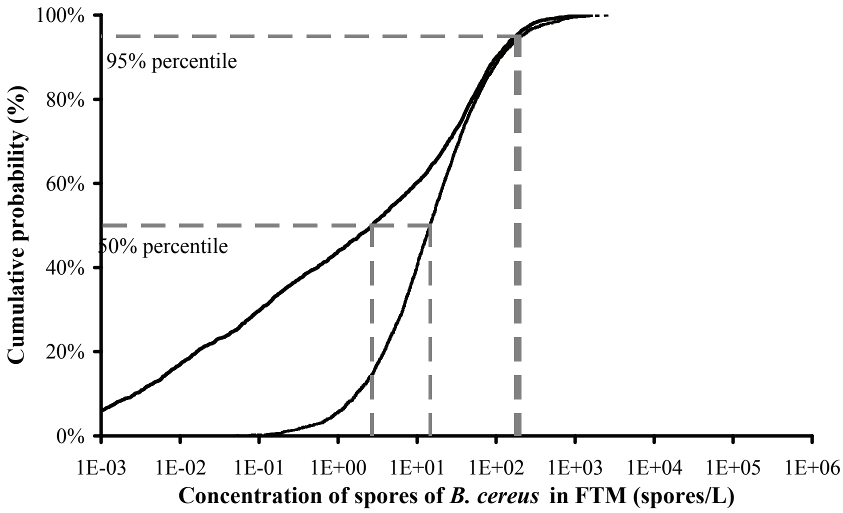

Figure 6.5 Simulated cumulative probability distributions of the concentration of *Bacillus cereus*

concentration in FTM (spore concentration in soil and feed, Figure 6.4). Secondly, strategies were identified that, compared to the base level, could reduce the spore concentration in raw milk stored at the dairy processor by 99% during the grazing period and by 60% during the housing period, respectively.

6.4.1 Control of the concentration during the grazing period

The simulations confirmed the conclusion of Christiansson et al. (1999) that during the grazing period the spore concentration in FTM is related to the contamination of teats with soil (Figure 6.3). Teats of grazing cows can also be contaminated with spores from feed (via feces) but maximum spore concentrations in soil are higher than maximum spore concentrations in feces. Consequently during grazing high *B. cereus* spore concentrations in FTM are most probably due to the contamination of teats with soil. With respect to control of the FTM concentration this is problematic because the spore concentration in soil is uncontrollable. Minimization of the contamination of teats with soil and optimization of teat cleaning efficiency at all farms in the Netherlands are necessary to achieve a 99% reduction of the spore concentration in raw milk and to assure concentrations below the MSL. It is important that all farms take the necessary measures because the concentration of *B. cereus* spores in processed milk is determined by less than 15% of the FTM deliveries. In practice, implementation of the necessary measures at all farms is likely to be difficult and an alternative strategy should be considered.

An alternative would be to house cows when the concentration of *B. cereus* spores in soil is too high (above 4 $\log_{10}$ spores/g). Concentrations above the MSL are rare and easier to achieve when cows are housed (Figures 6.3 and 6.5, (Slaghuis et al., 1997, Te Giffel et al., 1995). However, currently farmers do not know the concentration of *B. cereus* spores in soil. Farmers can obtain information concerning the spore concentration in soil via regular sampling of soil and analysis of the samples for the *B. cereus* spore concentration. Another option is to identify factors that are indicators of a high spore concentration in soil and to develop a "soil concentration predictor". For example, Christiansson et al. (1999) detected a negative correlation between FTM concentrations and soil dry matter content. Based on this observation it could be beneficial to house cows only during moist and rainy days.

Bactofugation of raw milk during the grazing period is another alternative to reduce the concentration of *B. cereus* spores in milk. Bactofugation removes approximately 98% of the spores (Te Giffel and Van der Horst, 2003), a similar reduction as can be achieved via measures at the farm level. An economic evaluation should be performed to establish the most cost-effective control option: minimizing teat contamination and optimization of teat cleaning at all farms, moving cows inside when the *B. cereus* spore concentration in soil is above 4 $\log_{10}$ spores/g or bactofugation of raw milk at the dairy processor. Costs of communicating strategies to farmers, welfare costs of housing (relative to remaining on pasture) and practical aspects such as the frequency of moving cows in and outside should also be included in the economic evaluation.

6.4.2 Control of the concentration during the housing period

If the milking equipment is cleaned properly, feed are the main source of spores during housing of cows (Figure 6.3). Currently during the housing period the concentration of spores of *B. cereus* in raw milk is almost always less than the MSL (Slaghuis et al., 1997, Te Giffel et al., 1995) and Figure 6.3). However, simulations show that higher concentrations can occur when the initial concentration in the feed is above 3 $\log_{10}$ spores/g and when the spore concentration in the ration can increase due to growth of *B. cereus* (Figure 6.4B). To keep concentrations low, most attention should be paid to ensiled feed because concentrations above 3 $\log_{10}$ spores/g are found more frequently in silage than in other feed. In addition, the pH of silage offered to the cows can increase to levels above 5 (at a pH below 5 growth of *B. cereus* is prevented). An increase of the pH occurs when silage is exposed to air and yeasts start to grow and consume the preservative acids (Pahlow et al., 2003). However, high concentrations are also possible in other feed, e.g. Christiansson (1999) measured in hay an average of 3.5 $\log_{10}$ spores/g with a maximum of 4.6 $\log_{10}$spores/g. In feed concentrates spore concentrations are almost always less than 3 $\log_{10}$spores/g and *B. cereus* spores in concentrates are generally mesophilic (Christiansson et al., 1999, Slaghuis et al., 1997, Te Giffel et al., 1995).

6.4.3 Accuracy of the models developed

The validation (Figure 6.3) and additional simulations showed that the models developed were accurate and reliable. The assumptions made for model development did not affect the conclusions that were drawn from the simulations. An important assumption was made with regard to the increase of the spore concentration in the ration and FTM due to growth of *B. cereus*. It was assumed that the spore concentration increases at the same rate as the concentration of vegetative cells. Since sporulation requires time this implies an overestimation of the increase of the spore concentration due to growth of *B. cereus*. However, no significant growth of *B. cereus* during storage of FTM was predicted. Consequently, the conclusion drawn with respect to the effect of improved cooling of the milk at the farm on the spore concentration in FTM, i.e. no positive effect on the concentration, is still valid. On the other hand, the feed-based model indicates that the spore concentration in the ration offered to the cows could increase significantly due to growth of *B. cereus* (see worst-case sensitivity bar for Feed Management in Figure 6.4B). It is possible that the concentration in the ration increases less than predicted but there are no data available to either support or reject the predicted increase. Therefore to be on the safe side, recommendations should be based on an overestimation of the effect of growth. Nevertheless, it is recommended to perform experiments to quantify a possible increase over time of the spore concentration in the ration offered to the cows.

With respect to the accuracy of the model it is also important that the variable that required expert opinion to estimate variable values (as no data were available) has only a minor effect on the predicted FTM spore concentrations. If these variables had a large impact on the simulation results, conclusions drawn from the simulation would have been largely affected by judgments of experts.

6.5 CONCLUSIONS

The concentration of *B. cereus* spores in raw milk stored in collection tanks at the dairy processors is determined by a limited number of FTM deliveries (15%) containing the highest concentration of spores. If milking installations are cleaned properly the highest concentrations occur during the grazing period when soil contains more than 4 $\log_{10}$ spores/g. A concentration of *B. cereus* spores in FTM below the MSL during the grazing period can be assured if the contamination of teats with soil is minimized and teat cleaning is optimized. Compared to the current situation in the Netherlands, implementation of these measures results in an approximately 99% reduction of the average concentration in raw milk during the grazing period. To assure a concentration in FTM below the MSL during the housing period, feed should initially contain less than the MSL and the pH of the ration should be below 5. At most Dutch farms these requirements with respect to the initial contamination of the feed and characteristics of ration are met.

CHAPTER 7

MONITORING THE CONTAMINATION OF FARM TANK MILK WITH *BACILLUS CEREUS* SPORES

In a year-long survey at 24 Dutch farms, *Bacillus cereus* spore concentrations were measured in farm tank milk (FTM), feces, bedding material, mixed grass/corn silage and soil from the pasture. The aim of this study was to determine, in practice, the most important factors affecting the concentration of *B. cereus* spores in FTM across the year. In addition, the results of the survey were used in combination with a previously published modeling study, to determine a strategy to ensure *B. cereus* spore concentration in FTM below the maximal acceptable spore level (MSL) of 3 $\log_{10}$ spores/L. The average *B. cereus* spore concentration in FTM was 1.2 $\log_{10}$ spores/L and in none of samples the concentration was above the MSL. The average spore concentration in soil (4.9 $\log_{10}$ spores/g) was more than a 100-fold higher than the average concentration in feces (2.2 $\log_{10}$ spores/g), bedding material (2.8 $\log_{10}$ spores/g) and mixed silage (2.4 $\log_{10}$ spores/g). Spore concentrations in FTM were increased between July and September compared to the rest of the year (0.5 $\log_{10}$ spores/L difference). In this period comparable increases of the concentrations in feces (0.4 $\log_{10}$ spores/g), bedding material (0.5 $\log_{10}$ spores/g) and mixed silage (0.4 $\log_{10}$ spores/g) were found. The increased concentration in FTM was not related to grazing of cows. No correlations or trends were observed between spore concentration in FTM and soil when cows were grazing during the daytime only or 24 h per day. On the other hand significant correlations were found between the spore concentrations in FTM and feces and between feces and mixed silage even when cows grazed. The increased concentrations during summer could be explained by an increased growth of *B. cereus* due to the higher temperatures. It was concluded that all year round *B. cereus* spores are predominantly transmitted from feed, via feces, to FTM. Therefore, farmers should take measures that minimize the transmission of spores via this route by ensuring low initial contamination levels in the feed (<3 $\log_{10}$ spores/g) and prevention of growth of *B. cereus* in the farm environment. In addition, because of the extremely high *B. cereus* spore concentrations occurring in soil, the contamination of teats with soil needs to be prevented.

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7.1 INTRODUCTION

Growth of the spore-forming *Bacillus cereus* often limits the shelf life of pasteurized dairy products kept at refrigerator temperatures. Dairy products spoiled by *B. cereus* show defects like off-flavors, sweet curdling and bitty cream (Overcast and Atmaram, 1971, Stone and Rowlands, 1952). *B. cereus* in dairy products originates from the farm environment and/or from recontamination during processing (Lin et al., 1998, Svensson et al., 2000, Te Giffel et al., 1996).

A chain management approach is important to prevent spoilage of pasteurized dairy products by *B. cereus*. Major control options comprise minimizing the *B. cereus* spore concentration in farm tank milk (**FTM**), removal of spores from raw milk via bactofugation or microfiltration and prevention of recontamination and growth during processing and storage. With respect to the shelf life of pasteurized dairy products, a maximum spore limit (**MSL**) for *B. cereus* of 3 log_{10} spores/L in FTM in necessary to achieve a shelf life for pasteurized milk of at least 7 days (Walstra, 2005). The highest concentrations are generally observed during summer and early autumn (Phillips and Griffiths, 1986, Sutherland and Murdoch, 1994, Te Giffel et al., 1995).

FTM can be contaminated with *B. cereus* spores originating from feed and soil via the exterior of teats or via surface of the milking equipment (Griffiths and Phillips, 1990, Saran, 1995, Slaghuis et al., 1997). After the initial contamination the concentration can increase further during (incorrect) storage of the milk at the farm due to growth. The soil-milk route is considered to be dominant during grazing of cows, the feed-milk route during housing of cows (Christiansson et al., 1999, Slaghuis et al., 1997).

Model simulations indicated that control of the *B. cereus* spore concentration in feed, below 3 log_{10} spores/g, and prevention of growth of *B. cereus* in the mixed silage offered to the cows in the barn are the most important measures to assure FTM concentrations below the MSL during housing of cows (Vissers et al., 2007b; Chapter 6). When cows graze and teats are contaminated with soil, the transmission of soil to FTM should be minimized in order to meet the criterion. Based on the model simulations, no increase of the *B. cereus* spore concentration during storage of FTM is expected when the farm tank meets regulation 5708 of the International Standard Office (ISO, 1983).

The aim of this study was to determine, in practice, the most important factors that affect the concentration of *B. cereus* spores in FTM via a year-long survey at 24 Dutch farms. In addition, the results of the survey were used in combination with a previously published modeling study, to determine a strategy to ensure *B. cereus* spore concentration in FTM below the MSL.

7.2 MATERIALS AND METHODS

7.2.1 Sample collection

Samples were taken at 24 farms spread across the Netherlands between January and December 2005. These farms were initially selected based on the contamination of FTM with butyric acid bacteria (**BAB**) spores in 2004. At 10 farms the Dutch requirements for BAB spores in FTM were met all year round in 2004, at 9 farms the BAB spore concentration was occasionally too high and 5 farms regularly produced FTM with too much BAB spores in 2004. However, no relation between the BAB spore concentration in FTM in 2004 and 2005 was found (Vissers et al., 2007c; Chapter 5). *B. cereus* spore concentration in FTM of these farms in 2004 was unknown.

Each farm was visited six times at a regular interval of eight weeks between January and December 2005. Each visit, samples were collected and a questionnaire was filled out to collect farm management data. Samples were collected by trained personnel, and each farm was visited by the same person on all occasions. The questionnaire contained questions relating to the ration fed to the cows, housing conditions and milking practices. The evaluated aspects of farm management were: barn type (loose vs tied housing of cows), feed, bedding material used (straw or sawdust), box-cleaning frequency (once a day vs less than once a day), presence of cows preferring to lie down on dirty patches, fore-stripping, teat cleaning method (dry vs moist towel) and frequency of an acid rinse of the milking equipment (once per week or more vs less than once per week). When cows grazed it was also noted whether the pasture was wet or dry.

The following sample types were collected: FTM (every visit), feces from the barn (when cows were housed half or all day), feces from the pasture (when cows were grazing all day), bedding material from the barn (when cows were housed half or all day), soil (when cows were at pasture half or all day) and mixed grass and corn silage fed to the cows in the barn (every visit when appropriate). All samples except the FTM sample were composite samples consisting of 10 mixed randomly collected sub samples. Samples were stored at 4-6 °C until microbial analysis within 24 h after sample collection.

7.2.2 Microbial analyses

Feces, bedding, soil, silage and feed extracts were prepared by adding 90 mL of peptone physiological salt solution (**PSS**) (1 g of neutralized bacteriological peptone and 8.5 g sodium chloride per liter) to 10 g of sample and homogenizing for 2 minutes in a laboratory blender (Stomacher, Seward Medical, London, UK). The concentration of *B. cereus* spores was determined according to the Dutch Standard 6875 (NEN ISO, 1994). FTM samples (100 mL) and extract (10 mL) were heated at 80 °C for 5 minutes. After cooling, pasteurized FTM samples were centrifuged for 30 min at 3,000 g. Serial dilutions of pellet and cooled pasteurized extracts in PSS were prepared. *B. cereus* spores were enumerated by surface plating on mannitol egg yolk polymyxin (**MEYP**) agar. MEYP plates were aerobically incubated at 30 °C for 24 h. Typical pink colonies surrounded by a zone of precipitation were counted. Pink colonies were isolated and confirmed as *B. cereus* by testing for anaerobic acid production from glucose. In addition, psychrotolerant and mesophilic strains were discriminated by PCR targeting 16S rDNA (Von Stetten et al., 1998) and cspA (Francis et al., 1998).

7.2.3 Statistical analyses

Statistical calculations were performed using Statistica© and Microsoft Excel. In order to perform statistical analyses, samples with a *B. cereus* spore concentration below the detection limit were assigned a concentration equaling half the detection limit. All statistical analyses were performed on $\log_{10}$ transformed data. The *Student t*-tests was used to detect significant differences between farms, environmental carriers and aspects of farm management. *Fisher-Exact*-tests were used to determine whether low or high concentrations occurred more frequently in a specific carrier or group. In addition, *Pearson* and *Spearman* correlation analyses were performed.

7.3 RESULTS

7.3.1 Concentration in environmental carriers

Figure 7.1 summarizes the *B. cereus* spore concentrations found in different environmental carriers. In total 142 feces, 114 bedding material, 117 mixed corn/grass silage and 59 soil samples were analyzed. Overall, bedding material contained most spores, with an average concentration of 2.8 $\log_{10}$ spores/g. Concentrations in feces (average of 2.2 $\log_{10}$ spores/g) and in mixed silage (average of 2.4 $\log_{10}$ spores/g) were significantly lower than in bedding material ($P<0.01$). In contrast to previous studies no accumulation of spores in feces was found (Te Giffel et al., 2002, Vissers et al., 2007c; Chapter 5); the average concentrations in feces and mixed silage did not significantly differ ($P=0.10$).

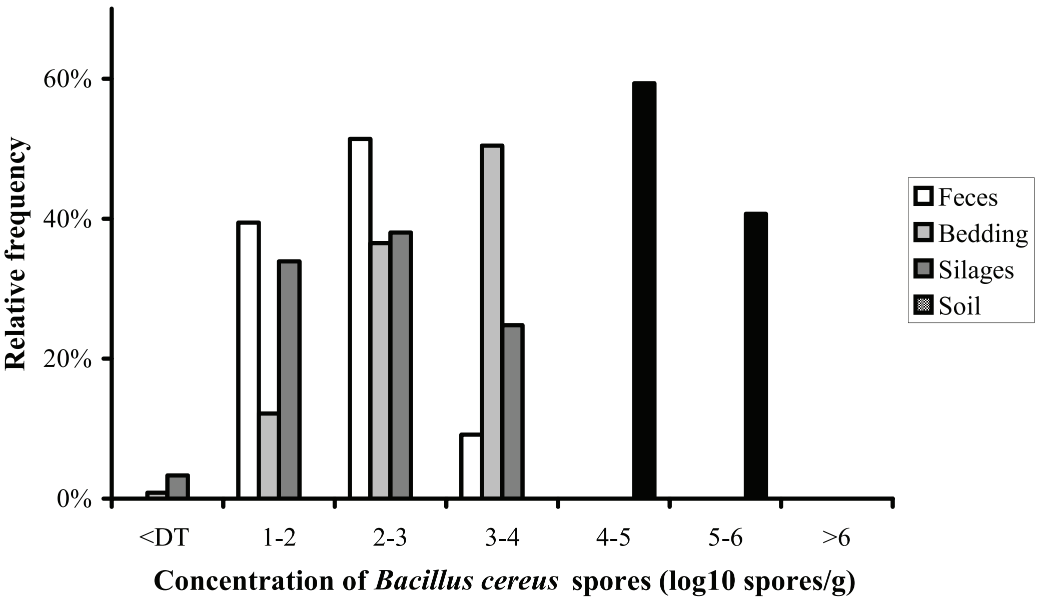

Figure 7.1 Distribution of concentrations of *Bacillus cereus* spores measured in feces, bedding material, mixed silage and soil. (DT = detection limit (1 $\log_{10}$ spores/g))

The average concentration in soil (4.9 $\log_{10}$ spores/g) was about 100 times higher than the concentration in the other environmental carriers. The concentration in soil also showed less variation, i.e. factor 100 vs. more than a factor 1,000 between minimum and maximum (Figure 7.1). Concentrations were independent of the type of soil (sand, clay or peat).

7.3.2 Concentrations in FTM

Figure 7.2 shows the distribution of the *B. cereus* spore concentration in FTM measured at the 24 farms during the survey and the predicted concentrations using model simulations assuming that all *B. cereus* spores in FTM originate from feed (Vissers et al., 2007b; Chapter 6). There was good agreement between measured and predicted concentrations. In the survey, none of the 140 FTM samples exceeded the MSL. The median concentration, 1.0 $\log_{10}$ spores/L, was a factor 100 below the MSL. The average concentration was 1.2 $\log_{10}$ spores/L.
Christiansson et al., (1999) found higher *B. cereus* spore concentrations in milk from individual cows when cows were grazing during wet weather. In our survey at 52 of the visits cows were grazing at least half of the day. At 8 of these 52 visits the pasture was moist due to rainfall. No differences between dry and moist conditions were found. The average *B. cereus* spore concentration measured in FTM on wet and dry days was 1.4 $\log_{10}$ spores/L (the median concentrations was under both conditions also equal; 1.3 $\log_{10}$ spores/L).
The field survey presented in this paper was also used to determine the contamination routes of BAB spores in FTM (Vissers et al., 2007c; Chapter 5). *B. cereus* and BAB spore concentrations in FTM were not correlated (P>0.2). Furthermore, the farms that produced FTM with the lowest average concentration of *B. cereus* spores were not the same farms that produced FTM with the lowest average BAB spore concentration.

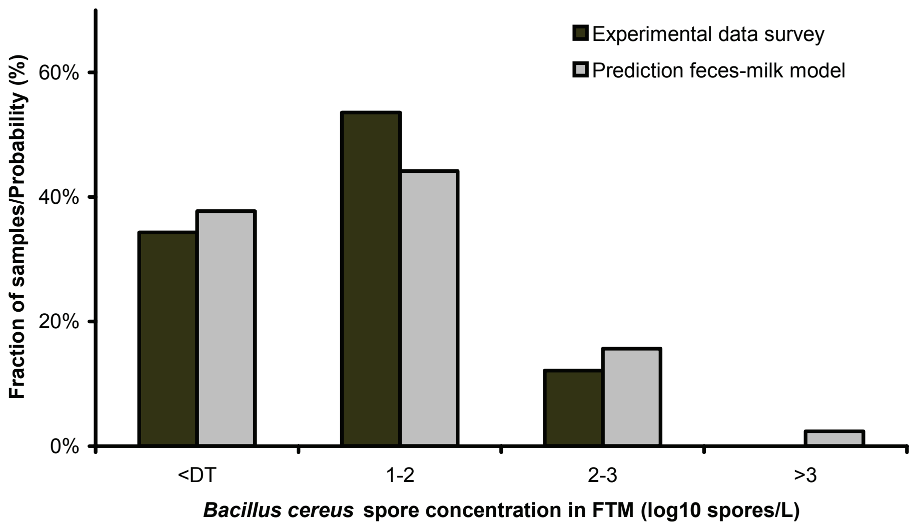

Figure 7.2 Distribution of the measured concentrations *Bacillus cereus* in farm tank milk (FTM) in the survey (Black bar) and concentrations predicted with a simulation model (grey bars, Vissers et al, 2007b; Chapter 6). DT=Detection limit (1 $\log_{10}$ spores/L)

7.3.3 Seasonal trends

During the summer months (July-September) the *B. cereus* spore concentrations in FTM were significantly higher than in the rest of the year (difference of 0.5 log_{10} spores/L; P<0,01). In the same period the spore concentrations in feces, bedding material and the mixed silage were increased too, by 0.4-0.5 log_{10} spores/g (P<0.01). In soil, this trend was not observed.

It has been postulated that increased *B. cereus* spore concentrations in FTM during the summer period are associated with grazing of cows and contamination of the cows' teats with soil (Christiansson et al., 1999, Slaghuis et al., 1997). This hypothesis was tested using data of from the survey. Data collected during the period that cows can be at pasture (May to October; grazing period) were divided into three groups according to the housing situations of the cows in that period: 1) cows were housed all day (27% of the visits), 2) cows were housed during the night and grazing during daytime (53% of the visits) and, 3) cows were grazing 24 h per day (20% of the visits). At five farms cows were kept inside during the entire grazing period and only at one farms cows were at pasture 24 h of the day during the entire grazing period. At the other 18 farms cows different housing situation occurred during the grazing period. A fourth group consisted of *B. cereus* spore concentrations collected in the period when at all farms cows were housed 24 h per day (November to April; housing period). The fourth group was distinguished to prevent a seasonal bias in the comparison of the different housing situations. Table 7.1 provides an overview of the *B. cereus* spore concentrations measured in FTM for the four groups. Table 7.2 summarizes the concentrations measured in the environmental carriers.

No grazing effect was observed. The spore concentration measured in FTM at the three housing situations during the grazing period were not statistically different (Table 7.1). The only significant differences detected in FTM were significantly lower spore concentrations during the housing period compared to some housing situations during the grazing period. However, this is likely related to the observed seasonal trend of the spore concentration. The *B. cereus* spore concentrations measured in feces, bedding material and mixed silage were also similar for the three housing situations during the grazing period (Table 7.2). Similarly as in FTM, the lowest concentrations were observed during the housing period.

The observed seasonal trend and data summarized in Tables 7.1 and 7.2 suggest that the *B. cereus* spore concentration in FTM is related to the spore concentration in feces and mixed silage. Table 7.3 shows the correlation coefficients between different carriers for the situation in which cows were most likely not in contact with soil (housed all day in the barn) and the situation in which cows were in contact with soil (grazing during day-time or grazing 24 h per day). In both situations significant correlations (P<0.001) between the spore concentrations in mixed silage and feces and between feces and FTM were found. In contrast, no correlation between spore concentrations in soil and FTM was observed.

Table 7.1 *Bacillus cereus* spore concentrations in FTM (log_{10} spores/L) at different housing situations (housing period: November to April; grazing period: May to October)

Housing situation	**Average[1]**	**Median**	**Range**	**Fraction of samples (%) with a concentration[2]**	
				<1 log_{10} spores/L	> 2 log_{10} spores/L
Barn all day (housing period) (N=66)	1.0[a]	1.0	<1.0-2.2	47[b]	3[a]
Barn 24 h per day (grazing period) (N=19)	1.3[a,b]	1.3	<1.0-2.4	37[a,b]	21[b]
At pasture during daytime (grazing period) (N=38)	1.5[b]	1.3	<1.0-2.9	13[a]	26[b]
At pasture 24 h per day (grazing period) (N=14)	1.3[a,b]	1.4	<1.0-2.3	21[a,b]	17[a,b]

1 *Student t*-test used to detect significant differences; Different superscript (a,b) indicate significant differences (P<0.05)

2 *Fisher-exact* test used to detect significant differences.

Table 7.2 Effects *Bacillus cereus* spore concentration in environmental carriers (log_{10} spores/g) in different housing situations (housing period: November to April; grazing period: May to October)

Housing situation	**Feces**		**Bedding material**		**Mixed silage**		**Soil**	
	Average	Range	Average	Range	Average	Range	Average	Range
Barn all day (housing period) (N=66)	2.0[a]	1.0-3.3	2.7[a]	1.0-4.0	2.2[a]	<1.0-4.0	No soil samples collected	
Barn 24 h per day (grazing period) (N=19)	2.2[a,b]	1.2-3.5	2.9[a,b]	<1.0-3.7	2.8[b]	1.5-3.7		
At pasture during daytime (grazing period) (N=38)	2.5[b]	1.4-3.7	3.1[b]	2.1-4.0	2.6[b]	<1.0-4.0	4.9[a]	4.1-6.0
At pasture 24 h per day (grazing period) (N=14)	2.5[b]	1.7-3.8	No bedding samples collected		2.4[a,b]	<1.0-3.9	5.0[a]	4.6-5.0

[1] *Student t*-test used to detect significant differences; Different superscript (a,b) indicate significant differences (P<0.05)

Table 7.3 Significant Pearson correlation coefficients between different carriers

	All data	**Cows in barn 24 h per day**	**Cows grazing during the daytime or 24 h per day**
Milk vs Feces	0.51[1]	0.45[1]	0.44[1]
Milk vs Bedding Material	0.26[2]		
Milk vs Mixed Silage	0.20[3]		
Milk vs Soil		No significant correlation found	
Feces vs Bedding Material	0.37[1]	0.33[2]	
Feces vs Mixed Silage	0.43[1]	0.38[1]	0.50[1]
Bedding Material vs mixed Silage	0.29[2]		0.45[2]

[1] P<0,001; [2] P<0,01 ; [3] P<0,05

7.3.4 Effects of farm management.

Significant differences in the average *B. cereus* spore concentration in FTM between farms were observed. Again, this appeared to be related to *B. cereus* spore concentrations in feces, bedding material and mixed silage. The average FTM concentration at the six farms with the lowest *B. cereus* spores concentrations was 0.5 $\log_{10}$ spores/L (P<0.01) lower than at the six farms with the highest concentrations. Housing situations during the grazing period were similar for the farms with the highest and lowest average spore concentration. At the six farms with the lowest average concentration in FTM, the concentrations in feces (0.3 $\log_{10}$ spore/g difference; P=0.03), bedding material (0.4 $\log_{10}$ spores/g difference; P=0.03) and mixed silage (0.3 $\log_{10}$ spores/g difference; P=0.04) were also significantly lower. The average concentrations in the environmental carriers at the other 12 farms were in between the average concentrations measured at the six farms with the lowest and the six farms with the highest average *B. cereus* spore concentration in FTM.

At each visit different aspects of farm management were evaluated via a questionnaire. These comprised: barn type (loose vs tied housing of cows), bedding material used (straw or sawdust), box-cleaning frequency (once a day vs less than once per day), presence of cows preferring to lie down on dirty patches, fore-stripping, teat cleaning method (dry vs. moist towel) and frequency of an acid rinse of the milking equipment (once per week or more vs. less than once per week). However, for none of the evaluated aspects a significant effect on the *B. cereus* spore concentration in FTM was demonstrated.

7.4 DISCUSSION

7.4.1 Dominant contamination pathway

The *B. cereus* spore concentrations measured in FTM, feces, bedding material and mixed silage at the 24 farms were comparable with previous reported results (Christiansson et al., 1999, Slaghuis et al., 1997, Te Giffel et al., 1995). The concentrations measured in soil (4-6 $\log_{10}$ spores/g) correspond with concentrations measured in an unpublished survey held in 2002-2003. However compared to the investigations of Te Giffel et al. (1995) and Slaghuis et al. (1997) the range of spore concentrations measured was much more limited. They measured concentrations between 1 and 6 $\log_{10}$ spores/g, using the same method for enumeration. With the information available in the publications, it is not possible to identify the cause of the observed difference.

In contrast to Slaghuis et al. (1997) and Christiansson et al. (1999) no grazing effect due to the contamination of teats with soil was observed (Tables 7.1 and 7.2). Furthermore no effect of wet weather on the concentration in FTM was shown. This could be due to the limited data collected under wet conditions. Our data indicate that all year round the *B. cereus* spore concentrations measured in FTM mostly depends on the spore concentrations in mixed silage offered to the cows and the transmission of feces to FTM. Firstly, similar seasonal trends for FTM, feces and mixed silage were observed (e.g. Tables 7.1 and 7.2). Secondly, farms with the lowest average spore concentration in FTM also fed silages with the lowest concentration. And thirdly, significant correlations between FTM and feces and between feces and mixed silage were found even when cows were grazing during daytime or 24 h per day (Table 7.3). The correlation coefficients were relatively low. Presumably due to disturbances such as differences in the fecal contamination of the exterior of teats, passage time of feed through the gastrointestinal tract and occurrence of *B. cereus* spores in feed other than silage (e.g. concentrates, brewer's grain and beet pulp). On the other hand, no correlations or trends were observed between spore concentrations in FTM and soil, even when cows grazed all day. In addition, the correspondence between the *B. cereus* spore concentrations measured in FTM in the survey and *B. cereus* spore concentrations predicted by a simulation model underline that in the survey spores in FTM primarily originated from feed (Figure 7.2). For the model predictions was assumed that all *B. cereus* spores in FTM originate from feed and are transmitted to FTM via feces. If a significant amount of spores was transmitted from soil to FTM deviations between the measured and predicted distribution of *B. cereus* spore concentration in FTM should have been found (Vissers et al., 2007b; Chapter 6).

Assuming that the transmission of *B. cereus* from feed, via feces, to FTM is the dominant route all year round, the increased spore concentration detected during the summer months suggests that at some stage or stages in the contamination pathway growth of *B. cereus* occurred, for instance in mixed silage and bedding material. During the summer temperature is higher and as a result microbial growth rates are most likely higher than during the winter. Consequently, *B. cereus* spore concentrations in the silage or bedding material may increase, resulting in higher spore concentrations in FTM. Additionally, during grazing the spore concentration in feces can be increased due to the digestion of soil by the cows during grazing (Christansson et al., 1999).

Although this study shows that transmission of *B. cereus* spores to FTM via feces is the dominant pathway, it does not imply that the transmission of *B. c*ereus spores via soil is irrelevant. Table 7.4 shows how much feces and soil, based on the observed median and maximum concentration in our survey, has to be transmitted to 1 L of FTM in order to obtain a concentration in FTM of 1 log_{10} spores/L (observed median concentration) and 3 log_{10} spores/L (MSL). Table 7.3 demonstrates that transmission of small amounts of soil, 1 to 13 mg/L, already affects the microbial quality of FTM signficangtly. In an earlier study it was concluded that at least 2 mg/L (average of approximately 40 mg/L) is transmitted to milk via the exterior of teats during housing of cows (Stadhouders and Jørgensen, 1990). Nevertheless, the survey results obtained indicate that generally less than 1 mg of soil per L of FTM is transmitted. If more than 1 mg of soil was regularly transmitted to 1 L of FTM concentrations above the MSL should have been measured occasionally. These data indicate that in situation where the risk of contamination of teats with soil is high care should be taken to keep teats clean. Moist weather and dirty walking paths have been associated with the contamination of teats with soil and increased concentration in FTM (Christiansson et al., 1999). However, they also did not measure concentrations in milk above the MSL.

From the results of this survey it is concluded that all year round, during housing and grazing, *B. cereus* spores from feed, via feces, are transmitted to FTM, but that concentrations in FTM above the MSL are more likely due to the transmission of spores from soil to FTM. This implies that although the feed-feces-FTM pathway is generally dominant, control of the soil-FTM pathway is most important for the dairy industry.

Table 7.3 Amount of feces respectively soil that has to be transmitted to FTM to give a FTM *B. cereus* spore concentration of 1 and 3 log_{10} spores/L.

			FTM concentration	
			1 log_{10} spores/L	3 log_{10} spores/L (MSL)
Concentration in Feces	Observed median	2.1 log_{10} cfu/g	71 mg/L	7100 mg/L
	Observed max.	3.8 log_{10} cfu/g	2 mg/L	170 mg/L
Concentration in soil	Observed median	4.9 log_{10} cfu/g	0.1 mg/L	13 mg/L
	Observed max.	6.0 log_{10} cfu/g	0.01 mg/L	1 mg/L

7.4.2 Contamination via the milking equipment

Contamination via the milking equipment was not addressed specifically in this survey but the surfaces of the milking equipment have been mentioned as an additional source of *B. cereus* spore in FTM (Griffiths and Phillips, 1990). *B. cereus* remaining in the equipment can grow in the period between two milkings, and during milking spores can be transmitted to the milk (Vissers and Driehuis, 2007). However, in measurements at a limited number of farms build up of *B. cereus* in the milking equipment was not experimentally established (Christiansson et al., 1999, Te Giffel et al., 1995).

To complete the picture with respect to the contamination of FTM with *B. cereus* spores, some basic calculations are performed to quantitatively assess the possible relevance of this route. Milking equipment is made of similar material as processing equipment. On surfaces of equipment that has not been properly cleaned equipment up to 4 $\log_{10}$ *B. cereus* per cm^2 have been found (Faille et al., 2001). In milking equipment in the period between two milkings, the number of adhered spores may increase further due to growth and sporulation of *B. cereus* if residues are left. At an average Dutch farm with a farm tank of 6000 L the transmission of 6 $\log_{10}$ *B. cereus* spores per milking will give rise to 3 $\log_{10}$ spores/L of FTM. So if about 10-100 cm^2 of the surface of the milking equipment is covered with *B. cereus* (spores), recontamination of FTM can result in spore concentrations around the MSL. This is not an unlikely scenario. Consequently in practice, recontamination of milk via the milking equipment in practice should not be underestimated, although it presumably a rare incident.

7.4.3 Control strategy

A major objective of the study was to develop a strategy to ensure a *B. cereus* spore concentration in FTM below the MSL. To meet this objective, it is important to control the dominant contamination pathway (feed-feces-FTM). Model simulations have shown that this can be achieved by maintaining an initial contamination level of all feed, including silages, below 3 $\log_{10}$ spores/g and prevention of growth in the mixed silage (Vissers et al., 2007b). Options to prevent of limit the possibility of growth are a pH of the mixed silage (and bedding material) below 5 and relatively high frequency of refreshing mixed silage offered to the cows in the barn and bedding material (at least once a day). These requirements are generally met and consequently *B. cereus* spore concentrations in FTM are usually below the MSL.

Nevertheless spore concentrations above the MSL are possible. Firstly when substantial amounts of soil (>1 mg/L) are transmitted to FTM (Figure 7.1). Although in our study no effect of wet conditions was observed, presumably under wet conditions concentration above the MSL are possible (Christiansson et al., 1999). A second theoretically possible cause of concentration in FTM above the MSL could be contamination of FTM via surfaces of the milking equipment (see 7.4.2). However, the relevance of the last route has never been confirmed. The frequency and thus relevance of the transmission of substantial amounts of spores from soil to FTM and of contamination via surfaces of the milking equipment could not be established from the study presented in this paper or previous papers (e.g. Christiansson et al., 1999, Slaghuis et al., 1997, Te Giffel et al., 1995) This is due to the limited data collected under specific conditions or the limited number of farms that took part in the surveys. Therefore, it is essential to perform targeted measurements on a larger group of farms with observed elevated *B. cereus* spore concentrations in FTM. Such a survey should also focus on the identification of possible control measures, especially during wet weather.

With the current knowledge it is advisable to implement measures at the farm that minimize the risk of transmission of soil or build-up of *B. cereus* in the milking equipment.

7.5 CONCLUSIONS

This study showed that during housing and grazing *B. cereus* spore in FTM most likely originate from feed and are transmitted to FTM via feces and the exterior of teats. However, in the survey none of the FTM samples contained a *B. cereus* spore concentration above the MSL (3 $\log_{10}$ spores/L), indicating that transmission of *B. cereus* spores via this route seems under control under Dutch circumstances. Nevertheless, it is highly important to prevent the contamination of teats with soil during grazing because *B. cereus* spore concentrations in soil are roughly 100-fold higher than in other environmental carriers (feed, feces and bedding material). This implies that the transmission of relatively small amounts of soil, 1 to 13 mg/L, can already lead to a concentration in FTM exceeding the MSL.

CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

The concentration of microorganisms and bacterial spores in farm tank milk (**FTM**) depends on the introduction of the species into the farm environment, transmission from this source of contamination to FTM and the growth, microbial inactivation, or removal along the pathway from to FTM (Figure 8.1). Farm management and uncontrollable factors affect these aspects and determine the final concentration occurring in FTM. Good farm management comprises measures that effectively reduce the contamination of FTM with microorganisms (Chapter 1).

The objective of the work described in this thesis was to quantify the effectiveness of measures to control the contamination of FTM with spores of butyric acid bacteria (**BAB**) and *Bacillus cereus*. Based on common practice, available data, expert knowledge and existing predictive models, simulation models describing the contamination pathway at the farm were developed for spores of BAB (Chapter 2) and *B. cereus* (Chapter 6). The results derived using model simulations were in agreement with results obtained a year-long field survey at 24 Dutch farms (Chapters 5 and 7). Based on model development two more detailed studies into specific parts of the contamination pathway were performed (Chapters 3 and 4).

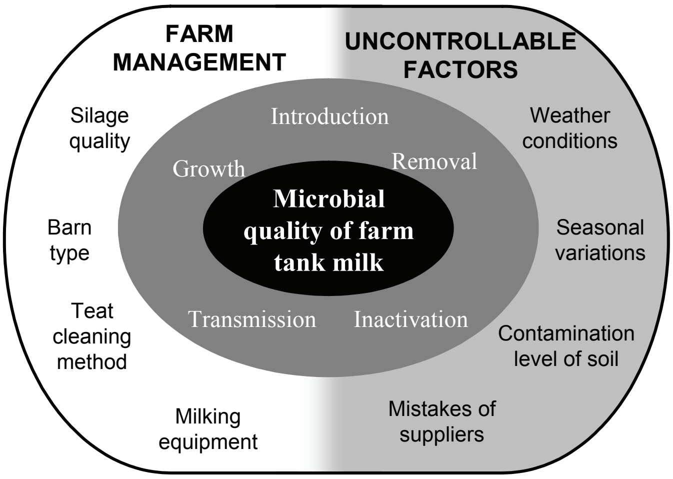

Figure 8.1 Control of microbial quality of farm tank milk (mentioned aspects of farm management and uncontrollable factors are examples and not all inclusive).

Spores of BAB and *B. cereus* are introduced into the farm environment via the same sources: soil and feed. Silage was identified as a highly important source of contamination for both types of bacteria (Chapters 2 and 6). Silage is contaminated via inclusion of soil during ensiling (Pahlow et al., 2003). The results of the field survey (Chapters 5 and 7) showed that the dominant transmission route (or contamination pathway) for both types of bacterial spores is similar: spores from grass and corn silage are transmitted to FTM via mixed silage offered to cows in the barn, feces, bedding material (partly), and the exterior of the cow's teats. However, despite the same sources of contamination and the same transmission paths, BAB and *B. cereus* spore concentrations in FTM measured in the survey were not correlated (correlation coefficient of 0.10; P>0.2). The lack of correlation between spore concentrations in FTM is related to the differences in behavior of BAB and *B. cereus* along the contamination pathway (schematically shown in Figure 8.2). The concentration of BAB spores can increase significantly (up to about 1,000 times) due to growth in silage of poor quality, whereas no growth in the other carriers occurs (e.g. Chapters 2 and 3). In contrast, *B. cereus* seems to be able to grow in different carriers (silage in silo, mixed silage, bedding material, and FTM (in case of incorrect cooling)), but only to a limited extent. As a consequence, the control strategies identified for BAB and *B. cereus* spores have the same basis, control of the spore concentration in mixed silage and feed. A difference is that, BAB spore concentration in FTM can be most effectively reduced by improving silage preservation. The *B. cereus* spore concentration in mixed silage and feed is generally under control. Another difference is that to control *B. cereus* spore concentrations in FTM below the maximum allowed spore limit (**MSL**) it is important to prevent the transmission of soil to FTM (Chapters 6 and 7), whereas this is not relevant for control of the BAB spore concentration in FTM below the MSL (Chapter 2).

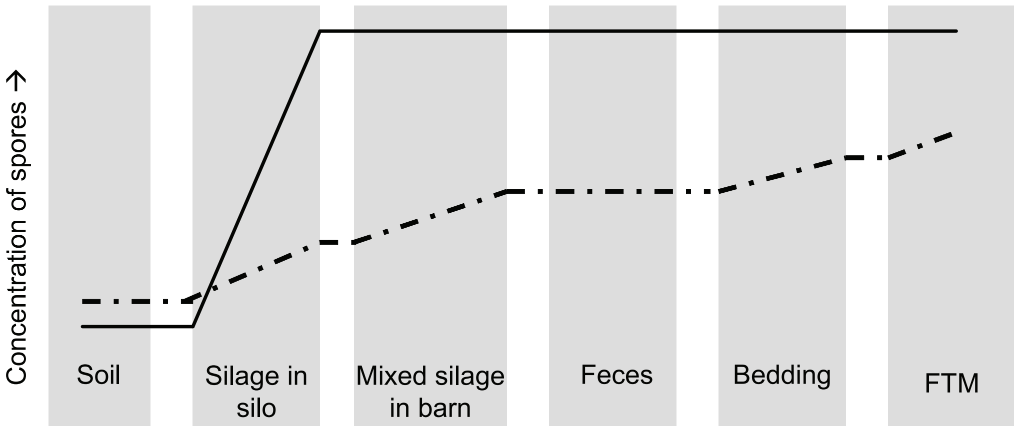

Figure 8.2 Passage of BAB (solid line) and *B. cereus* (dashed line) spores through the farm environment; increasing lines indicate the possibility of growth within the specific carrier.

8.2 CONTROL OF BAB SPORE CONCENTRATION IN FTM

8.2.1 Control strategy

At the start of the project representatives from the dairy industry defined a MSL for BAB spores in FTM of 3 $\log_{10}$ spores/L (Chapter 2). It turned out that to control the BAB spore concentration in FTM below the MSL, the most important measure is to minimize the BAB spore concentration in mixed grass/corn silage fed in the barn (Chapters 2 and 5). Measures to reduce the contamination of teats with dirt material and teat cleaning prior to milking are advisable, but not effectively. For example the difference between good and bad quality silage can be a factor 10,000 whereas teat cleaning only reduces the transmission of spores to FTM a factor 2 to 10 (Chapter 2, Chapter 5, Vissers and Driehuis (2007) and references therein).

Traditionally, control of BAB spore concentration in FTM was mainly focused on prevention of growth of BAB in grass silage. Growth of BAB in grass silage is associated with anaerobically unstable silage (Pahlow et al., 2003). However, Chapter 3 clearly demonstrated that, nowadays, growth of BAB is also associated with aerobically unstable silage, in particular corn silage. In corn silage high concentrations of BAB spores (more than 5 $\log_{10}$ spores/g) were found in local pockets, so-called hot spots, mostly present in the top layers of the silage. The occurrence of hot spots is associated with the penetration of oxygen into the silage followed by aerobic deterioration by yeasts and molds. Therefore concluded that to achieve the desired BAB spore contamination levels in mixed silage (below 3 $\log_{10}$ spores/g) farmers should take measures to limit the penetration of oxygen into silage and prevent growth of yeasts and molds. An aspect that requires further investigation is whether aerobic deterioration in the surface layer, and the formation of hot-spots, mainly occurs before or after the silage silo is opened. Obviously, it remains important to achieve and maintain anaerobically stable silages after ensiling (Pahlow et al., 2003).

Table 8.1 summarizes measures to control the BAB spore concentrations in mixed silage that were identified in this thesis (Chapters 2, 3 and 5) and in previous studies. It is possible to implement the measures listed in Table 8.1 without affecting daily farm practice too much. The survey results showed that it is possible to feed mixed silage with less than 3 $\log_{10}$ spores/g. Thus in general, it should be feasible to achieve BAB spore concentrations in FTM below the MSL.

8.2.2 Impact of measures at the farm on risk of late-blowing of Gouda-type cheese

The benefit for the dairy industry of a reduction of the BAB spore concentration in FTM is a reduced risk of late-blowing of Gouda-type cheeses. The risk of late-blowing depends on the BAB spore concentration in pasteurized cheese milk and the concentration of preservatives added to the pasteurized cheese milk inhibiting growth of BAB. Examples of inhibitors of growth are nitrate and lysozyme (Lodi, 1990, Stadhouders, 1990).

Figure 8.3 shows a standardized process of cheese manufacturing from arrival of FTM at a cheese manufacturer to pasteurized cheese milk. FTM of about 50 farmers is collected in one silo tank (Lankveld, 2002). After a short storage period, the raw milk from the silo tank is pasteurized. In the Netherlands, generally one bactofugation step is incorporated in the process. Bactofugation removes between 75% and 99% of the spores (Waes and Van Heddeghem, 1990). Scenarios to reduce the BAB spore concentration in pasteurized cheese milk are:

1. decreasing the BAB spore concentration in raw milk via measures at the farm level (subject of this thesis),
2. implementation of an additional bactofugation step in series, and
3. a combination of both measures.

Table 8.1 Measures needed to achieve a butyric acid bacteria (BAB) spore concentration in mixed grass/corn silage fed to cows below 3 $\log_{10}$ spores/L (generally resulting in concentration in farm tank milk (FTM) below 3 $\log_{10}$ spores/L (Chapter 2)).

Aspect of farm management	Objective	Measures	Ref.
Harvesting & ensiling of grass and corn	• Initial contamination below 3 $\log_{10}$ BAB spore/g	➢ Include less than 1% of soil in the silage	Ch2, Ch5.
	• Sufficiently low pH at achieved dry matter content (DM), criterion: DM (g/kg) > 450-WSC/BC[1] or pH <4.4	➢ Wilting ➢ Harvesting during dry weather ➢ Addition of silage additives (e.g. starter culture, fermentable sugars, chemical preservatives)[2,3]	a,b
	• Achieve and maintain anaerobic conditions	➢ High degree of compaction ➢ Immediate sealing ➢ Airtight seal ➢ Weight on top of seal (e.g. layer of sand)[2]	Ch3,a
Storage of silage	• Prevent penetration of oxygen	➢ Prevent damage to seal	Ch3,c
Feed-out of silage	• Prevent penetration of oxygen	➢ High feed-out rate (>1.5 m/week) ➢ Protect open face from air[3]	Ch3,d,e
	• Prevent contamination of bulk of silage with BAB spores from local pockets with high concentrations of BAB spores	➢ Remove molded areas and areas showing signs of deterioration	Ch3

[1] WSC = Fermentable sugar concentration (g/kg DM), BC=Buffering capacity of crop ensiled (g lactic acid/kg DM needed to lower pH to 4.0)
[2] Optional measures, not always necessary
[3] Needs further research

Ch2. Chapter 2 of this thesis　Ch3. Chapter 3 of this thesis　Ch5. Chapter 5 of this thesis　a. Pahlow (2003) and references therein.
b. Thylin et al. (1995)　c. Jonsson (1991)　d. McGechan (1994)　e. Parsons (1991)

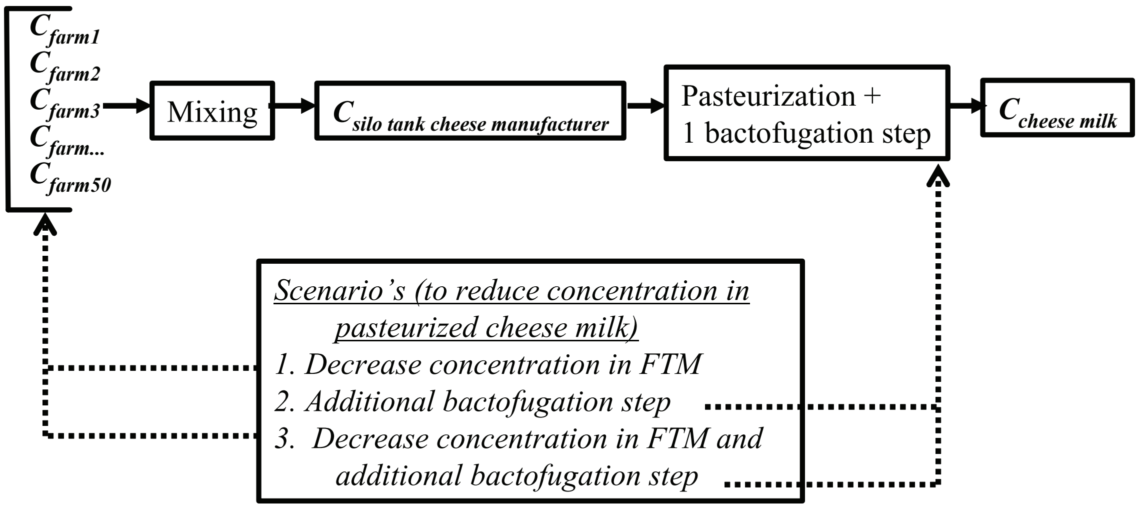

Figure 8.3 Standardized process for the production of pasteurized cheese milk with scenario's to reduce the butyric acid bacteria (BAB) spore concentration in pasteurized cheese milk.

In this section, the effects of the three scenarios on the probability of exceeding the breakthrough concentration for late-blowing in Gouda-type cheeses are estimated given a specific nitrate concentration ("breakthrough risk"). If the BAB spore concentration in pasteurized cheese milk is above the breakthrough concentration, there is a risk of late-blowing of Gouda-type cheeses (Stadhouders and Jørgenson, 1990).

First, the breakthrough risks for the standard process (base scenario; Figure 8.3) are calculated using Monte Carlo simulations. For each iteration, the BAB spore concentrations in the 50 FTM deliveries were simulated using historical data from the Netherlands Milk Control Station (MCS, 2003) and data collected in the field survey (Chapter 5). Subsequently, the BAB spore concentration in the raw milk in the silo tank was calculated by averaging the 50 simulated concentrations in FTM, assuming that each delivery had the same volume. Next, the concentration in the pasteurized cheese milk was simulated using a Poisson distribution and reported spore removal efficiencies of bactofugation (Waes and Van Heddeghem, 1990).

The breakthrough risks for three scenarios were calculated in a similar way as for the base scenario with one adjustment in the scheme. For scenario 1 the BAB spore concentrations in the FTM deliveries were simulated using the model presented in Chapter 2 with adjustments conforming to Table 8.1 (resulting in BAB spore concentration below 3 $\log_{10}$ spores/g in the mixed silages fed). This assumes that all Dutch farms implement the measures of Table 8.1. For the second scenario (additional bactofugation step) an additional removal of BAB spores from raw milk was simulated. The third scenario was a combination of the above.

The simulation results are presented in Table 8.2. The data show that after implementation of measures at the farm and implementation of an additional bactofugation step (Scenario 3) even without the addition of nitrate, the risk of late blowing is very low (probability of exceeding the breakthrough concentration is less than 1%). Table 8.2 also shows that an additional bactofugation step is about as effective as implementation of the identified measures at all farms. An economic evaluation should be performed to establish which option is most attractive in practice.

Table 8.2 The relative frequency of exceeding the breakthrough concentration ("breakthrough risk") given the addition of a specific concentration nitrate for a base case and 3 scenarios

Nitrate added to cheesemilk (g/100 L)	**Breakthrough concentration (BAB spores/L)[1,2]**	**Simulated breakthrough risk (%)**			
		Base scenario (Figure 8.2)	Scenario 1: Measures at the farm	Scenario 2: Additional bactofugation step	Scenario 3: Combination of scenarios 1 and 2
0.0	5-10	96.2	23.7	18.7	0.7
1.0	100	21.8	0.1	0.3	0.0
2.5	250-1,000	9.5	0.0	0.0	0.0
7.5	5,000-10,000	0.0	0.0	0.0	0.0

[1] Stadhouders (1990)

[2] Worst case estimate used for estimate of breakthrough risk (underlined concentrations)

8.3 CONTROL OF *BACILLUS CEREUS* SPORE CONCENTRATION IN FTM

8.3.1 Control strategy and necessary additional research

The *B. cereus* spore concentration in FTM depends on the transmission of spores via the exterior of teats and via surfaces of the milking equipment to FTM (Chapter 1). In addition growth of *B. cereus* during storage of FTM could increase the concentration. Chapter 7 confirmed that spores of *B. cereus* are generally transmitted to FTM via the exterior of teats. Spores transmitted via the exterior of teats predominantly originate from feed. However, when the *B. cereus* spore concentration in FTM exceeds the MSL, soil is most likely the main source (Chapter 6 and 7). In this thesis limited attention has been paid to the transmission of *B. cereus* spores via surfaces of the milking equipment to FTM. This is a rare route, but a basic calculation showed that this route should not be neglected without consideration (Chapter 7). However, growth of *B. cereus* during storage of FTM is negligible when the farm tank is designed and operated according to ISO 5708 (Chapter 6).

Table 8.4 lists measures needed to achieve *B. cereus* spore concentrations in FTM below the MSL. Firstly, the transmission of *B. cereus* spores via the most important transmission route (feed-feces-FTM) has to be minimized. Essential measures are a low initial contamination level of the feed (below 3 $\log_{10}$ spores/g) and prevention of growth in the transmission route The best way to prevent growth of *B. cereus* is a pH below 5 (Chapter 6). Experiments should be conducted to establish whether and to what extent *B. cereus* can grow in amongst others mixed silage and bedding material. Besides control of the dominant contamination pathway, incidents, defined as rare situations that lead to *B. cereus* spore concentration in FTM above the MSL, should be prevented. Typical incidents that may occur are contamination of teats with substantial amounts of soil (transmission of more than 1 mg soil/L of FTM, Chapter 7) and build-up of *B. cereus* in improperly cleaned milking equipment (Griffiths and Phillips, 1990, Chapter 7). In order to establish whether it is necessary to take measures that reduce the probability of spore transmission via these incidents, the actual frequency and impact of these incidents need to be assessed first.

8.3.2 Impact of measures at the farm on the shelf life of pasteurized consumer milk

Reducing the *B. cereus* spore concentration in FTM is an option to extend the shelf life of pasteurized consumer milk. Figure 8.3 shows a standardized process for the production of 1 L packages with pasteurized consumer milk. After collection from the farms, raw milk from the silo tank is pasteurized and filled into packages. The packages are kept at temperatures below 7 °C in the retail chain and, in case of proper handling, also at the consumer. At the end of shelf life the *B. cereus* concentration should be below 8 $\log_{10}$ cells/L to prevent intoxication (Langeveld et al, 1996). Besides reducing *B. cereus* spore concentrations in FTM, implementation of bactofugation or microfiltration steps in the production process are alternative scenarios to extend shelf life.

The impact of the three options to extend the shelf life of pasteurized consumer milk, as mentioned in Figure 8.4, can be quantitatively assessed and compared using model simulations. A difficulty compared to the BAB spore case (section 8.2.2) is that there is no extensive dataset with *B. cereus* spore concentrations in FTM. An alternative was to use the simulation models (feed based and soil based model) and farm management data described in Chapter 6. With these models it is possible to determine a distribution of expected *B. cereus* spore concentrations in FTM when feed or soil is the only source (see also Chapter 6). For these estimations, data presented in Chapters 4 and 7 can be used.

An additional difficulty is that the frequency with which substantial amounts of soil are transmitted to FTM is unknown. To get around this, an assumption can be made for the frequency that soil is the main source of spores. Based on this frequency the number of FTM deliveries with spores mainly originating from soil and with spores mainly originating from feed can be made. To obtain information about the relevance of the frequency that soil instead of feed is the main source of spores, on the expected shelf-life three series of simulations were performed. In the first simulations it was assumed that soil is the main source of *B. cereus* in 0% of the FTM deliveries. In the second and third series of simulations it was assumed that this frequency was 1% and 10%. The maximum frequency of 10% was based on the results presented in Chapters 6 and 7 and discussions with the Dutch dairy industry. In the simulations, shelf life was defined as the time in days that less than 0.01% of the packages reach a *B. cereus* concentration of 8 $\log_{10}$ cells/L.

Table 8.4 Measures needed to control *B. cereus* spore concentration in FTM below 3 log_{10} spore/L.

Aspect of farm management	**Objective**	**Measures**	**Ref.**
Ensiled grass and corn	• Initial concentration <3 log_{10} spores/g	➢ Inclusion of less than 0.1% soil during ensiling	Ch6, Ch7
	• Prevent growth	➢ pH<5.0	
Other feed	• Initial concentration <3 log_{10} spores/g	➢ Mainly quality issue for feed supplier ➢ Dry storage	Ch6
Barn/facility management	• Prevent growth in mixed silage	➢ pH<5.0 ➢ Refresh feed regularly[1]	Ch6
	• Prevent growth in bedding	➢ pH<5.0 ➢ refresh bedding regularly[1]	Ch6, Ch7
	• Prevent contamination of teats with soil	➢ E.g: keep cows walking paths clean[1]	Ch6, Ch7,a
Milking equipment	• Prevent build-up of *B. cereus* in equipment[1]	➢ Proper design, cleaning and maintenance of equipment ➢ Replace cracked and decayed parts[1]	Ch7,b
Cooling of milk	• Prevent growth during storage of FTM	➢ Design and operation of the tank according to ISO-5708[c]	Ch6

[1] Requires additional research

Ch6. Chapter 6 of this thesis Ch7. Chapter 7 of this thesis a. Christiansson et al. (1999) b. ISO 5707 (2001) c. ISO 5708 (1983)

Figure 8.4 represents the base case scenario. Per iteration, first the number of FTM deliveries with *B. cereus* spores mainly originating from soil was simulated using a binomial distribution and the assumption that 50 FTM deliveries are collected in one silo tank. Based on the outcome of this calculation the *B. cereus* spore concentrations in the 50 FTM deliveries were simulated using the feed-based and soil-based model (Chapter 6). As for BAB spores, the concentration in the raw milk stored in the silo tank was calculated as the average of the simulated concentrations in FTM. It was assumed that no growth of *B. cereus* or recontamination occurs in the processing environment. Since *B. cereus* spores are not inactivated during pasteurization, *B. cereus* spore concentrations in raw milk stored in the silo tank and in pasteurized milk are equal. The *B. cereus* spore concentration in 1 L packages after filling was simulated using the Poisson distribution. Based on this concentration and a storage temperature of 7 °C the time to reach a concentration of 8 $\log_{10}$ cells/L was then calculated using equation 8.1 derived from Zwietering et al. (1996):

$$\text{If } t \le \frac{2}{0.00125 \cdot T} \text{ then } C(t) = C_0 \text{ else } C(t) = C_0 \cdot e^{0.00125 \cdot T \cdot (t - 2/0.00125 \cdot T)} \qquad [8.1]$$

With

C_0	Concentration of *B. cereus* after filling of the package (cfu/L)
$C(t)$	Concentration of *B. cereus* after time *t* (cfu/L)
t	Time (h)
T	Storage temperature (°C)

The effects of the different scenarios shown in Figure 8.4 were simulated using the base case model with one adjustment per scenario, similar to the BAB case in the previous section. Data of the spore removal efficiency of microfiltration were obtained from Te Giffel and Van der Horst (2003).

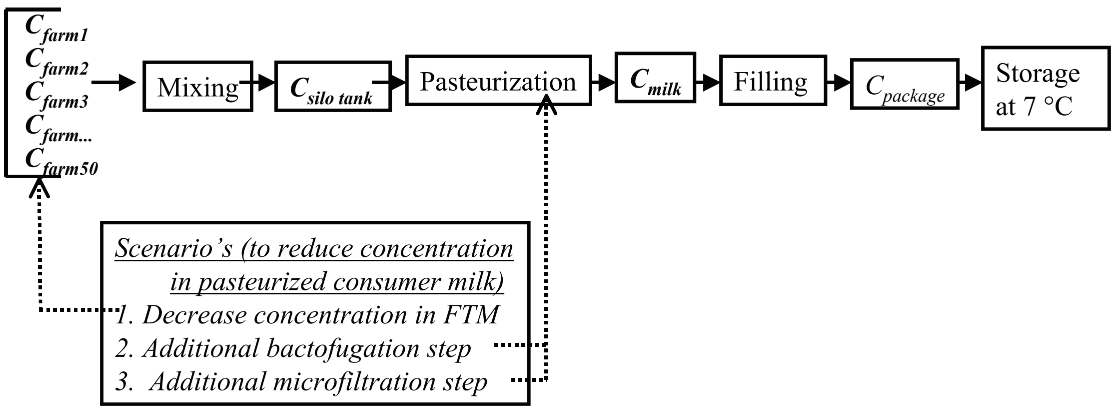

Figure 8.4 Standardized process for the production of pasteurized consumer milk with options to reduce the *Bacillus cereus* spore concentrations.

Table 8.4 shows the predicted shelf life for the different scenarios. It shows that prevention of the transmission of *B. cereus* spores from soil to FTM increases shelf life by approximately 10% (or 1 day). Implementation of the other scenarios extends shelf life by 1 to 2 days. Based on these results, microfiltration seems the most attractive option because it has the most effect on the shelf life and because it is most likely the easiest scenario to implement. However, the simulations performed did not account for the transmission of spores from surfaces of the milking equipment to FTM. Currently no experimental data are available about the relevance of this route and the degree of contamination that can occur via this route. Based on the calculations performed in Chapter 7 it is likely that the transmission of spores from milking equipment surfaces has more impact on the concentration in FTM than the transmission of spores from soil. Consequently, it could be that prevention of contamination via milking equipment surfaces has more effect on the shelf life of pasteurized milk than microfiltration.

Table 8.4 Estimated shelf life for base and three scenario's. Shelf life was defined as the time that less than 0,01% packages has reached a *B. cereus* concentration of 8 $\log_{10}$ cells/L.

Scenario	**Estimated shelf life (days)**		
	Relative frequency that soil is the main source of **Bacillus cereus** ***spores in FTM deliveries***		
	0%	1%	10%
Base scenario (Figure 8.4)	9	8	8
Scenario 1: Measures at farm	10	NR	NR
Scenario 2: Additional bactofugation step	10	10	9
Scenario 3: Additional microfiltration step	11	10	10

NR: Not relevant. Measures at the farm imply that no soil is transmitted to FTM (Chapters 6 and 7). Consequently simulations were only performed for 0% soil.

8.4 CONCLUSIONS

The quality of FTM depends mainly on the concentration of BAB and *B. cereus* spores. In order to control the quality of farm tank milk many factors regarding farm management have been identified in the past. In this study, the relative impact of these factors was successfully quantified using modeling approaches and verified by year-long field surveys (Chapters 5 and 7) Simulations and survey results corresponded, implying that that the microbial contamination of FTM is well described by the models and that the models can be used to derive control strategies. The most important advantage of modeling is that mathematical models give the opportunity to perform "in-silico experiments" to quantitatively assess and compare the impact of specific factors. This enables the identification of effective and ineffective control measures. In addition, results of model simulations can be and were used to optimize the experimental design of field experiments and to prevent putting efforts into investigating aspects that have marginal impacts on bacterial spore concentration in FTM.

With respect to BAB the spore concentration in FTM is determined predominantly by the spore concentration in the mixed grass/corn silage fed to cows in the barn. Local small pockets in silage and corn silage in particular, so called hot spots, have been identified as important factors affecting the BAB spore concentration in mixed silage (Chapter 3). The results of this thesis indicate that it is possible to achieve BAB spore concentrations in FTM below the MSL at all farms. Implementation of the measures identified to control BAB spore concentration in mixed silage below 3 $\log_{10}$ spores/g seems feasible in practice. In addition, the results of the surveys (Chapters 3 and 5) indicate that farmers are able to feed mixed silage meeting this criterion. Therefore, based on these results and the assessment of the impact of measures at the farm level on the risk of late blowing (section 8.2.2) it can be concluded that is beneficial to encourage farmers, for example via the milk payment system, to implement the measures identified. Measures at the farm level are approximately as efficient as an additional bactofugation step.

The *B. cereus* spore concentration in FTM generally also depends on the spore concentration in mixed silage (and other feed). Contamionation of FTM with *B. cereus* spores originating from feed seems to be under control and concentrations in FTM are generally below the MSL. However, incidents like the transmission of substantial amounts of soil to milk and presumably contamination of milk via surfaces of the milking equipment are more likely causes of *B. cereus* spore concentration above the MSL. Implementation of measures at the industrial level are more attractive to extend the shelf life of pasteurized consumer products than measures at the farm level.

In summary, the research showed that mathematical modeling is a very useful tool to identify measures that most effectively control the contamination of FTM with bacterial spores. The following general conclusions can be drawn:

- To minimize the concentration of BAB spores in FTM, it is by far most important to prevent growth of BAB in grass- and corn silage. Farmers should aim for a concentration in grass and corn silage fed to cows below 1,000 spores/g. To achieve this, it is essential to prevent oxygen penetration into the silage silo and to remove molded and deteriorated silage parts from the ration fed to the cows. Measures aimed at other parts of the contamination pathway, such as measures to keep teats clean or teat cleaning prior to milking are much less effective;
- The concentration of *B. cereus* spores in FTM is normally below 1,000 spores/L. During housing and pasturing spores of *B. cereus* in FTM originate from feeds. However, if the concentration in FTM is above 1,000 spores/L, it is more likely that most spores originate from other sources, most likely soil and/or the milking equipment.
- Implementation of measures identified in this study could make late-blowing of Gouda-type cheeses a rare incident and prolong the shelf-life of refrigerated pasteurized consumer milk with approximately 10%.

SUMMARY

The concentration of bacterial spores in farm tank milk (**FTM**) is an important for the microbial quality and shelf life of pasteurized milk and dairy products. For example, lower concentrations of butyric acid bacteria (**BAB**) spores in FTM decreases the risk of production losses during cheese storage due to late-blowing and lower concentrations of *Bacillus cereus* spores extend the shelf life of refrigerated pasteurized dairy products. As a consequence, the dairy industry aims at spore concentrations in FTM higher below 3 log_{10} spores/L. Farmers can take measures to reduce the transmission of spores to FTM, but often they do not know which measures are most effective. In order to help the farmers improve the quality of FTM there is clearly a need for quantification of the impact of several measures. In other words: what are the right measures to focus on. It is more and more common practice to use predictive models. Predictive modeling is a tool to quantify the effectiveness of different control measures. Application of predictive modeling to identify measures that most efficiently reduce the microbial contamination of FTM is new, but likely beneficial for both farmer and dairy industry. The objectives of in this thesis were to quantify the effectiveness of measures for controlling the contamination of FTM with spores of butyric acid bacteria (**BAB**) and *Bacillus cereus* using model simulations.

Chapter 1 contains a general introduction of the background of this thesis. Chapter 2 describes a model predicting BAB spore concentration in FTM. BAB spores in FTM originate from feed, especially silage, and soil. From these sources, spores are transmitted via feces and the exterior of the cows' teats to FTM. The path from source(s) to FTM is called the contamination pathway. The model developed was based on a translation of the contamination pathway into a chain of unit operations. Effects of factors related to feed quality, feed management, barn hygiene and milking practices on BAB spore concentrations in FTM were evaluated. Simulation results showed that control of the spore concentration in mixed grass/corn silage fed to cows in the barn is, by far, most important. To consistently produce FTM with a BAB spore concentration below 3 log_{10} spores/L, farmers should aim for a BAB spore concentration in mixed silage below 3 log_{10} spores/g. Mixed silage containing more than 5 log_{10} spores/g should not be fed, because at these concentrations it is impossible to meet the demands of the dairy industry.

In Chapter 3 it was demonstrated that the BAB spore concentration in mixed silage is determined by a small fraction of silage that hosts a high concentration (between 5 and 7 log_{10} spores/g). These so called hot spots were detected in surface layers of grass and corn silage, in particular in visibly molded areas. The occurrence of hotspots was higher in corn silage than in grass silage. Based on these results it was concluded that corn silage currently is a more important source of BAB spores than grass silage.

A relationship was found between increased spore concentrations and aerobic deterioration of silage. In corn silage signs of aerobic deterioration, such as increased concentrations of yeast and molds (above 5 log_{10} spores/g), an increased temperature (more than 5 °C), and especially a pH above 4.4, were associated with increased BAB spore concentrations. It was postulated that increased BAB spore concentrations in corn silage are the result of oxygen penetrating into the silage, resulting in aerobic deterioration and formation of anaerobic niches with an

increased pH. Therefore, farmers should take measures that limit the penetration of oxygen (e.g. high density). Furthermore, the use of additives inhibiting growth of yeasts and molds will probably be beneficial.

Development of the model for BAB spores (Chapter 2) revealed that information on the amount of dirt (consisting of feces, bedding, and/or soil) transmitted via the exterior of teats to FTM was limited and originated from 20 years ago. To be able to make more accurate predictions of transmission to FTM, Chapter 4 describes a survey in which new data were collected. It was shown that during housing of cows, the amount of transmitted dirt per farm varied between 3 and 300 mg/L, with an average of 59 mg/L.

To validate the outcome of the models, a field survey was carried out. During one year, samples of FTM and environmental carriers (i.e. mixed silage, feces, bedding material, and soil) were collected six times at 24 farms and analyzed for BAB and *B. cereus* spore concentrations. In addition, a questionnaire was used to gather farm management information. Chapter 5 describes the results of the field survey with respect to BAB spores. The average BAB spore concentration in FTM was 2.7 $\log_{10}$ spores/L. In 33% of the FTM samples the concentration was above 3 $\log_{10}$ spores/L. The results of the survey confirmed the results of the BAB model. Mixed silage was proven to be the main source of BAB spores, not only in autumn/winter when cows were housed but also in spring/summer when they were partly grazing. This was explained by the fact that the ration of grazing cows was supplemented with silage. The field survey indicated that the main difference between well and poor performing farms is the ability of farmers to prevent incidental occurrence of high BAB spore concentrations in mixed silage (above 5 $\log_{10}$ spores/g). These high concentrations in mixed silage are related to the aerobic deterioration processes discussed in Chapter 3.

Chapter 6 describes two simulation models developed for *B. cereus* spores. Both models are based on a translation of a contamination pathway into a chain of unit operations. The models account for growth during storage of FTM. The first model describes the transmission of *B. cereus* spores from feed, via feces, bedding material, and the exterior of teats to FTM. The second model describes the transmission of spores from soil via the exterior of teats to FTM. Simulations showed that to control the transmission of spores from feed to FTM, the feed must contain less than 3 $\log_{10}$ spores/g and growth in mixed silage should be prevented via a pH below 5.0. To control the transmission of spores from soil to FTM, measures that prevent the contamination of teats with soil should be taken. No significant growth of *B. cereus* occurs when the farm tank is in conformity with ISO 5708.

B. cereus spore concentrations measured in the field survey are described in Chapter 7. An important conclusion of this study that feed is the main source of *B. cereus* spores in FTM, not only in autumn/winter when cows are housed, but also in spring/summer when cows are grazing. Nevertheless, when the *B. cereus* spore concentration in FTM is above 3 $\log_{10}$ spores/L, the spores in FTM more likely originate from soil than from feed. Measured *B. cereus* spore concentrations in feces and bedding were too low to result in too high concentrations in FTM, whereas the transmission of a relatively small amount of soil (1-13 mg/L) can be sufficient to result in BAB spore concentrations above 3 $\log_{10}$ spores/L. However, the fact that none of the FTM samples exceeded 3 $\log_{10}$ *B. cereus* spores/L suggests that transmission of substantial amounts of spores originating from soil to FTM only occurs occasionally.

In Chapter 8 the impact of measures at the farm level and during processing (e.g. bactofugation) on production losses during cheese storage and the shelf life of pasteurized milk were quantitatively assessed. Controlling the BAB spore concentration in silage below 3 $\log_{10}$ spores/g and the implementation of two bactofugation steps in the process should make late-blowing of Gouda-type cheeses a rare incident. Control of the *B. cereus* spore concentration in FTM below 3 $\log_{10}$ spores/L mainly depends on the prevention of the (occasional) transmission of substantial amounts of spores originating from soil to FTM and perhaps the transmission of spores from surfaces of the milking equipment. Depending on the frequency of these incidents and the efforts needed to prevent them it could be more effective to extend the shelf life of pasteurized consumer milk via the implementation of an additional microfiltration or bactofugation step in the production process.

In conclusion, the study showed that mathematical modeling is a very useful tool to identify measures that most effectively control the contamination of FTM with bacterial spores. The following general conclusions can be drawn:

- To minimize the concentration of BAB spores in FTM, it is by far most important to prevent growth of BAB in grass- and corn silage. Farmers should aim for a concentration in grass and corn silage fed to cows below 1,000 spores/g. To achieve this, it is essential to prevent oxygen penetration into the silage silo and to remove molded and deteriorated silage parts from the ration fed to the cows. Measures aimed at other parts of the contamination pathway, such as measures to keep teats clean or teat cleaning prior to milking are much less effective;
- The concentration of *B. cereus* spores in FTM is normally below 1,000 spores/L. During housing and pasturing spores of *B. cereus* in FTM originate from feeds. However, if the concentration in FTM is above 1,000 spores/L, it is more likely that most spores originate from other sources, most likely soil and/or the milking equipment.
- Implementation of the measures identified in this study could make late-blowing of Gouda-type cheeses a rare incident and prolong the shelf-life of refrigerated pasteurized consumer milk with approximately 10%.

SAMENVATTING

De concentratie bacteriesporen in boerderijtankmelk is een belangrijke parameter voor de microbiële kwaliteit en houdbaarheid van gepasteuriseerde melk en zuivelproducten. Zo neemt bijvoorbeeld door het verlagen van de concentratie boterzuurbacteriesporen (**BZB**) in tankmelk het productieverlies tijdens de kaasbereiding af. Het verlagen van de concentratie *Bacillus cereus* sporen in tankmelk zal bijdragen aan een langeren houdbaarheid van gepasteuriseerde zuivelproducten. De industrie wil een sporenconcentratie in tankmelk onder de 1.000 sporen/L.

Veehouders kunnen maatregelen nemen die de overdracht van sporen naar melk verminderen, maar onduidelijk is welke maatregelen het meest effectief zijn. Voor industriële processen worden vaak voorspellende modellen gebruikt om effecten van mogelijke beheersmaatregelen te kwantificeren. Op eenzelfde manier zouden modellen, die de sporenconcentratie in tankmelk voorspellen als functie van het veehouderijmanagement, een bijdrage kunnen leveren aan het verlagen van de sporenconcentratie in tankmelk. Het doel van dit proefschrift was om met behulp van dergelijke modellen beheersmaatregelen te identificeren die de besmetting van tankmelk met sporen van boterzuurbacteriën en *Bacillus cereus* verminderen.

Na een algemene inleiding (hoofdstuk 1), wordt in hoofdstuk 2 het model beschreven dat de besmetting van tankmelk met BZB sporen voorspeld. BZB sporen in tankmelk zijn afkomstig uit het voer, met name gras- en maïskuil, en grond. Vanuit uit deze bronnen worden de sporen via faeces en de buitenkant van spenen overgedragen naar tankmelk. De route van bron naar tankmelk wordt de besmettingsroute genoemd. Het ontwikkelde model is gebaseerd op een vertaling van de besmettingsroute in een keten van processtappen. In modelsimulaties zijn de effecten van maatregelen gerelateerd aan de kwaliteit van het voer, de omgang met het voer in de stal, de bedrijfshygiëne en de handelingen tijdens het melken op de concentratie BZB sporen in tankmelk kwantitatief vergeleken. Het is veruit het belangrijkst om het besmettingsniveau in de in de stal aangeboden gras- en maïskuil te beheersen. Het doel moet zijn kuilvoer aan te bieden met minder dan 1.000 sporen/g. Het aanbieden van kuilvoer met meer dan 100.000 sporen/g moet voorkomen worden. Het wordt dan bijna onmogelijk om tankmelk te produceren met minder dan 1.000 sporen/L. Andere maatregelen, zoals het schoonmaken van spenen tijdens het melken hebben, in vergelijking met de concentratie in het aangeboden kuilvoer nauwelijks invloed op de sporenconcentratie in tankmelk.

Hoofdstuk 3 beschrijft de resultaten van een onderzoek naar de concentratie BZB sporen in gras- en maïskuil. Een verband werd gevonden tussen hoge BZB sporenconcentraties in kuil en broeiverschijnselen. Hoge concentraties BZB sporen in het aangeboden kuilvoer in de stal werden veroorzaakt door een zeer kleine fractie van de gras- of maïskuil met een sterk verhoogde concentratie BZB sporen. De concentraties in deze zogenaamde hot-spots waren vaak dusdanig hoog (meer dan 10.000.000 sporen/g) dat ze bepalend waren voor de gemiddelde concentratie van het gemengde kuilvoer. Verhoogde sporenconcentraties werden vooral gevonden in randlagen en beschimmelde plekken van de gras- en maïskuil. Hoge concentraties werden vaker gevonden in maïskuil dan in graskuil.

Het onderzoek liet ook zien dat lokaal verhoogde concentraties BZB sporen in maïskuil vaak gepaard gaan met tekenen van broei zoals verhoogde concentraties van gisten en schimmels

(meer dan 100.000 sporen/g), een verhoogde temperatuur (meer dan 5 °C) en met name een pH boven de 4.4. Op basis van de resultaten wordt daarom verondersteld dat hot-spots het gevolg zijn van het binnendringen van zuurstof in de kuil. Dit leidt tot groei van gisten en schimmels. Het gevolg hiervan zijn zuurstofarme niches met een verhoogde pH. In deze niches kunnen BZB hoge concentraties bereiken. Veehouders moeten daarom maatregelen nemen die het binnendringen van zuurstof verminderen (bijv. een hoge kuildichtheid). Daarnaast zal het toevoegen van middelen die de groei van gisten remmen een bijdrage leveren aan het voorkomen van hot-spots.

Bij de ontwikkeling van het model dat is beschreven in hoofdstuk 2 bleek dat er slechts beperkt data beschikbaar is over de hoeveelheid vuil die via de spenen overgedragen wordt naar melk. Om de overdracth van vuil naar melk beter te kunnen inschatten beschrijft Hoofdstuk 4 onderzoek dat is uitgevoerd om nieuwe data te verzamelen. Het bleek dat de hoeveelheid overgedragen vuil per boederij varieerde van 3 tot 300 mg/L, met een gemiddelde van 59 mg/L.

Om de resultaten van modelsimulaties te valideren werd een veldonderzoek uitgevoerd. In dit onderzoek werden de 24 veehouders zes keer bezocht om monsters te nemen van onder meer melk, het aangeboden kuilvoer, feces, strooisel en grond. In deze monsters werd de concentratie BZB and *B. cereus* sporen bepaald. Bij elk bezoek werd gevraag een vragenlijst in te vullen om informatie over de actuele bedrijfsvoering te verzamelen. Hoofdstuk 5 beschrijft de resultaten van het veldonderzoek met betrekking tot BZB sporen. In 33% van de tankmelkmonsters was de concentratie te hoog (meer dan 1.000 sporen/L). Dit bevestigt de resultaten van de modelsimulaties. Het aangeboden kuilvoer was zowel in de winter als in de zomer de belangrijkste bron van BZB sporen. Oorzaak hiervan is dat ook aan grazende koeien kuilvoer aangeboden wordt (voor en na het melken). Wanneer geen kuilvoer gevoerd werd, was de concentratie altijd lager dan 40 BZB sporen/L. Het veldonderzoek toonde ook aan dat goede en minder goede bedrijven waarschijnlijk verschillen in het vermogen om incidenteel hoge BZB sporenconcentraties in het gemende kuilvoer te voorkomen. Het bleek namelijk dat veehouders met een laag gemiddelde concentratie in tankmelk in een enkel geval de kritische sporenconcentratie van 3 $\log_{10}$ spores/L overschreden. Veehouders met de hoogste gemiddelde concentratie in tankmelk voldeden in ongeveer de helft van de gevallen aan de gestelde eis. Uitschieters in concentraties in de tankmelk worden waarschijnlijk veroorzaakt door een groot aantal hot spots in het verstrekte kuilvoer.

In hoofdstuk 6 worden twee modellen besproken die de besmetting van tankmelk met *B. cereus* sporen beschrijven. Het eerste model beschrijft de overdracht van sporen afkomstig uit het voer, via faeces, strooisel en de buitenkant van de spenen naar tankmelk. Het tweede model beschrijft de overdracht van sporen uit grond naar melk. Beide modellen nemen ook de potentiële groei van *B. cereus* tijdens opslag van melk mee. Modelsimulaties lieten zien dat om de besmetting van tankmelk met sporen afkomstig uit voer te beheersen, het belangrijk is om ervoor te zorgen dat de verschillende voercomponenten (kuilvoer, krachtvoer, bierbostel etc.) minder dan 1.000 sporen per gram bevatten en om groei van *B. cereus* in het gemengde kuilvoer te voorkomen. Het laatste kan bereikt worden door de pH van het voer beneden de 5 te houden. Om de overdracht van sporen vanuit grond naar tankmelk te beheersen dient voorkomen te worden dat spenen in aanraking komen met grond. Indien de boerderijtank voldoet aan de ontwerprichtlijnen van de internalionale standaard organisatie (ISO) en de melk volgens de richtlijnen van ISO en de zuivelindustrie gekoeld wordt, treedt geen groei van *B. cereus* op tijdens opslag van tankmelk.

In hoofdstuk 7 worden de resultaten van het veldonderzoek die betrekking hebben op de besmetting van tankmelk met *B. cereus* sporen besproken. Een belangrijke conclusie is dat het voer zowel in de winter (wanneer de koeien op stal staan) als in de zomer (wanneer de koeien ook grazen) de belangrijkste bron van sporen is. Desalniettemin is het bij een sporenconcentratie in tankmelk boven de 3 $\log_{10}$ sporen/L waarschijnlijker dat de sporen afkomstig zijn uit grond dan dat de sporen afkomstig zijn uit het voer. Dit komt omdat de *B. cereus* sporenconcentraties in grond gemiddeld een factor 100 hoger liggen dan de concentratie in faeces en strooisel. Het gevolg hiervan is dat de overdracht van kleine hoeveelheden grond (1-13 mg/L) al kan leiden tot een te hoge concentratie in tankmelk, terwijl veel meer faeces en strooisel (>150 mg/L) overgedragen moet worden om deze concentratie te bereiken. De overdracht van een significante hoeveelheid sporen vanuit grond naar tankmelk lijkt een zeldzaam incident aangezien in het veldonderzoek de concentratie *B. cereus* sporen in tankmelk altijd onder de kritische sporenconcentratie lag.

In hoofdstuk 8 is de effectiviteit van maatregelen op de boerderij en maatregelen tijdens productie op verliezen tijdens de kaasopslag en de houdbaarheid van gepasteuriseerde melk berekend. Beheersing van BZB sporenconcentraties in aangeboden kuilvoer onder de 1.000 sporen/g in combinatie met 2 bactofugatiestappen leidt er toe dat 'laat-los' van Gouda kaas een zeldzaam incident wordt. De beheersing van de *B. cereus* sporenconcentratie in tankmelk het voorkomen van incidenten. Meest relevante incidenten zijn de overdracht van sporen afkomstig uit grond en mogelijk de overdracht van sporen afkomstig uit de melkwinningsinstallatie naar melk. Afhankelijk van de frequentie van deze incidenten en de noodezakelijke maatregelen om de incidenten te voorkomen, kan het effectiever zijn om in plaats van maatregelen op de boerderij een extra processtap in de fabriek in te bouwen.

Dit onderzoek heeft aangetoond dat voorspellende modellen zeer bruikbaar zijn om maatregelen te identificeren die effectief de concentratie bacteriesporen in FTM verminderen. De volgende algemene conclusies kunnen uit het onderzoek getrokken worden:

- Het voorkomen van groei van BZB in gras- en maïskuil is verreweg het belangrijkst om een lage concentratie BZB sporen in tankmelk te garanderen. Veehouders moeten streven naar een concentratie in het gemengde kuilvoer onder de 1.000 sporen/g. Andere maatregelen zijn veel minder effectief.
- In het algemeen zijn *B. cereus* sporen in tankmelk afkomstig uit het voer en is de concentratie in tankmelk laag. Wanneer de concentratie in tankmelk hoger is dan 1.000 sporen/L, dan zijn de sporen hoogstwaarschijnlijk afkomstig uit grond of van de oppervlakken van een slecht gereinigde melkwinningsinstallatie. Deze incidenten dienen voorkomen te worden om aan de eisen van de zuivelindustrie te voldoen.
- Het implementeren van de geïdentificeerde maatregelen op de boerderij kan ertoe leiden dat "laat-los" van Gouda kaas nauwelijks meer voorkomt en dat de houdbaarheid van gekoelde gepasteuriseerde consumptiemelk met 10% toeneemt.

ABBREVIATIONS

a_w	Wateractivity
BAB	Butyric acid bacteria
BC	Buffering capacity
BZB	Boterzuurbacteriën
CPD	Cumulative probability distribution
DM	Dry matter content
DT	Detection limit
FTM	Farm tank milk
HACCP	Hazard analysis and critical control points
ISO	International standardization organization
MAB	Mesophilic aerobic bacteria
MCS	Netherlands Milk Control Station
MEYP	Mannitol egg yolk polymyxin
MPN	Most probable number
MSL	Maximum spore limit in milk
NR	Not relevant
PPS	Physiological salt solution
WSC	Concentration fermentable sugars
Y&M	Yeast and molds

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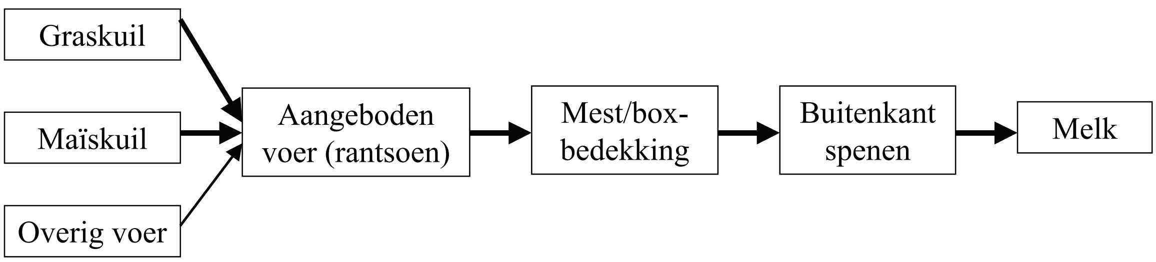
PRAKTIJK ADVIES

INLEIDING

Versleep van microorganismsen en hun sporen vanuit de boerderijomgeving naar tankmelk beïnvloed de productie en houdbaarheid van melk en zuivelproducten. De overdracht van boterzuurbacteriesporen vanuit de boerderijomgeving naar tankmelk kan leiden tot productverliezen tijdens de kaasbereiding. De houdbaarheid van gepasteuriseerde melk wordt mede bepaald door de concentratie *Bacillus cereus* sporen in tankmelk. Veehouders kunnen bijdragen aan een verlaging van het risico op productieverliezen tijdens de kaasbereiding en aan de houdbaarheid van gepasteuriseerde melk door het nemen van gerichte maatregelen. Streven van de zuivelindustrie is een concentratie boterzuurbacterie- en *B. cereus* in tankmelk beneden de 1.000 sporen/L. In opdracht van de Nederlandse zuivelindustrie heeft NIZO food research een onderzoek uitgevoerd om beheersmaatregelen te identificeren, die de overdracht van boterzuurbacterie- en *B. cereus* sporen naar tankmelk effectief verminderen.

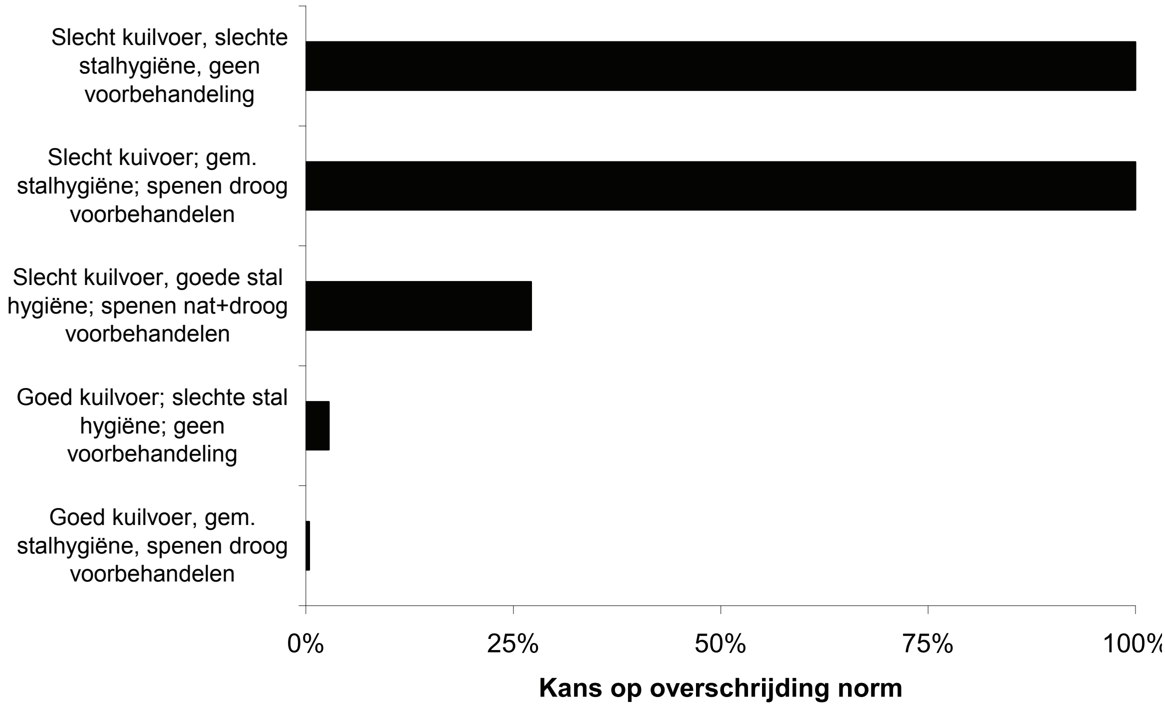
BOTERZUURBACTERIESPOREN

Uit al de analyses blijkt dat, op de boerderij, de gras- en maïskuil de belangrijkste bronnen van boterzuurbacteriesporen zijn. Via het voer in de stal, mest, strooisel en de buitenkant van de spenen komen de sporen in de melk terecht (Figuur 1). Sporen uit voer eindigen in de mest omdat ze het verteringskanaal van de koe intact passeren. Tot dus ver werd alleen graskuil gezien als een relevante bron. Het onderzoek heeft echter aangetoond dat op dit moment maïskuil een zeker zo belangrijke bron is. Als gevolg van broei, kan maïskuil lokaal zeer hoge concentraties boterzuurbacteriesporen bevatten (>10.000.000 sporen/g).



Figuur 1 De besmettingsroute van melk met boterzuurbacteriesporen

Het verlagen van de concentratie boterzuurbacteriesporen in de aangeboden gras- en maïskuil is veruit de effectiefste maatregel om de concentratie in tankmelk laag te houden (Figuur 2). Sterker nog, indien de aangeboden gras- en maïskuil van slechte kwaliteit zijn en gemiddeld meer dan 100.000 sporen per gram bevat, loopt zelfs het meest hygiënische bedrijf grote kans (meer dan 25%) de norm van de zuivelindustrie te overschrijden (Figuur 2). Dit betekent dat bijvoorbeeld een schone stal of het voorbehandelen van spenen tijdens het melken geen effectieve maatregelen zijn. Voor een effectieve beheersing van de overdracht van boterzuurbacteriesporen naar melk, dienen veehouders te streven naar een concentratie sporen in de aangeboden gras- en maïskuil beneden de 1.000 sporen/g. Als dit gerealiseerd wordt, is de kans op overschrijding van de norm zelfs bij een slechte stalhygiëne en het niet voorbehandelen van de spenen zeer laag (minder dan 3%, Figuur 2).



Figuur 2 Effect kuilvoer (gras en maïs), stalhygiëne en voorbehandeling van de spenen op het risico om de norm van de zuivelindustrie voor de norm concentratie boterzuurbacteriesporen in boerderijtankmelk (1.000 sporen/L) te overschrijden.

Tabel 1 bevat een overzicht van de maatregelen die nodig zijn om een concentratie boterzuurbacteriesporen onder de 1.000 sporen/g in de aangeboden gras- en maïskuil te realiseren. Het is essentieel om groei van boterzuurbacteriën in de kuil te voorkomen. Dit wordt in de eerste plaats bereikt door te zorgen voor een snelle en goede fermentatie van het gewas na inkuilen. Door de fermentatie daalt de pH van de kuil, wanneer de pH lager is dan 4.5 kunnen boterzuurbacteriën niet meer groeien.

Om groei te voorkomen is het daarnaast belangrijk dat zuurstof niet de kuil binnendringt. Dit leidt tot groei van gisten en schimmels, met een pH stijging tot gevolg. Vervolgens kan lokaal groei van boterzuurbacteriën optreden. Uit het onderzoek blijkt dat met name de randlagen van de gras- en maïskuil (buitenste 20 cm) gevoelig zijn voor dit proces. Een goede verdichting van de kuil tijdens het inkuilen vermindert de kans op broei en groei van boterzuurbacteriën in de randlagen aanzienlijk.

Het voeren van kleine fracties van het kuilvoer met een hoge concentratie sporen kan leiden tot een gigantische toename van de gemiddelde concentratie in het aangeboden voer. Om minder dan 1,000 sporen/g in het aangeboden voor te garanderen is het daarom belangrijk om beschimmelde delen (bijvoorbeeld “blauwe ballen”) en randlagen, die broei vertonen, te verwijderen voordat de gras- en maïskuil aan het melkvee wordt aangeboden. Deze delen van de kuil kunnen meer dan 10.000.000 sporen/g bevatten. Dit betekent dat, indien slecht 1% beschimmelde gras- of maïskuil gevoerd wordt, de gemiddelde sporenconcentratie in het aangeboden voor al boven de kritische concentratie van 100.000 sporen/g zal liggen. De kans dat de norm van de zuivelindustrie overschreden wordt is dan groot (Figuur 2).

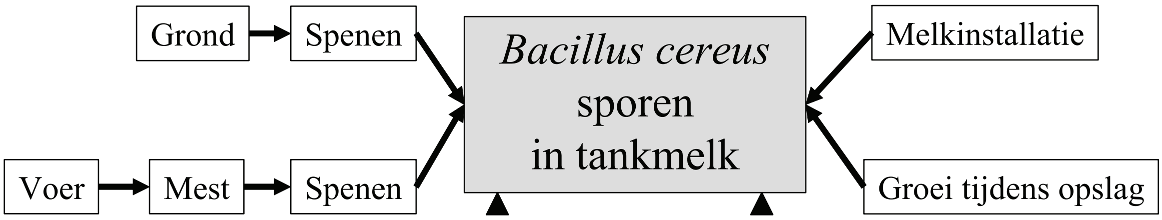
Tabel 1 Maatregelen nodig om concentratie boterzuurbacteriesporen in tankmelk onder de norm van de zuivelindustrie (1.000 sporen/L) te houden.

Doel	Maatregelen
Snelle en goede fermentatie van de gras- en maïskuil (pH onder de 4.5)	• Oogsten bij droog weer • Het gebruik van toevoegmiddelen als melasse en bacterieculturen • De gras- of maïskuil op de dag van oogsten afdekken met landbouwplastic
Voorkomen dat zuurstof de kuil binnendringt	• Tijdens het inkuilen de kuil goed aanrijden zodat een goede verdichting bereikt wordt • Het voorkomen van luchtkanalen in de kuil. Dit betekent dat het landbouwplastic goed aan moet sluiten op het gewas • Grondlaag op de kuil • Voorkomen dat er gaten in de afdekplastic komen • Voldoende hoge voersnelheid (>1.5 m/week)
Voorkomen van besmetting van goed geconserveerde delen van de gras- en maïskuil met boterzuurbacteriesporen uit slecht geconserveerde delen	• Verwijderen van schimmelbollen en beschimmelde delen van de kuil • Verwijderen van randlagen (buitenste 20 cm) die teken van broei vertonen

BACILLUS CEREUS SPOREN

Op de boerderij zijn drie potentiële bronnen van besmetting van tankmelk met *B. cereus* sporen, namelijk: voer, grond en de melkinstallatie, namelijk voer, grond en de melkinstallatie (Figuur 3). Daarnaast kan tijdens opslag van de melk groei van *B. cereus* leiden tot een toename van de sporenconcentratie.

Uit het onderzoek blijkt dat *B. cereus* sporen in tankmelk meestal afkomstig zijn uit het voer. Net als boterzuurbacteriesporen worden *B. cereus* sporen vanuit het voer via mest, strooisel en de buitenkant van de spenen naar melk overgedragen. Indien het voer minder dan 1.000 sporen/g bevat wordt de norm van de zuivelindustrie niet snel overschreden. De veehouder dient dan wel groei van *B. cereus* in het voer dat in de stal ligt en in strooisel te minimaliseren. Dit wordt bereikt door een pH van voer onder de 5.0 en door voer en strooisel regelmatig te verversen. Uit het veldonderzoek blijkt dat veehouders besmetting via deze route in het algemeen onder controle hebben.



Figuur 3 Besmettingroutes die leiden tot *Bacillus cereus* sporen in boerderijtankmelk

Grond kan zeer hoge concentratie *B. cereus* sporen bevatten, tot 1.000.000 sporen/g. Dit betekent dat de overdracht van een geringe hoeveelheid grond naar melk (1 tot 13 mg per L melk) al kan leiden tot overschrijding van de norm van de zuivelindustrie. Het is daarom van essentieel belang dat veehouders de besmetting van spenen met grond voorkomen. Met name beweiding tijdens nat weer vormt een risico. Besmetting van tankmelk met *B. cereus* sporen uit grond kan leiden tot een overschrijding van de norm met factor 10.

B. cereus kan zich ophopen in slecht ontworpen en slecht gereinigde melkinstallaties. Ophoping kan met name optreden in versleten rubber onderdelen en dode hoeken. Tijdens het melken kunnen *B. cereus* sporen vanuit deze ophoping overgedragen worden naar de melk. Theoretisch kan een ophoping van B. cereus op een oppervlak van 10-100 cm2 tijdens warme dagen (>25 °C) leiden tot een overschrijding van de norm van de zuivelindustrie met een factor 100. Echter, in de praktijk is het bestaan van deze route nog niet aangetoond. Desalniettemin, wordt gezien de grote impact die ophoping van *B. cereus* in de installatie kan hebben, geadviseerd ophoping van sporen in de installatie te voorkomen. Dit wordt bereikt door een goed(e) ontwerp, onderhoud en reiniging van de installatie

B. cereus is een bacterie die kan groeien bij zeer lage temperaturen (< 7 °C). Dit betekent dat bij slechte koeling van melk tijdens opslag de *B. cereus* sporenconcentratie toe kan nemen. De toename is sterk temperatuur afhankelijk. Uit het onderzoek blijkt dat geen relevante groei van *B. cereus* valt te verwachten, wanneer melk van het eerste melkmaal binnen 2 uur gekoeld is tot 4 °C en de lage temperatuur vervolgens gehandhaafd blijft gedurende de rest van de opslagtijd. Echter, uitvallen van de koeling na het 1ste, 2de of 3de melkmaal kan leiden tot een relevante toename van de concentratie *B. cereus* sporen in tankmelk.

Tabel 2 geeft de maatregelen die nodig zijn om melk te produceren met een concentratie *B. cereus* sporen onder de norm van de zuivelindustrie (1.000 sporen/L). In de praktijk blijkt de besmetting van tankmelk met *B. cereus* sporen bij de meeste boeren onder controle; in het veldonderzoek werden nooit meer dan 1000 *B. cereus* sporen/L tankmelk gevonden. Echter incidentele uitschieters door overdracht van sporen uit grond of vanuit de melkinstallatie naar melk kunnen grote gevolgen hebben. De houdbaarheid van gepasteuriseerde consumptiemelk kan met 10% verkort worden wanneer bij 1 op de 20 veehouders sporen uit grond naar tankmelk overgedragen worden. Een zelfde verkorting van de houdbaarheid kan bereikt worden wanneer bij 1 op de 100 veehouders, *B. cereus* opgehoopt is in de melkinstallatie ten gevolge van slecht ontwerp, onderhoud of reiniging.

Tabel 2 Overzicht maatregelen nodig om concentratie *Bacillus cereus* sporen in tankmelk onder de norm van de zuivelindustrie te houden

Doel	**Maatregel**
Voorkom besmetting van tankmelk met *B. cereus* sporen uit	• Gevoerde gras- en maïskuil, krachtvoer, vochtrijkebijproducten en andere voeders dienen minder dan 1.000 sporen/g te bevatten (in het algemeen wordt aan deze eis voldoen) • Zorg voor een voldoende lage pH van het voer en strooisel dat in de stal ligt (pH bij voorkeur lager dan 5). Dit voorkomt groei van *B. cereus* • Ververs voer en strooisel bij voorkeur elke dag (dit voorkomt significante groei van *B. cereus*)
Voorkom besmetting van tankmelk met *B. cereus* sporen uit grond	• Voorkom bevuiling van spenen met grond • Houdt de looproutes van het vee vrij van grond • Houd koeien bij nat weer bij voorkeur binnen
Voorkom ophoping van *B. cereus* in de melkinstallatie	• Zorg voor een hygiënisch design van de apparatuur (bijv. zo min mogelijk dode hoeken) • Zorg voor goed onderhoud van de melkinstallatie (bijv. regelmatig vervangen rubber onderdelen) • Reinig de installatie volgens de voorschriften
Voorkom groei van *B. cereus* tijdens opslag van de melk	• Zorg dat de melk van het eerste melkmaal binnen 2 uur gekoeld is naar 4 °C • Zorg dat de temperatuur van de melk in de periode tussen 2 melkmalen niet boven de 5 °C uitkomt • Voorkom storing van de koelapparatuur

CONCLUSIES

Dit onderzoek heeft aangeoond dat maatregelen op de boerderij een aanzienlijke bijdrage kunnen leveren aan de beheersing van de concentratie microorganismen in tankmelk. Voor de besmetting met boterzuurbacteriesporen is het essentieel dat groei van boterzuurbacteriën in gras- en maïskuil voorkomen wordt en dat de aan de koeien aangeboden gras- en maïskuil minder dan 1.000 sporen/g bevat. Om de besmetting van tankmelk met *B. cereus* op een laag niveau te houden dient met name voorkomen te worden dat spenen bevuild raken met grond en dat de melkinstallatie goed ontworpen, onderhouden en gereinigd wordt. Strikte uitvoering van deze maatregelen op alle boerderijen in Nederland kan de houdbaarheid van gepasteuriseerde consumptiemelk met zo'n 10% kunnen verlengen.

NAWOORD

Dat was het dan, 130 en nog wat pagina's leesplezier. Het geeft me een trots gevoel te kunnen zeggen dat ik 1 cm boekenkastvulling zelf heb geproduceerd. Aan deze cm heb ik de afgelopen jaren overwegend met veel plezier en enthousiasme aan gewerkt. Zoals bij de meeste promoties was de start langzaam en oriënterend, maar werd besloten met een heuse eindsprint. Erg leuk was de link met de praktijk. Termen als "blauwe ballen" en "een zooitje" zijn lekker ontnuchterend naast alle interessante wetenschap en Excel sommetjes.

Dit "boekje" is natuurlijk niet alleen mijn verdienste. Velen hebben mij geadviseerd, geholpen en ondersteund tijdens die 4 jaar promotieonderzoek. Zonder iemand te kort te willen doen wil ik een aantal mensen persoonlijk bedanken.

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Dit "boekje" bevat een berg aan experimentele data. Dataverwerking ging me goed af, maar voor het verzamelen van data heb ik veel hulp gehad. Theo, Douwe en Reinier, ik vond het leuk jullie te begeleiden en het is mooi dat jullie werk twee hoofdstukken heeft opgeleverd. De veehouders die deelgenomen hebben aan de monitoring: nogmaals bedankt. Steef, Jos, Gerben, Ellen en Jan bedankt voor de hulp bij het organiseren van de monitoring en het verzamelen van de monsters. Arjen, Elly, Jos, Nico, Jan, Jan en Jan bedankt voor het uitplaten, tellen en nog wat meer. Ik hoop dat de mestgeur inmiddels uit jullie kleren is verdwenen.

Zonder arbeid geen resultaat, maar bakkies koffie met collega's op zijn tijd doen wonderen. Alle NIZO collega's van Processing en van Health and Safety bedankt hiervoor. Annemarie, Paula, Rianne en Rob voor jullie een aparte regel omdat jullie me tijdens mij laatste schrijferijen regelmatig uit mijn vissekom wisten te trekken voor koffie of lunch. Na afloop van onze labpauzes vloeiden er vaak vlot enkele nieuwe alineas uit mijn pen.

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CURRICULUM VITAE

Marc Vissers werd geboren op 27 januari 1976 in Veghel. In 1994 behaalde hij zijn Atheneum diploma aan het Zwijsen College in Veghel. In september van dat jaar begon hij aan zijn opleiding levensmiddelentechnologie aan Wageningen Universiteit. Na een afstudeervak bij het IMAG (Wageningen) en stages bij Massey University (Nieuw-Zeeland) en Cargill (Bergen op Zoom) studeerde hij in september 1999 af met als specialisatie proceskunde. Van december 1999 tot op heden is hij werkzaam bij NIZO food research in Ede. Hij was werkzaam als projectleider, eerst bij de afdeling Processing, Modeling & Control en van 2004 tot en met 2006 bij de afdeling Health & Safety. Van januari 2003 tot december 2006 heeft hij binnen het gemeenschappelijke research programma, dat NIZO food research uitvoert voor de Nederlandse zuivelindustrie, gewerkt aan het project "Modeleren van microorganismen op de boerderij". Dit project heeft uiteindelijk geresulteerd in dit proefschrift. Vanaf januari 2007 is hij werkzaam als business development manager voor het Processing Centre van NIZO food research.

TRAINING ACTIVITIES

Discipline specific activities

- Congress on microbial fermentation and preservation, Wageningen, 2002
- Advanced issues in Neurocomputing, ASCI, Amsterdam, 2003
- Second international workshop on microbial risk assessment, National Veterinary School of Altfort, Parijs, 2003
- NIZO Dairy Conference, Papendaal, 2003
- Food Safety Risk Analysis, David Vose, Wageningen, 2003
- SAFE seminar on Contaminant in agricultural practice, Brussel, 2004 (presentation)
- NvvM sympsosium "Eigen werk", Wageningen, 2004
- FoodSim 2004, Wageningen, 2004 (presentation)
- SFAM winter meeting 2005, Norwich, 2005
- Applied Statisitics, WBS, Wageningen, 2005
- Model-it Symposium, Leuven, 2005 (presentation)
- 4th Mastitis Congress, Maastricht, 2005 (presentation)
- GPROMS modeling environment, Ede, 2006

General courses

- Project management I, Leeuwendaal, 2002
- Commercial Skills I, Kralendonk, 2002
- Commercial Skills II, Kralendonk, 2003
- Business marketing, STEM, 2004
- Persoonlijk presenteren, De Baak, Driebergen, 2005
- Projectmatig creeëren, NIZO, Ede, 2006
- Carrier assessment, Ranger, Nijmegen, 2006

Optionals

- Writing projectplan, 2002
- Half year progress meetings with representatives from the Dutch dairy industry
- Half year progress meetings with Product Desing and Quality Management Group and Laboratory of Food Microbiology of Wageningen University

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