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Bacterial residues after cleaning in place in an aseptic milk processing system

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ABBREVIATIONS

ABBREVIATION	NAME
BCG	*Bacillus Cereus* Group
BSG	*Bacillus Subtilis* Group
°C	Degree Celsius
$C_2H_4O_3$	Peracetic Acid
$C_6H_8O_7$	Citric Acid
CA	Columbia Agar
CIP	Cleaning In Place
CFU	Colony Forming Unit
ClO_2	Chlorine Dioxide
cm	Centimeter
COD	Chemical Oxygen Demand
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic Acid
DVG	Deutsche Veterinärmedizinische Gesellschaft
EU	European Union
GSP	Glutamate Starch Phenol Red Agar
H_3BO_3	Boric Acid
HNO_3	Nitric Acid
H_2O_2	Hydrogen Peroxide
H_3PO_4	Phosphoric Acid
HACCP	Hazard Analysis Critical Control Points
HDPE	High Density Polyethylene
HPLC	High Performance Liquid Chromatography
HTST	High Temperature Short Time
KOH	Potassium Hydroxide Test
MAP	*Mycobacterium avium spp. paratuberculosis*
min	Minute
MRS	De Man, Rogosa And Sharpe Agar
MYP	Mannitol Egg Yolk Polymyxin
ml	Milliliters
Na_2CO_3	Sodium Carbonate
NaOH	Sodium Hydroxide
$Na_2S_2O_3$	Sodium Thiosulfate
ng	Nanogram
Nr.	Number
%	Percent
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PET	Polyethylene Terephthalate
pkat	Petakatal
QS	Quorum Sensing
RCF	Relative Centrifugal Force
rRNA	Ribosomal Ribonucleic Acid
s	Seconds

S1-S3	Sampling 1- Sampling 3
SCC	Somatic Cell Count
SIP	Sterilisation In Place
SLB	Sample Loading Buffer
SPC	Standard Plate Count
SrRNA	Small-Subunit Ribosomal Ribonucleic Acid
TBE	Tris Borat Edta
TSAY	Tryptic Soy Agar Plus 0.6 % Yeast
Tris HCl	Trishydroxymethylaminomethanhydrochlorid
UHT	Ultra-High Temperature
UV light	Ultra-Violette Light
V	Volt
VRBG	Violet Red Bile Glucose Agar
µm	Micrometer
xg	Force X Gravity

INHALTSVERZEICHNIS

1. INTRODUCTION 7

1.1. MILK AND MILK PRODUCT PROCESSING 7

1.2. RAW MILK QUALITY AND MICROBIAL COMPOSITION OF RAW MILK, PASTEURISED MILK AND UHT MILK 11

1.3. SPOILAGE ASSOCIATED BACTERIA 14
I. ENZYME PRODUCING GRAM NEGATIVE BACTERIA 15
II. SPORE FORMING BACTERIA 18
III. OTHER SPOILAGE ASSOCIATED BACTERIA 23

1.4. PROCESS HYGIENE AND SAFETY CRITERIA 23

1.5. AIM OF THE STUDY & STUDY design 29

2. MATERIALS AND METHODS 30

2.1 PHASE I: SAMPLING, PHYSICOCHEMICAL MONITORING AND STERILE FILTRATION 30

2.2 PHASE II: BACTERIAL GROWTH, COLONY CHARACTERISATION AND CLASSICAL BACTERIAL DIFFERENTATION 31

2.3 PHASE III: DNA EXTRACTION 32

2.4 PHASE IV: PCR, GELELECTROPHORESIS AND 16S rNA GENE SEQUENCING 32

3. RESULTS 34

4. DISCUSSION AND CONCLUSION 40

5. EXTENDED SUMMARY 46

6. ZUSAMMENFASSUNG 48

7. REFERENCES 50

8. ACKNOWLEDGEMENTS 65

9. SUPPLEMENTS 66

LIST OF TABLES

Table 1: Requirements for heat treatment of raw milk in accordance with Annex III Section IX Chapter II of EU Regulation No 853/2004 as amended by Commission Regulation No 1662/2006. 8
Table 2: Microbiological criteria in bovine raw milk, pasteurized milk and UHT milk according to EU Regulation 853/2004and 2073/2005. 14
Table 3: Product changes of UHT milk samples during storage at 20°C caused by heat resistant *Pseudomonas* peptidases. 18
Table 4: Summary of highly heat resistant spore formers isolated from raw milk and dairy farm environments. 20
Table 5: Sequence of a full recovery CIP cycle. 24

LIST OF FIGURES

Figure 1: Workflow of UHT processing. 9
Figure 2: Relevant bacteria in association with spoilage of UHT milk and its products. 16
Figure 3: Schematic diagram showing water treatment for CIP in an Austrian scale dairy. 25
Figure 4: Principle steps of process assessment. 28
Figure 5: Workflow during the screening process of bacterial residues in CIP rinse water. 30
Figure 6: Simplified illustration of the sampling stations ($n_{sterile}$= 12, $n_{non\text{-}sterile}$= 1). 31

1. INTRODUCTION

1.1. MILK AND MILK PRODUCT PROCESSING

In EU Directive No 1308/2013 drinking milk is defined as raw milk or heat treated milk which is intended for delivery without further processing to the consumer. Raw milk means milk which is not heated above 40°C or treated with another process aimed to reach an equal effect. Heat treated milk complies fat content requirements and is divided into whole milk with an fat content of at least 3.50%, semi-skimmed milk (1.50-1.80%) and skimmed milk (less than 0.50%) (https://www.legislation.gov.uk/eur/2013/1308/annex/vii/part/iv; accessed on 17-01-2021).

Regulation (EC) No 178/2002, Regulation (EC) No 852/2004, Regulation (EC) No 853/2004, Regulation (EC) form the legal base for the public health rules for trade and introduction into the EU (https://ec.europa.eu/food/animals/animalproducts/milk_en; accessed on 22-12-2020).

Pasteurized milk has an unopened shelf life of up to twelve days at refrigeration temperatures (maximum 8°C). ESL (extended shelf-life) milk has a "longer shelf life" of 21 to 30 days. The following processes are available for the production of ESL milk: 1. indirect heating by tubes or plates 2. direct heating by steam injection/steam infusion 3. membrane process (microfiltration) 4. depth filtration 5. sterilization separators (https://www.dlg.org/fileadmin/downloads/lebensmittel/themen/publikationen/expertenwissen/ernaehrung/e_2014_4_Expertenwissen_ESL.pdf; accessed on 17-01-2021).

In 2014, two thirds of the sold drinking milk products were ultra-high temperature (UHT) milk. The purpose of UHT milk processing is to produce a product, which will remain virtually, sensory and nutritional unchanged for a shelf life up to 12 months while kept at ambient temperature. EU Food Hygiene Regulations require defined temperature time combinations for heat treated milk products, these are listed in Table 1. For UHT milk and milk products a temperature treatment from at least 135°C is necessary. Diaries have to ensure these legal requirements with their HACCP schedule (MÄRTLBAUER and BECKER, 2016; KARLSSON et al., 2019).

UHT milk and milk products are referred to be commercially sterile. As bacterial cells are reduced exponentially through heat treatment, it is not possible to reach complete sterility after UHT processing. Therefore, commercial sterilisation aims to reduce the spoilage rate of UHT products to less than 1 in every 10 000th product (TAMIME, 2017). According to CERF and DAVEY (2001), the lack of sterility in UHT products could be explained statistically on the basics of residence time distribution of heat resistant spores in the high temperature holding tube. GRIFFITHS (2010) and

SARKAR (2015) named post sterilisation contamination to be the major reason for product spoilage after UHT treatment.

Table 1: Requirements for heat treatment of raw milk in accordance with Annex III Section IX Chapter II of EU Regulation No 853/2004 as amended by Commission Regulation No 1662/2006.

HEAT TREATMENT	TEMPERATURE (°C)	HOLDING TIME (SECONDS)	SHELF-LIFE
Pasteurisation	72-75	15-30	refrigerated at max. 8°C 6-12 days
HTST	127	1-3	refrigerated at max. 8°C 21-30 days
UHT	≥ 135	1-2	stored at room temperature up to 12 weeks
Sterilised, aseptic milk	110-120	10-30 min	stored at room temperature up to 12 months

Abbreviations: HTST, high temperature short time heating; UHT, Ultra-high temperature processing.
Source: MÄRTLBAUER and BECKER,2016; BURTON, 2012;
https://www.dlg.org/fileadmin/downloads/lebensmittel/themen/publikationen/expertenwissen/ernaehrung/e_2014_4_Expertenwissen_ESL.pdf;
https://foodsafety.foodscience.cornell.edu/sites/foodsafety.foodscience.cornell.edu/files/shared/documents/CU-DFScience-Notes-Milk-Pasteurization-UltraP-10-10.pdf; both accessed on 21-12-2020.

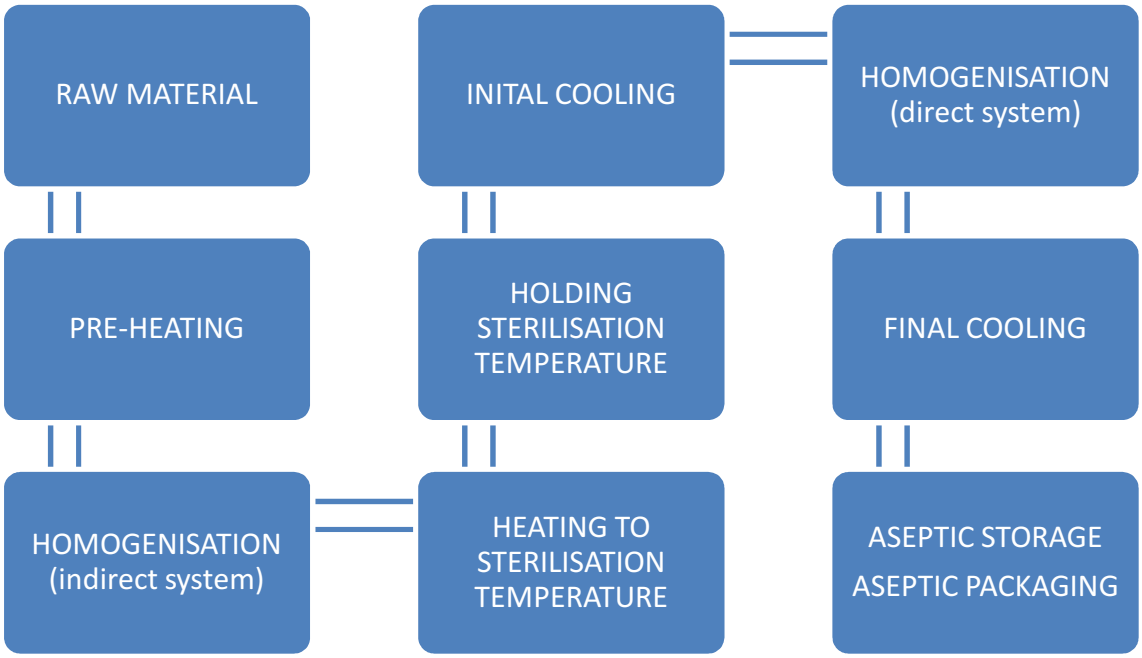
Different parameters enable the comparison of different temperature time combinations and their effect on bacterial destruction (TOKUŞOĞLU and SWANSON, 2014). According to DEETH and LEWIS (2017), the D-value is defined as the time required at a selected temperature to reduce the bacterial population by 1 log cycle. This means the higher the initial count of bacteria in the raw milk, the higher will be the count of remaining bacteria after heat treatment. The change in temperature which is necessary to produce a tenfold change in the decimal reduction time (D-value) of bacteria is called z-value. The lower the z-value, the higher will be the destruction of bacterial population caused by the temperature raise in heat treatment. Specific parameters for high temperature treatments are Q_{10} value and bacterial indices B* and $F_{0.}$ Q_{10} value describes the increase of bacterial destruction with a temperature change of 10°C.

While the z-value is given in °C, Q_{10} value is dimensionless. Q_{10} value is in relation with z-value as follows:

$$z\ (°C) = 10/\log Q_{10} \text{ or } Q_{10} = 10^{(10/z)}$$

The bacterial indices B* and F_0 allow to measure the bactericidal effect of a heating process, whereas the major one of relevance for UHT processing is B*. B* is a measure of bacterial destruction in comparison with a heat treatment at 135°C. A process with B* = 1 causes a nine decimal reduction of thermophile spores and is equivalent to holding the product at 135°C for 10.1 seconds (s) (TOKUŞOĞLU and SWANSON, 2014). UHT processes are required to have a B* value of at least 1, in practice most commercial UHT plants work with B* values from 2-20 (TRAN et al., 2008). The UHT process is an integrated series of united processing steps. The performance level of a UHT plant depends on the quality of each processing step, sterility of the heat treated milk, the production equipment and the aseptic packaging material has to be maintained equally. Figure 1 illustrates the major steps of UHT processing.

Figure 1: Workflow of UHT processing.



Source: https://www.newfoodmagazine.com/article/8203/uht-processing-of-milk/; accessed on 21-12-2020.

Due to different Q_{10} values for bacterial destruction, a high-temperature short-time (HTST) combination results in less chemical change than a low-temperature long time combination. Minimum time and temperature requirements are determined by the need to inactivate heat

resistant bacteria spores, while an upper limit for time and temperature treatments in UHT processes avoids great chemical alterations of the milk product. B* = 1 is considered to be the desired lower limit (F_0 = 3), while C* = 1 is representing as upper limit 3% reduction of thiamine in milk. This is the maximum acceptable amount of chemical change to the components of UHT treated milk (DEETH and LEWIS, 2017; LALIĆ, 2014). Homogenisation improves product stability and is used in the UHT processes primarily to avoid fat separation during storage and sediment formation in milk based beverages. It reduces thermal stability of milk proteins, therefore in direct UHT plants the homogeniser is placed downstream after product sterilisation to avoid reassociation of fat globules. The homogeniser performs under aseptic conditions if placed downstream, nevertheless it has to be regarded as a common source of bacterial contamination (DATTA and DEETH, 2003).

Two major energy sources for preheating the milk to sterilisation temperature are used, direct heating with culinary steam or indirect heating through conduction and convection from steam or hot water. In direct heating process, the milk is heated very rapidly with a rise of temperature of 60-70°C within 0.5s through steam injection or steam infusion. The condensed water mixed with the processed product is removed after sterilisation in vacuum flash down chamber. In indirect UHT plants, the milk is heated with a counter current flowing heating medium in tubular or plate heat exchangers, the heating can take several seconds to minutes. After reaching sterilisation temperature, the milk passes a temperature holding tube. The time the milk takes to pass the holding tube is referred to be the nominal sterilisation condition which is usually cited for a UHT process (DEETH and LEWIS, 2017; RASANE et al., 2020). The heating to sterilisation temperature and cool down parts of an UHT process are underestimated in indirect UHT plants, as they have a major contribution to B* and C* at temperatures from 75-90°C (TRAN et al., 2008). To combine an economical operation with a tolerable chemical change and a maximum extent of bacterial destruction, direct-indirect combination plants have been engineered (GRIFFITHS, 2010).

1.2. RAW MILK QUALITY AND MICROBIAL COMPOSITION OF RAW MILK, PASTEURISED MILK AND UHT MILK

"Raw milk means milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40°C or undergone any treatment that has an equivalent effect" (EU Regulation No 853/2004; https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32004R0853&from=en; accessed on 21-12-2020).

Raw milk quality is not only influenced by natural factors, but also by operational factors such as storage and transportation. Food business operators must ensure that in the case of daily collection the raw milk has to be cooled down immediately to a temperature of no more than 8°C and if not collected daily, the raw milk must be cooled down to no more than 6°C (EU Regulation No 853/2004; https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32004R0853&from=en; accessed on 21-10-2020). Raw milk which is selected for UHT processing has to be in a good microbiological condition. EU Directive No 326/2015 establishes microbiological quality criteria for bovine raw milk. The standard plate count (SPC) and the somatic cell count (SCC) are quality indicators to classify the raw milk in these different microbiological quality criteria. There are three microbiological quality categories bovine raw milk can be classified: S class (SPC ≤50 000/ml, SCC ≤250 000/ml), first class (SPC ≤100 000/ml, SCC ≤400 000/ml) and second class (SPC ≥100 000/ml, SCC ≥400 000/ml) (https://www.ris.bka.gv.at/GeltendeFassung.wxe?Abfrage=Bundesnormen&Gesetzesnummer=20009330; accessed on 22-12-2020).

The SPC is a good hygienic indicator for raw milk quality as it includes microbiological contamination occurred on the milk producing farm and the milk processing dairy (HOLM et al., 2004; JAYARAO et al., 2004; MURPHY et al., 2016). The SCC can be used to evaluate udder health of the dairy herd, as high counts of SCC results from high subclinical or clinical mastitis rates (ZADOKS et al., 2004). Due to increased spoilage and reduced shelf life stability of UHT products, raw milk should not contain high counts of heat resistant spores and heat resistant enzymes from non-spore forming psychrotrophic bacteria (BARBANO et al., 2006). Heat resistant spores in raw milk are identified by enumerating those microorganisms, which survive 80°C for 10 min. Other protocols use 100°C for 10 min. or 100°C for 30 min. resulting in a hundred-fold difference in reported estimated spore counts (TAMIME, 2017). These more stringent heating conditions are recommended since non-spore forming bacteria such as *Coryneformes* can survive heating at 80°C for 10 min (DEETH and LEWIS, 2017). The milking hygiene, environmental and cow hygience factors influence the bacterial spore counts in bulk tank

raw milk already on the dairy farm. The microbial composition of raw milk is highly diverse and may contain spoilage microorganisms, food borne pathogens as well as bacteria with beneficial or technological properties (MARTIN et al., 2019).

The initial, i.e., immediately after milking, predominance of mesophilic, Gram-positive, lactic acid-forming bacteria (*Lactococcus, Streptococcus, Lactobacillus, Leuconsostoc, Enterococcus* species) in raw bovine milk (QUIGLEY et al., 2013) shifts over the course of storage until processing, as these organisms show absent or very slow growth under refrigeration conditions (FRICKER et al., 2011). The microbial profile of longer refrigerated raw milk consists mainly of psychrotrophic, non-spore-forming Gram-negative bacteria, with *Pseudomonas* species predominating (ZHANG et al., 2019a).

Generally, vegetative cells are destroyed by heat treatment such as high-temperature short-time (HTST) pasteurisation (MACHADO et al., 2015), but produce heat stable spoilage enzymes, which may lead to a reduced shelf life stability of UHT milk products. The SPC of stored cooled raw milk is a reasonable guide to the probability of heat resistant enzymes being present as *Pseudomonas* species constitutes the majority of bacterial population (VELÁZQUEZ-ORDOÑEZ et al., 2019). The constitution of bacteria population in raw milk has also an impact on sensory quality of pasteurised milk products. DING et al. (2020) investigated the relation between sensory quality of pasteurised dairy products and microbial contamination in raw milk. *Pseudomonas*, *Omithimimicrobium*, *Cyanobacteria* and *Corynebacterium* had positive correlations with the flavour substances, whereas the Gram-positive *Streptococcus* and *Paeniclostridium* had significant negative correlations with these substances.

Before heat treatment of raw milk, bacteria population can be reduced through bactofugation. RIBEIRO-JÚNIOR et al. (2020) stated that the number of *Bacillus (B.) licheniformis*, *B. toyonensis, Micrococcus aloeverae* and *Aestuariimicrobium kwangyangense* could be reduced by 33, 43, 86 and 92% by bactofugation. *Macrococcus caseolyticus*, *Lysinibacillus varians*, *Carnobacterium divergens*, *Microbacterium hominis*, *Kocuria indica*, *Micrococcus yunnanensis*, *Gordonia paraffinivorans*, *B. invictae* and *Kocuria kristinae* were reduced by bactofugation to undetectable levels before pasteurisation. The microfiltration of raw milk aimed for UHT processing increases storage stability of UHT milk at room temperature, as microfiltered UHT milk is less prone to proteolysis during storage and prevents fat creaming (D'INCECCO et al., 2018).

The primary purpose of pasteurisation is to inactivate foodborne pathogens and to render the milk safe for human consumption. Foodborne pathogens associated with the consumption of raw milk or inadequate pasteurised milk are *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella* spp.,

Escherichia (E.) coli including *E. coli* O157:H7, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Coxiella burnetti* (CLAEYS et al., 2013; DE BUYSER et al., 2001; DUYNHOVEN et al., 2009; FARROKH et al., 2013; FOX et al., 2009; TEH et al., 2015). *Mycobacterium avium spp. paratuberculosis* (MAP) has raised concern because it may survive pasteurisation and is pathogen of Crohn's disease in humans (GREENSTEIN, 2003). Foodborne pathogens contaminate the bovine raw milk at primary milk production as these bacteria are associated to dairy cattle (HEREDIA and GARCÍA, 2018).

Dairyborne disease in low- and middle-income countries (LMIC) are highly related to bacterial hazards as *Mycobacterium bovis*, *Campylobacter* spp. and *Salmonella enterica* and chemical hazards as mycotoxins, dioxin and heavy metals (GRACE et al., 2020).

Raw milk may also contain thermotolerant bacteria which survive pasteurisation temperature including spore forming microorganisms such as *Bacillus (B.)*, *Geobacillus*, *Paenibacillus* and *Clostridium* species and non-spore forming microorganisms like coryneformes, *Micrococcus* and *Streptococcus* species (DEETH and LEWIS, 2017). MUIR (1996b) named *Pseudomonas* species and *Bacillus* and *Bacillus*-like spore formers as microbial raw milk contaminants with the most concern for dairy industry in regard to the keeping quality of high temperature processed milk products. UHT treatment is designed to destroy all non-spore forming and most spore forming bacteria. Yet highly heat resistant spore formers like *Geobacillus stearothermophilus*, *B. sporothermodurans*, *B. subtilis*, *B. megaterium* and *Paenibacillus* lactis are enabled to survive common UHT heating conditions (HASSAN et al., 1993; INTARAPHAN, 2001; MUIR, 1990; PETTERSSON et al., 1996; SCHELDEMANN et al., 2004). In EU Regulation No 2073/2005 and No 853/2004 the criteria for bacterial contamination in bovine raw milk aimed for UHT processing are listed (Table 2).

Table 2: Microbiological criteria in bovine raw milk, pasteurized milk and UHT milk according to EU Regulation 853/2004and 2073/2005.

MICROBIOLOGICAL CRITERIA	LIMIT	TIME OF EXAMINATION
BOVINE RAW MILK		
SPC at 30°C incubation temperature	< 300 000 CFU/ml	immediately before processing
DRINKING MILK AND PRODUCTS THEROF		
Listeria monocytogenes	not detectable in 25g	leaving the dairy factory
Enterobacteriaceae	M≤10 CFU/ml Two-class plan with n = 5, c = 0	at the end of processing
UHT MILK AND AND PRODUCTS THEROF		
Listeria monocytogenes	not detectable in 25g	leaving the dairy factory
SPC after incubation of 15 d at 30°C	stable pH value and <1 CFU/ml	at the end of processing
SPC after incubation of 7 d at 55°C	stable pH value and <1 CFU/ml	at the end of processing

Abbreviations: SPC, Standard plate count; M, maximum count; n, number of product samples; c, number of product samples ≥ 10 CFU/ml; CFU, Colony forming unit
Source: EC 853/2004: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32004R0853&from=EN; EC 2073/2005: https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32005R2073&from=EN; both acessed on 17-01-2021.

1.3. SPOILAGE ASSOCIATED BACTERIA

Milk processing lines offer a wide variety of microenvironments where hygiene indicator bacteria and potential pathogens are able to proliferate, to form biofilms and potentially recontaminate heat treated milk products (MARCHAND et al., 2012; TEH et al., 2015). Spoilage microbiota in milk are of important concern for the dairy industry as the growth of spoilage microorganisms and the production of metabolic by-products and extracellular enzymes decreases sensory quality of the final milk product. Extracellular enzymes cause deterioration during storage, reduce shelf life and in severe cases the yield of final milk products fit for sale (DEETH and LEWIS, 2017; DOYLE, 2009). Figure 2 shows an overview of bacteria associated with the spoilage of UHT processed milk products. Psychrophiles (e.g. *Arthrobacter*, *Psychrobacter*, *Halomonas, Pseudomonas, Hyphomonas* and *Sphingomonas*) are cold-adapted with a growth optimum of 15°C or lower and a maximal growth potential at about 20°C. Psychrotrophs (e.g. *Pseudomonas, Psychrobacter*, and *Arthrobacter*) are cold-tolerant with an optimal growth above 15°C (FURHAN, 2020; MOYER and MORITA, 2007). Both are relevant spoilage causing microorganisms for dairy processing. Many species are able to grow at refrigeration temperatures, although they show optimum growth under mesophilic conditions (TEH et al. 2015). Bacteria able to grow at temperatures lower than 7°C are psychrotrophic and account for 10% of the total bacterial population in milk produced under good hygiene conditions and for 75% in poor-hygienic-quality milk (ODEYEMI et al., 2020).

I. ENZYME PRODUCING GRAM NEGATIVE BACTERIA

Pseudomonas (P.) the most important non spore forming psychrotrophic bacterial genus in cooled raw and pasteurised milk, has optimum growth temperatures between 25-30°C (DE SANTANA et al., 2020).

YUAN et al. (2018a) studied the enzymatic degradation of raw milk by the lipolytic and proteolytic spoilage flora. *Yersinia intermedia* followed by *P. fluorescens* indicated the highest proteolytic activity, whereas *Acintetobacter* in detail *Acinetobacter guillouiae* was highly lipolytic.

β-D galactosidase and phospholipase activity was observed for certain spoilage candidates. The bacterial population within the same species expressed different proteolytic and lipolytic enzyme activity.

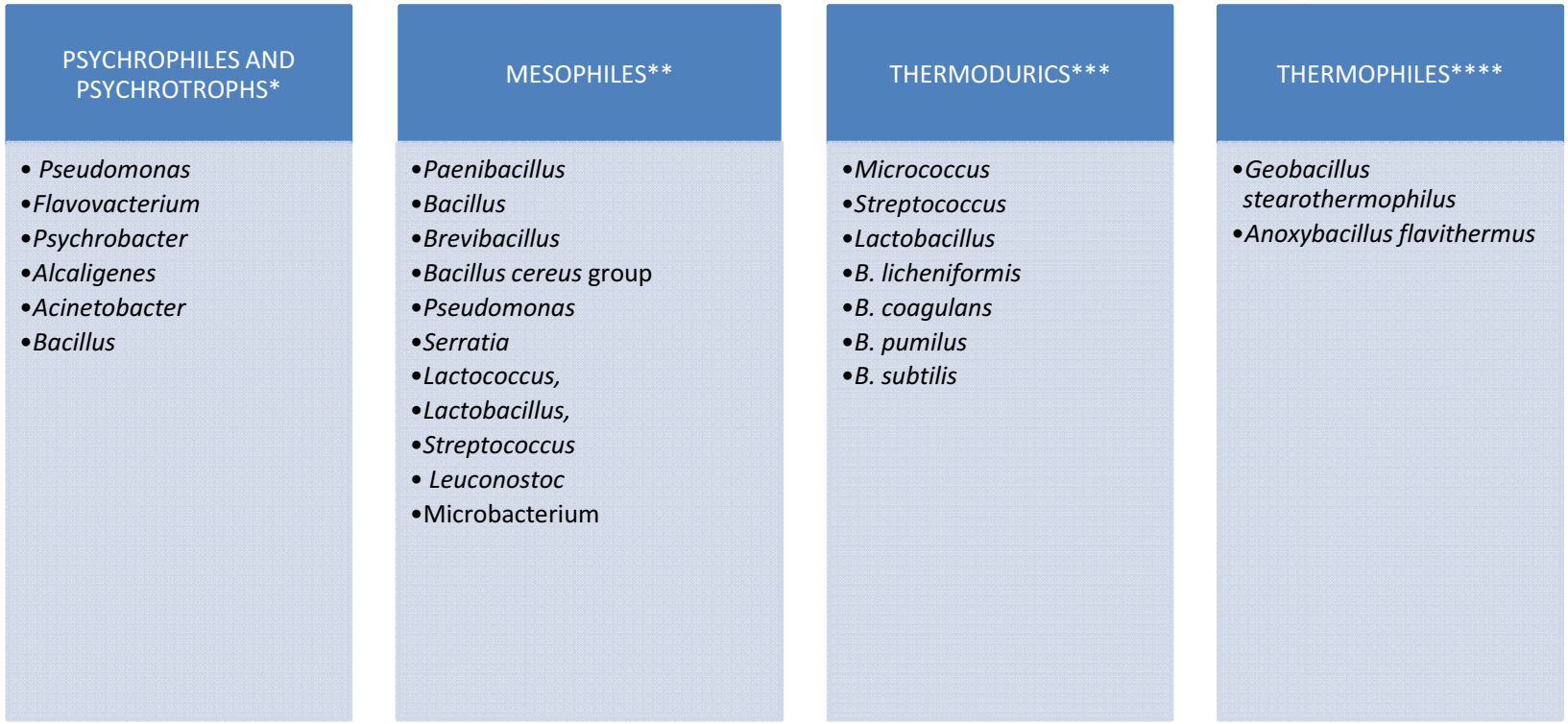
Pseudomonadaceae grow either as planktonic cells or within biofilm attached to processing surfaces and produce heat resistant extracellular enzymes (NÖRNBERG et al., 2011; TEH et al., 2011). While pasteurisation inactivates vegetative cells of psychrotrophic bacteria, heat-stable enzymes survive the heat treatment and remain active. Their heat stability increases when multiple heat-stable enzymes, such as proteases are present (CHOPRA and MATHUR, 1985). The latter enzymes have been found to reduce the shelf life of UHT milk during storage at ambient temperature (BARBANO et al. ,2006).

Especially, AprX molecules produced by proteolytic *Pseudomonas* strains hydrolyze κ-, β-, and α-caseins. AprX is particularly involved in inducing solid and compact gels in UHT milk by hydrolyzing κ-casein (ÅKERSTEDT et al., 2012; ZHANG et al., 2019b).

Heat resistant bacterial lipases in UHT milk products cause lipolysis of milk fat and reduce the sensory quality and the stability of milk foam in beverages (HUPPERTZ, 2010). The proteolytic and lipolytic activity of bacteria within dairy biofilms is greater than that of bacteria in a planktonic state (TEH et al., 2012, 2013).

TEH et al. (2014) studied the effect of biofilms attached to the surface of raw milk tankers on the quality of final UHT milk. They demonstrated that more proteolysis occurred in UHT milk made from biofilm exposed raw milk compared to controlled raw milk which had not been exposed to biofilms.

Figure 2: Relevant bacteria in association with spoilage of UHT milk and its products.



Abbreviations: * grow rapidly at 7°C and below; ** grow at 20-40°C; *** survive, but do not grow at pasteurisation temperature; **** grow optimally at 55°C

Source: BURGESS et al., 2010; DEETH and LEWIS,2017, MACHADO et al., 2017; TEH et al. 2015.

Heat resistant enzymes produced by psychrotrophic bacteria growing in biofilms can remain attached to the biofilm matrix, be trapped within or can be released from the biofilm (KHAJANCHI et al., 2009; RAJENDRAN et al., 2010). Biofilms and quorum sensing (QS) have been identified as important factors in the deterioration process of milk. Production and heat stability of enzymes are enhanced in biofilms which contain protective shields of extracellular polymeric substances (EPS). QS modulates the expression of hydrolytic enzymes and the construction of the biofilm matrix (YUAN et al., 2018b).

The pores within the biofilms constitute a microenvironment for enzymatic activities and provide protection against hazardous environmental conditions such as high heat treatment for both the bacterial cells and the extracellular enzymes (FLINT et al., 2020; YUAN et al., 2018b).

Stress response may facilitate the enzyme production, whereas the accumulation of extracellular enzymes in biofilms increases the ability of planktonic cells to survive (SPECTOR and KENYON, 2012; THOMASON et al., 2012).

The enzyme secretion of psychrotrophic bacteria is influenced by environmental and bacterial population factors such as temperature, phase of bacterial growth, nutrient availability and bacterial communication and usually peaks during mid to late exponential or early stationary phase of growth (LU and WANG, 2017; OLIVEIRA et al., 2015;TEH et al., 2015).

Pseudomonas spp. varies considerably in their propensity to produce proteases and lipases. Besides members of the *P. fluorescens* group the species *P. fragi* and *P. gessardii*-like are related to milk spoilage. Furthermore, *P. proteolytica* or *P. brenneri* accumulated in raw milk, and *P. peli*-like in pasteurized milk. *P. lundensis, P. helleri* and *P. weihenstephanensis* are novel species detected in cow milk (MACHADO et al., 2017).

The amount of heat stable *Pseudomonas* peptidases after UHT processing influences the dimension of the final milk product defect, this correlation between residual proteolytic activity and the decrease in sensory quality is summarised in Table 3. Product defects due to bacterial peptidases in indirectly heated UHT milk during shelf life at 20°C correlate with proteolytic activity. The product defects bitterness - particle - creaming - sedimentation - gelation occurred in one experiment for all samples containing peptidases (apparent enzyme activity ≥ 0.03 pkat/ mL) (STOECKEL et al., 2016).

In the literature different microbial criteria for the heat stable enzyme contamination in raw milk aimed for UHT processing exist. Raw milk should contain less than 0,3 ng/ml of protease to be stable at least four months at room temperature (MITCHELL and EWINGS, 1985) or should have a total bacterial count of less than 10^5-10^6 CFU/ml (DEETH and LEWIS, 2017), according to ELLNER (2015) 10^4-10^5 CFU/ml. The significance of the total bacterial count as indicator for the risk of contamination

through extracellular enzymes is controversial because different psychrotrophic bacteria have different propensities to heat resistant enzyme production (HARYANI et al., 2003).

The influence of milk fat on the proteolytic activity of *Pseudomonas* is high. Whole milk compared to skim milk is more vulnerable to proteolytic activity (ZHANG et al., 2020).

Table 3: Product changes of UHT milk samples during storage at 20°C caused by heat resistant *Pseudomonas* peptidases.

RESIDUAL ENZYME ACTIVITY (PKAT/ ml)	STORAGE (MONTHS)	PRODUCT DEFECTS
0.05		Bitterness
0.07	1	Bitterness
0.07	2-4	Creaming (cream layer ≥1cm)
≥0.07	3-4	Sedimentation >5%
>0.16	4	Gelation

Abbreviations: pkat, petakatal ; Source: STOECKEL et al. (2016).

II. SPORE FORMING BACTERIA

Another important group of microbiota associated with the spoilage of UHT milk products are aerobic spore forming bacteria, particularly *Bacillus* and *Bacillus* like species. Common characteristics of bacteria associated to the genus *Bacillus* are Gram positive, motile, rod shaped, occurring in chains and harbouring a central or terminal spore and include psychrotrophic, mesophilic and thermophile species (GRUMEZESCU and HOLBAN, 2018; RAY and BHUNIA, 2007; https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/spore-forming-bacteria; accessed on 24-01-2021). Vegetative cells are destroyed by pasteurisation temperatures, but their spores are heat resistant with some spores surviving heating conditions at 100°C for 30 minutes (TEH et al. 2015) and having the ability to germinate after sterilisation when conditions are ideal (LOPEZ-BREA et al., 2018).

Thermoduric and thermophilic sporeformers (*B. coagulans, B. megatherium, Anoxybacillus flavithermus, B. sporothermodurans, B. licheniformis*, and *Geobacillus stearothermophilus*) can survive milk pasteurization and cause spoilages of products made thereof (GLEESON et al., 2013; KHANAL et al., 2014). Thermophilic spore formers can contaminate UHT milk and its products both in their vegetative and spore state and cause off-flavours or curdling of milk in final milk products due to their ability to grow >55 °C (DE JONGHE et al. 2010; RANIERI et al. 2009a; TEH et al. 2015).

The contamination route of *Bacillus* and *Bacillus* like species into the milk can be either directly on the dairy farm or in a second step on the dairy plant through growth within the milk storage, survival of spores after pasteurization and post-pasteurisation contamination. *Bacillus* population and prevalence in milk increase steadily along the dairy processing chain (ORTUZAR et al., 2018).

MARTINEZ et al. (2017) observed that the spore former population found in raw, pasteurized and concentrated milk was similar. *Paenibacillus* was primarily associated with concentrated milk and can cross-contaminate and recontaminate other dairy products. Other *Bacillus* species found in concentrated milk were *B. clausii, B. subtilis, Lysinibacillus*, *B. safensis, B. licheniformis, B. sonorensis*, and *Brevibacillus,* with the last three additionally having a thermophilic profile.

The teats of cows appear to be one of the primary routes by which bacteria and their spores (bacilli and *Clostridia*) enter raw milk. Spore forming bacteria are ubiquitous in the farm environment and can be isolated from a wide variety of materials, including feed, bedding materials, manure, silage, soils and milking shed wash water (MARTIN et al., 2019). Udder hygiene influences the presence of spore formers on teat skin and entrance into the milking pipelines and storage tanks (BAVA et al., 2017; EVANOWSKI et al., 2020).

The species of *Bacillus* contaminating in raw milk is influenced by the season. *B. cereus* usually found in raw milk during grazing period as this spore former is associated with soil, while *B. licheniformis* is usually found in winter and associated with bedding material (GOPAL et al., 2015; SCHELDEMAN et al., 2006). In Table 4 different *Bacillus* species according to their isolation on dairy farm are depicted, these sporeformers were isolated in raw milk, dairy farm environment or both. Summer temperatures and conditions may favor proliferation of sporeforming bacteria. BUEHNER et al. (2014) identified *B. licheniformis* as the major contaminant regardless of season. In this experiment, corn silage was the major environmental source of sporeformers with higher concentrations in summer. Nevertheless, the reports on the seasonal effect on the incidence of *B. cereus* spores in raw milk in literature are inconsistent.

Some *B. circulans*, *B.cereus* and *Paenibacillus* sp. strains are able to grow at temperatures < 7°C although their optimum temperature for growth is 20-30°C (DEETH, 2017; UBONG et al., 2019).
This ability is particularly relevant for refrigerated stored milks such as extended shelf life milk (ESL). MEER et al. (1993) isolated 12 different psychrotrophic *Bacillus* species from refrigerated milk

including *B. licheniformis*, which is considered to be mesophilic or thermophile (DELAUNAY et al., 2020).

Table 4: Summary of highly heat resistant spore formers isolated from raw milk and dairy farm environments.

SPORE FORMER	% ISOLATES FROM RAW MILK	ISOLATES FROM ANIMAL FEED AND MILKING EQUIPMENT	
		> 10% of total isolates	> 4% of total isolates
B. licheniformis	22.3	*B. pallidus*	*B. subtilis* group
B. pallidus	15.1	*B. licheniformis*	*B. farraginis*
Brevibacillus	10.8		*Brevibacillus agri*
Paenibacillus	10.2		Other *Brevibacillli*
Other *Bacilli*	9.6		*Geobacillus* spp.
Virgibacillus	9.0		*B. smithii*
Ureibacillus	6.6		
Aneuribacillus	1.2		
Bacillus barbaricus	1.2		
Bacillus fortii	1.2		
Bacillus smithii	1.2		
Bacillus subtilis	1.2		

The *Bacillus* species are colored corresponding to their isolate origin. Source: SCHELDEMANN et al. (2005), DEETH and LEWIS (2017).

The generation and lag times of psychrotrophic spore formers at refrigeration temperatures (2-7°C) are longer than those of *Pseudomonas* (MC KELLAR, 1989).

The heat stability of proteinases, lipases and phospholipases produced by *Bacillus* species is comparable to the corresponding enzymes from *Pseudomonas* species (SHIEH et al., 2009; MACHADO et al., 2017).

Spore formers producing highly heat resistant spores (surviving 125°C for 30 min) are mostly thermophiles (*Geobacillus* spp.) and occasionally mesophilic species as *Brevibacillus (Br.) brevis*. SADIQ et al. (2016) reported that *Paenibacillus macerans* showed the highest proteolytic activity besides *B. cereus* group, *Br. brevis, B. subtilis, G. thermoleovorans* and *Virgibacillus proomii*. The highest lipase activity was observed from *B. licheniformis*. Phospholipase activity was exclusively observed from *B. cereus* sensu lato and *Br. parabrevis*.

Paenibacillus has become very important for dairy industry as it is produces highly heat resistant spores and can growth both at refrigeration and ambient temperatures (MUGADZA et al., 2018), some strains are able to survive HTST and UHT treatment (SADIQ et al., 2018). BENO et al. (2020) isolated *Paenibacillus* spp. from fluid milk with a high interspecies diversity and *Paenibacillus odorifer* as predominant species. *Paenibacillus odorifer* harbors a adaption rich portfolio of nitrate/nitrite reduction pathways and cold-shock proteins enabling the growth at refrigeration temperatures.

Due to the increased international trade in UHT milk with the transport across tropical climate zones and the intermediate storage at high ambient temperatures spoilage problems with these thermophilic bacteria may be a raising problem (DEETH and LEWIS, 2017).

Mesophilic spore formers like *B. subtilis*, *B. sporothermodurans* and *B. cereus* can cause UHT product spoilage at ambient temperatures as they have the ability to produce highly heat resistant spores and optimum growth temperatures from 20-40°C (VYLETELOVA et al., 2002; PINTO et al., 2018).

In a study by MEHTA et al. (2019) the highest levels of proteolysis at 24°C were shown by *B. mojavensis, B. cereus, B. subtilis* and *Paenibacillus polymyxa* identified by the non-casein nitrogenat content.

From a public health perspective, *B. cereus* is considered the most pertinent microbial hazard in UHT products (PUJOL et al., 2015). *B. cereus* is not only an important spoilage associated microorganism for dairy industry, but also a pathogenic spore former potentially producing cereulid and enterotoxins (EHLING-SCHULZ et al., 2019). The *B. cereus* group comprises at least eight species, which are difficult to distinguish phenotypically*: B. anthracis, B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. cytotoxicus, and B. toyonensis* (EHLING-SCHULZ et al., 2019; LIU et al., 2015). Based on the analysis of 16S rRNA gene sequences LIU et al. (2017) assigned novel strains which share over 97 % similarity with the known species (*B. paranthracis*, *B. pacificus*, *B. tropicus*, *B. albus*, *B. mobilis*, *B. luti*, *B. proteolyticus*, *B. nitratireducens* and *B. paramycoides).* CAROLL et al. (2020) proposed rather to concentrate on eight genomospecies in the *B. cereus* taxonomy: *B. pseudomycoides, B. paramycoides, B. mosaicus, B. cereus s.s., B. toyonensis, B. mycoides, B. cytotoxicus, B. luti*) that correspond to resolvable clusters obtained at a ≈92.5 average nucleotide identity (ANI).

Management factors (udder hygiene, husbandry, feeding) have a major influence on *B. cereus* spore counts at farm level (FEI et al., 2019). O´CONNELL et al. (2013) observed higher counts when cows had beed housed in comparison to extensive farming (201 versus 50 CFU/ml).

In the Netherlands, the average *B. cereus* spore concentration is 1.2 log/l and the limit of *B. cereus* spores in farm tank milk of 3 log/l is required, to achieve a shelf-life for pasteurized milk of at least seven days (HEYNDRICKX, 2011; VIDIC et al., 2020).

B. cereus is one of the most important spoilage microorganisms and biofilm former in the dairy environment and its growth leads to dairy product deterioration. Especially, *B. cereus* spores and also *Bacillus* spores in general survive the Cleaning-in-place (CIP) regimes in biofilms (OSTROV et al.,2019). Only, alkali-based CIP decreases *B. cereus* contamination events of dairy products (KUMARI and SARKAR, 2016). SHAHEEN et al. (2010) observed that spores highly resistant to hot 1% sodium hydroxide may be effectively inactivated by hot 0.9% nitric acid during the cleaning of dairy silo tanks.

Several strains of *B. cereus* are able to produce toxins, which have the potential to cause diarrhoea and emetic syndromes (MESSELHÄUßER and EHLING-SCHULZ, 2018). The emetic syndrome is caused by cereulid which is heat stable (90 min at 126°C), pH resistant (pH 2-11) and is not destroyed by cooking or digestion. An amount of > 5 log *B. cereus* cfu/g food leads to toxin production which is harmful, but also few cells may cause intoxication. Entertoxins are rather heat-labile, therefore larger amounts of *B. cereus* containing HBL, Nhe or CYTK have to be ingested (3-5 log cfu/g) (HUANG et al., 2020; JESSBERGER et al., 2020; https://www.fil-idf.org/wp-content/uploads/2016/12/Bacillus-cereus-in-Milk-and-Dairy-Products.pdf; accessed on 24-01-2021). At optimal storage of milk (4°C), the *B. cereus* population remains stabile, but under suboptimal conditions (8°C) *B. cereus* grow. Many mesophilic and psychrotrophic *B. cereus* isolates originating from milk carry enterotoxin genes (*nheA* and *hblA, cytK2*) (PORCELLATO et al., 2021). In UHT milk (LIN et al., 2017) *B. cereus* strains were associated to 17 genotypes by multi-locus sequence typing. These strains harbored to a majority the enterotoxin gene *nhe* (74%) and the emetic toxin gene (48%) *ces*. Some strains were able to tolerate hot-acid or hot-alkali when grown in biofilms.

In pasteurised milk, sweet curdling (coagulation of the milk without acidification) is mainly caused by *B. cereus* proteolytic behaviour. Sweet bitterness is caused by *B. cereus* lecithinase activity that hydrolyses phospholipids in the milk fat globule membrane (POLTRONIERI et al., 2017).

III. OTHER SPOILAGE ASSOCIATED BACTERIA

Gram-positive cocci can be either thermoduric or may recontaminate drinking milk (Figure 2), *Microbacterium lacticum* can be isolated from micro-filtered milk. BELLASSI et al. (2020) revealed low proteolytic and lipolytic activity, but the ability to form biofilms. The strains are able to grow to high cell numbers and perform an acidification in heat-treated milk that could pose a potential risk to the final quality.

CHAJĘCKA-WIERZCHOWSKA et al. (2020) found that enterococci [*Enterococcus faecium* (53.4%) and *Enterococcus faecalis* (34.4%)] are widely present in retail ready-to-eat dairy products in Poland. Many isolated strains are antibiotic resistant (e. g. streptomycin, erythromycin, tetracycline) and carry transferable resistance genes (*tet*(M) *tet*(L)*, erm*(A) and *erm*(B)), which pose a risk of transmission of multidrug-resistant bacteria to consumers.

Streptococcus thermophilus is highly adapted to the dairy environment, forms undesired biofilms which contribute to an ecological benefit for its survival and persistence (BASSI et al., 2017)

Pasteurization induces a Viable-but-not-culturable (VBNC) state in staphylococci. VBNC is of major concern for *Staphylococcus aureus*, which is able to produce heat-stable enterotoxins and is well described for antimicrobial resistance (*blaZ, mecC* and *tetK* plasmid-mediated AMR genes) (TAHER et al., 2020).

1.4. PROCESS HYGIENE AND SAFETY CRITERIA

A high standard in dairy plant hygiene is necessary to produce an UHT milk product with a good microbiological quality, which results in a stable shelf life. Cleaning in place (CIP) is defined as “The cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. The process involves the jetting or spraying of surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity” (CHAVAN and GOYAL, 2018; ROMNEY, 1990). The main target of CIP is a continuable effective cleaning and disinfection of the dairy plant with less economical effort (VERRAN, 2002). In the dairy industry the full recovery, reuse or three tank system with an optional heated rinse tank is commonly in practice. The central CIP station of the full recovery system consists of a water buffer tank, a detergent recovery tank and a rinse recovery

tank (TAMIME, 2008). In Table 5 the principle cleaning operations of a fully recovery CIP system are listed.

Table 5: Sequence of a full recovery CIP cycle.

CYCLE	DESCRIPTION
1	Purge out residual products in the plant with air or water
2	Establish a return flow using cold water
3	Pre-rinse to drain
4	Purge out the pre-rinse water with dilute detergent
5	Return dilute detergent to tank and recirculate for a set time
6	Monitor temperature and conductivity of the detergent
7	Recover the detergent
8	Intermediate rinse to recovery tank
9	Inject acid
10	Circulate the acid solution
11	Intermediate rinse to drain
12	Inject disinfectant
13	Recirculate disinfectant
14	Final rinse

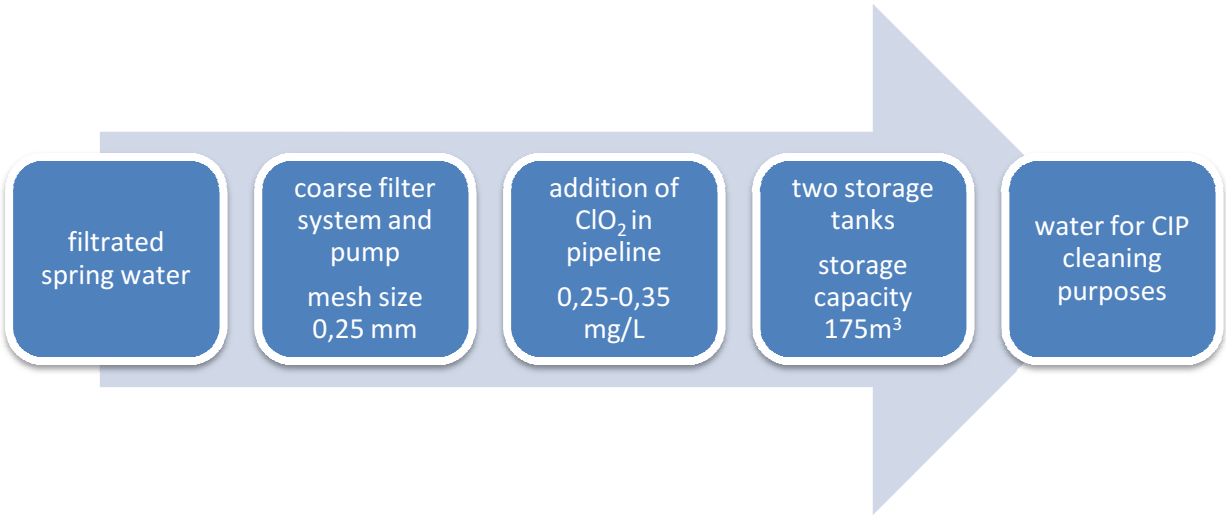
Source: TAMIME (2008).

At the start of a cleaning sequence, the detergent is recirculated through a make-up loop circuit, which contains conductivity and temperature probes to ensure the detergent is dissolved in the optimum concentration and held at its temperature optimum, as these physicochemical parameters are important to ensure an effective cleaning process (CHMIELEWSKI and FRANK, 2003; FRYER et al., 2006).The water buffer tank is used to establish a hydraulic loop before the pre-rinse cycle to purge the residual product out of the pipelines, which can be recovered in a suitable vessel (https://www.controleng.com/articles/control-design-for-cip-systems/; accessed on 24-01-2021).

The following pre-rinse cycle is important for an effective CIP cycle: Recovered water from the intermediate or final rinse stage of the previous CIP cycle is reused to utilise heat energy and residual detergent solution (https://www.resourceefficient.eu/pt/node/139; accessed on 24-01-2021). Detergents residues in the final rinse water lead to a more effective pre-rinsing effect and in addition water consumption can be reduced reusing the recovered water (DHAGE and DHAGE, 2016). In the first alkaline detergent circulation, the main task of cleaning takes place resulting in the organic soil being solved from the plant surface and held suspended in the detergent solution. Alkaline sodium salts like sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), silicates and phosphates are used to

attack the organic components of milk residues in the processing plant (https://www.sciencedirect.com/topics/food-science/clean-in-place; accessed on 24-01-2021). After an intermediate rinse with hot potable water, a second acidic detergent circulation follows to attack inorganic product debris (WILDBRETT, 2006a; ROBINSON, 2014). The most common acid detergents to remove water and milkstone scale are phosphoric acid (H_3PO_4), nitric acid (HNO_3) and citric acid ($C_6H_8O_7$) (PAUGAM et al., 2013). In the second intermediate rinse cold potable water is used, the water quality in this cleaning step has to be observed critical if there is no following disinfection stage with oxidising biocides or hot steam (TAMIME, 2008). Also the final rinse after optional disinfection requires a high microbiological water quality to avoid post disinfection contamination of the dairy plant (THOMAS et al., 2016). The addition of chlorine dioxide (ClO_2) can be used to increase the microbiological quality of rinse water (DeQUEIROZ and DAY, 2007). Regarding economical and ecological aspects, DOGAN et al. (2020) investigated the microbial safety of wastewater reuse in CIP systems and the potential risk of *Listeria monocytogenes* infection through raw milk contamination. The study's results indicate that appropriate treated wasterwater could be an alternative to potable water for CIP applications. The tolerable limit of contamination in CIP water was estimated to be -2 log CFU/ml. The water supply and treatment for CIP of an Austrian scale dairy is schematically illustrated in Figure 3.

Figure 3: Schematic diagram showing water treatment for CIP in an Austrian scale dairy.



Abbreviations: ClO2, Chlorine dioxide.

The efficiency of a cleaning detergent and disinfectant used for CIP depends on the interaction of different factors: The contact time between detergent or disinfectant and the surface to be cleaned

has to be long enough for the substance to complete its cleaning effect (FRYER et al., 2006). The mechanical action of the solution caused by flushing with high pressure and turbulence supports the cleaning and disinfecting performance (BREMER et al., 2006). The adjustement of the detergent or disinfectant specific optimum concentration is very important (TEUFEL, 1984; MOERMAN et al., 2014) to reach a maximum cleaning or disinfecting effect combined with minimum economical effort. The concentration exponent of a disinfectant measures the effect of changes in its concentration on cell- death rate through determining the time needed for two different concentration of the disinfectant to produce the same cell-death rate in a bacterial suspension. The effectiveness of a disinfectant is in general exponentially related to its concentration (SCHIRONE et al., 2019). The temperature of the cleaning detergent and disinfectant used has to be monitored continuously during the CIP cycle as in general the higher the temperature, the more effective is cleaning or disinfection (CHMIELEWSKI and FRANK, 2003). Another important factor in the design of a CIP process is the detergent to soil ratio, which is influenced by the quality of the pre-rinse cycle (LAMBERT and JOHNSTON, 2001; TAMIME, 2008). In general, the performance of a disinfectant in a CIP cleaning cycle depends on the nature of the used disinfectant (PARKAR et al., 2003) and its antimicrobial effect on the predominant bacteria. SIKKEMA et al. (1995) named Gram-negative bacteria more resistant against disinfectants than Gram-positive bacteria. Product residues in the dairy plant pipelines results with a residual moisture in an increased resistance of unselective bacteria against disinfection than selective bacteria (EDELMAYER, 1983). LINDSAY et al. (2002) and STORGARDS et al. (1999) investigated the mutual influence of *Bacillus* sp. and *P. fluorescens* on their resistance against disinfection. *B. subtilis* is more resistant against disinfection than *P. fluorescens* because of its ability to form spores, whereas the disinfecting sensitivity of *P. fluorescens* increases in presence of *B. cereus* (WINTER, 2009). The composition of bacterial cell envelope and the presence of biofilms also influence the disinfecting performance of the CIP sequence. Biofilms are more resistant against disinfection than planctonic bacteria (CHMIELEWSKI and FRANK, 2003) and the resistance of bacteria attached in biofilms increases if the biofilm is microbiological diverse (LINDSAY et al., 2002). OSTROV et al. (2016) investigated the effectiveness of cleaning agents in removal of biofilm derived spores in milking system. In this study, the spore removal effect and the cleaning and disinfecting effect toward biofilm derived spores were evaluated. The study showed that besides the mechanical effect of flow turbulence (pipeline T-junctions with different length were installed in the

CIP model system) elevating the temperature from 35 to 50 °C leads to a 0.5 log improvement in the efficiency of cleaning out biofilm derived spores.

The DVG (Deutsche Veterinärmedizinische Gesellschaft) describes applications criteria and desinfecting suitability for chemical disinfectants used in the food industry (http://www.desinfektion-dvg.de/index.php?id=1801; accessed on 30-01-2021). The chemical disinfectants used in the CIP process must be controlled for antimicrobiological effectiveness, practical compatibility and biodegradability.

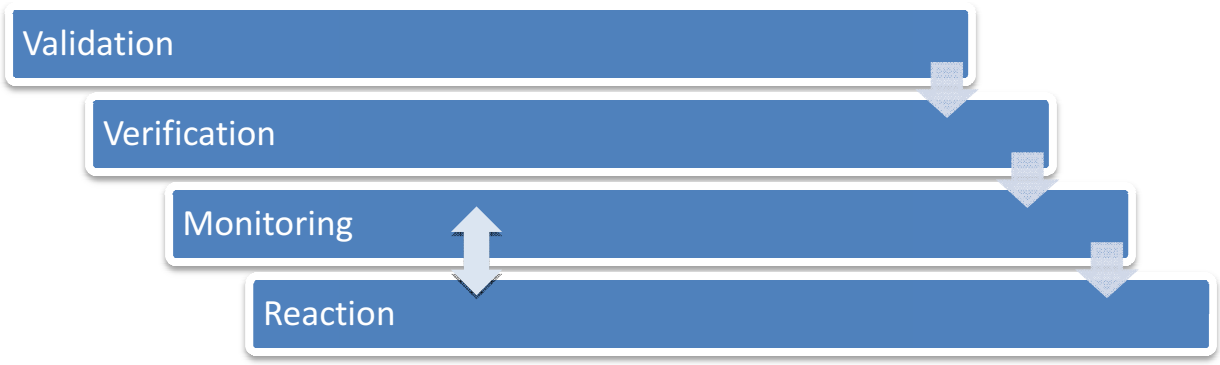
Regulation (EU) No. 528/2012 regulates the sale and supply and use of biocidal products throughout Europe. On a national level, biocides are regulated by the Federal Environment Agency (https://www.umweltbundesamt.de/themen/chemikalien/biozide/biozidprodukte; accessed on 30-01-2021).

Suitable and commonly used disinfectants for recirculation in CIP in dairy industry are chlorine-based and peroxide-based disinfectants like hydrogen peroxide (H_2O_2) and peracetic acid ($C_2H_4O_3$). Oxidising disinfectants react with the bacterial cell wall through oxidation and decrease its capability to absorb nutrients or rupture it (YOO, 2018). Peroxide-based disinfectants are effective against Gram-positive and Gram-negative bacteria, but less effective against *Mycobacteria* and ineffective against bacteria spores. Chlorine-based disinfectants are in addition to Gram-positive, Gram-negative bacteria and *Mycobacteria* effective against bacteria spores (BÖHM, 2002; WILDBRETT, 2006b; WINTER, 2009).

Air acts as a vehicle of microbial contamination in dairy industry. Therefore, besides monitoring microbial quality of process and cleaning water MASOTTI et al. (2019) suggest the ozonation and hydrogen peroxide aerosolization to reduce product spoilage (e.g. *Cladosporium, Alternaria* and *Penicillium*).

The assessment of cleaning efficiency of CIP procedures requires setting of process standards, establish reliable methods of performance measurement and recording and interpretation of results (TAMIME, 2008). Figure 4 illustrates the principle steps of process assessment.

Figure 4: Principle steps of process assessment.



Source: https://food.unl.edu/seven-principles-haccp; accessed on 30-01-2021.

Validation is the act of making the CIP process approved and takes place before implementation. Within validation the manufacturer ensures that the processing equipment is cleaned of product debris, bacterial contamination and chemical residues after the cleaning cycle (https://diversey.co.uk/en/blog/validation-challenge-cip-cleaning-protocols; accessed on 01-02-2021). It includes monitoring the CIP process parameters, bioluminescent Adenosintriphosphat (ATP) assay for indirect detection of bacterial cells, direct microbial counts in rinse waters and redox reactions to quantify residual organic matter in the dairy plant, e.g. the Nicotinamid-Adenin-Dinucleotid (NAD) test (GOLL et al. 2004; GRIFFITH 2016; WINTER, 2009). Verification determines whether the process agrees after validation with the required standard. It is a continuous assessment of the whole cleaning process, whereas monitoring evaluates specific process parameters (MEMISI et al., 2015). For evaluating the microbial status of the dairy plant surfaces, swabs, dip slides and replicate organisms detection and counting plates (RODAC) are suitable test methods (MOOR and GRIFFITH, 2002; PONTEFRACT, 1991). Swabs are preferred for quantitative results and to detect the presence of specific bacteria (http://www.foodefficiency.eu/system/resources/W1siZiIsIjIwMTYvMDEvMzEvMTgvMDQvMDMvOTcyL0Zvb2RfUHJvZHVjdGlvbl9DbGVhbmluZ19hbmRfSHlnaWVuZV9WYWxpZGF0aW9uX0d1aWRhbmNlXzIwMTYucGRmIl1d/Food%20Production_Cleaning%20and%20Hygiene%20Validation%20Guidance_2016.pdf; accessed on 03-02-2021), whereas contact plates are recommended for the detection of microorganisms on non-porous surfaces and can be used to record before-and-after colony counts (KRÖMKER, 2006; TEBBUTT, 1991). The areas chosen for swabbing may be either a representative sample of pipelines in regular use or specific areas that are suspected not to have been cleaned effectively (https://www.fda.gov/validation-cleaning-processes-793; accessed on 03-02-2021).

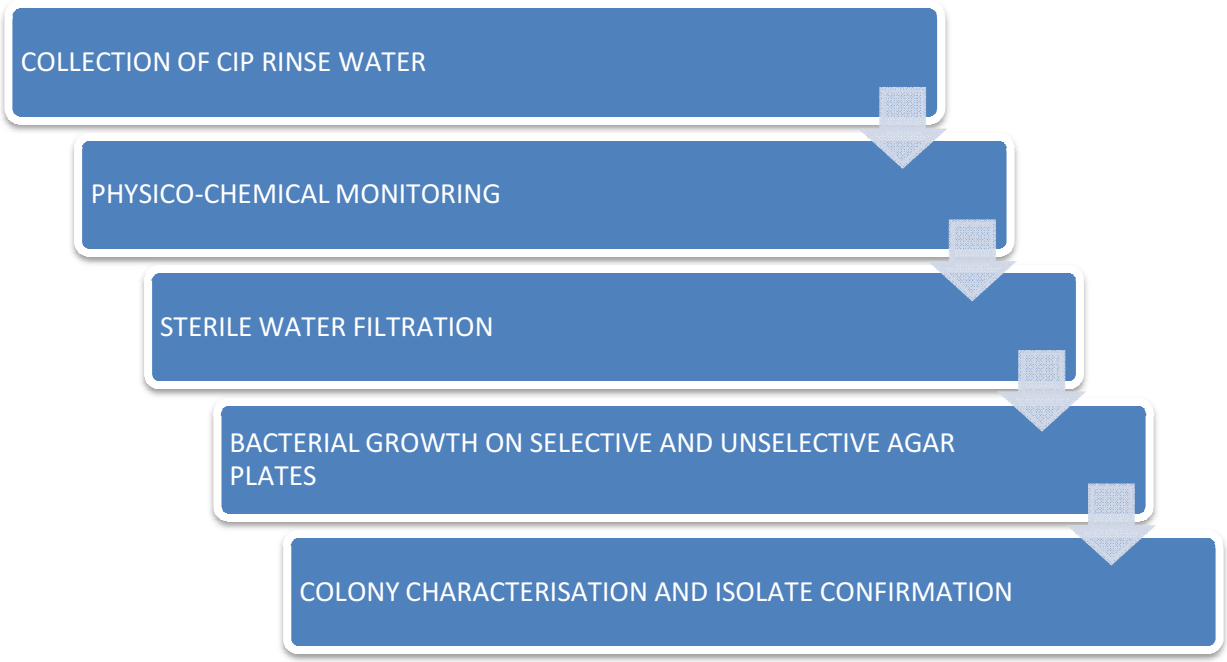
Within the verification process, it is important to test specific points in the dairy plant which are microbial at higher risk, e.g. bypasses, critical valves, dead ends, pipeline T-pieces, filler heads, heating or holding areas and recirculation pipes (TAMIME, 2008). If these critical control points are not properly cleaned, the following product passing the bacterial residues gets contaminated (ASTERIADOU et al., 2006). In an UHT plant, sterility after the cleaning process can also be evaluated by subjecting a sample of the first packaged product to total plate count agar and violet red bile glucose (VRBG) agar for specific detection of *Enterobacteriacae* (TAMIME, 2008). Monitoring refers to the regular measurements taken on the cleaning process that serve as indicators of whether the process is in state of control. Effectiveness must be monitored according to a sampling plan that specifies methods, sampling frequencies and target values to ensure the cleaning process minimises the risk of product and line contamination (TAMIME, 2008).

1.5. AIM OF THE STUDY & STUDY DESIGN

Milk processing lines offer a wide variety of microenvironments where hygiene indicator bacteria and potential pathogens are able to proliferate, to form biofilms and potentially recontaminate heat treated milk products. In this diploma thesis the bacterial residues in rinse water after cleaning in place (CIP) before sterilisation in place (SIP) in milk processing lines in an Austrian large-scale dairy were examined in order to evaluate the hygienic status. The microbiota in rinse water of twelve sterile equipments including the Asepto (ultra-high temperature) UHT heating system, eight aseptic tanks, two aseptic packaging machines for milk products and the aseptic fresh water tank were compared to the non-sterile milk pasteur. All sterile and unsterile equipments of this study are cleaned by one CIP station comprising three circuits (circuit 51 to 53). The rinse water was collected under sterile conditions and sample quality was monitored with physicochemical parameters before being filtrated by a sterile filtration unit. The water filters were placed directly on unselective and selective agar plates and incubated at temperatures corresponding to the optimal temperatures of hygiene indicators and potential pathogenic bacteria. For bacterial growth, unselective Tryptic soy agar plus 6% yeast (TSAY) extract was used for aerobic and anaerobic mesophilic counts, Lactobacilli agar according to deMan, Rogosa and Sharpe (MRS) for the isolation of fastidious lactobacilli, violet red bile glucose (VRBG) agar for *Enterobacteriaceae*, Mannitol Egg Yolk Polymyxin (MYP) agar and Glutamate Starch Phenol Red (GSP) agar for Pseudomonads. Single colonies were characterized by colony morphology and classical bacteria differentiation and purified bacteria colonies were

cryoconserved at -80°C. In parallel DNA of each purified bacterial colony was extracted including a fast Chelex Resin extraction protocol. Isolates were confirmed by 16S rRNA gene sequencing. The experimental workflow during the screening process is demonstrated in Figure 5.

Figure 5: Workflow during the screening process of bacterial residues in CIP rinse water.



2. MATERIALS AND METHODS

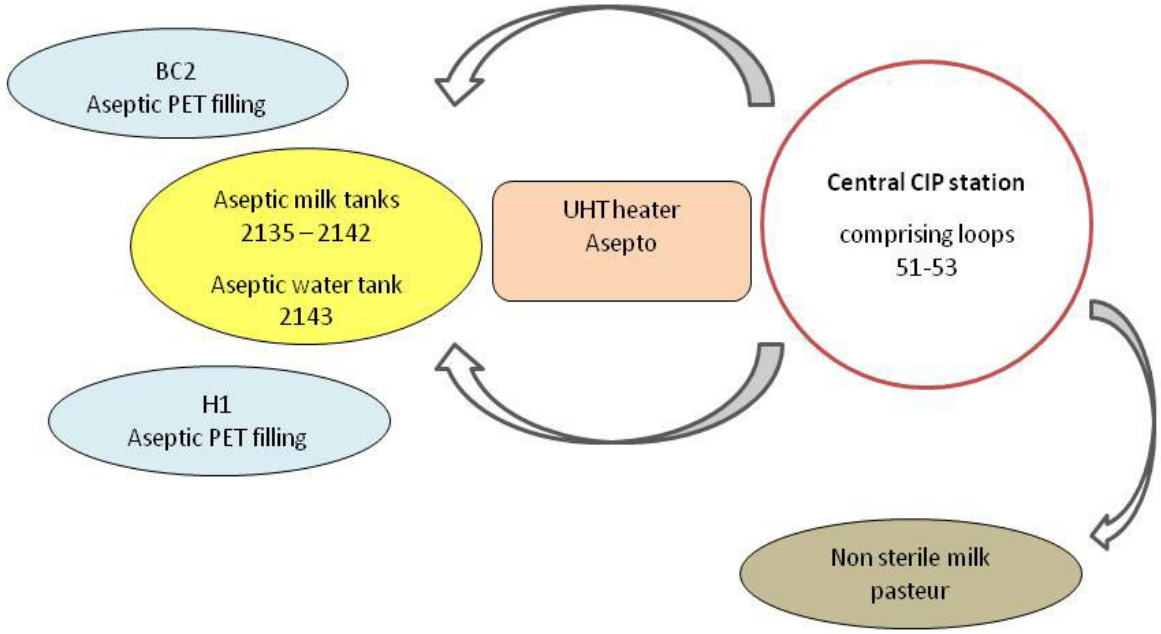
The equipment and consumables used in the actual study are depicted in Supplements Table 1.

2.1 PHASE I: SAMPLING, PHYSICOCHEMICAL MONITORING AND STERILE FILTRATION

The hygiene status of twelve aseptic equipments including the Asepto UHT heater, eight aseptic tanks (No 2135-2142), two aseptic PET filling machines for drinking milk products (BC2, H1) and the aseptic fresh water tank (No 2143) is compared to the non-sterile milk pasteur.

The aseptic sampling stations (n=12) and the non sterile milk pasteur (n=1) are examined for three times including a preliminary test (sampling 1, S1) and two regular samplings (sampling 2-3, S2-3). Four litres of final rinse flushing water are collected directly from the CIP pipe through a one way tap into a sterile sampling vessel at the end of the CIP cycles and stored for a maximum of 24 hours at 4°C. Figure 6 shows a simplified illustration of all sampling stations.

Figure 6: Simplified illustration of the sampling stations ($n_{sterile}$= 12, $n_{non\text{-}sterile}$= 1).



The collected rinsing water samples (n=39; sampling 1-3) are divided into sterile sampling vessels with a volume of 250-1000 ml. A total volume of 1.75 litres (7x250ml) per sampling and sampling station is needed for the sterile filtration.

The absence of cleaning detergent residues in sampling 2-3 (n=26/39) is controlled by pH and electrical conductivity measurement. The microbiological contamination of the samples is estimated before sterile filtration (µs/cm) by the evaluation of the chemical oxygen demand (COD) value (mg/L O_2) (COD measuring cuvette 15-150mg, 150-1000 mg). After physiochemical monitoring, sodium thiosulfate ($Na_2S_2O_3$) is added (320µl $Na_2S_2O_3$ per 250 ml water sample) to inactivate the microbicidal effect of ClO_2 in the cleaning water. Seven units of 250ml of each rinsing water sample are sterile membrane filtrated through a 45 µm pore size.

2.2 PHASE II: BACTERIAL GROWTH, COLONY CHARACTERISATION AND CLASSICAL BACTERIAL DIFFERENTATION

The water filters are placed directly on unselective (TSAY) and selective (GSP, MRS, MYP, VRBG) agar plates and are incubated at temperatures corresponding to the temperatures listed in Table 6. Tryptic soy agar plus 6% yeast (TSAY) agar is used for aerobic and anaerobic mesophilic counts, Lactobacilli agar according to deMan, Rogosa and Sharpe (MRS) for the isolation of fastidious

lactobacilli, violet red bile glucose (VRBG) agar for *Enterobacteriaceae*, Mannitol Egg Yolk Polymyxin (MYP) agar and Glutamate Starch Phenol Red (GSP) agar for Pseudomonades. Table 6 summarises incubation times and temperatures of the different agar types used in the actual study.

Table 6: Incubation times and temperatures of the different agar types .

	PCA	MRS	VRBG	MYP	GSP
Incubation time (hours)	72	72	24	24	48
Incubation temperature (°C)	30 9 4	30	30	30	30
oxygen condition	aerob	anaerob	aerob	aerob	aerob

After incubation, the bacterial colonies (n=217) are purified on Tryptic soy agar plus 6 % yeast (TSAY) extract (incubation at 30°C for 24-72 hours) through loop inoculation and are characterised by colony morphology and bacteria differentiation (Gram staining, potassium hydroxide -KOH test, Oxidase and Katalase reaction). The purified bacterial colonies are cryoconserved at -80°C.

2.3 PHASE III: DNA EXTRACTION

In parallel DNA is extracted including a fast Chelex Resin extraction protocol (Walsh et al. 1991). Colonies (n=2-3) of the purified bacteria are dissolved in 100µl 0.01M Trishydroxymethylaminmethanhydrochlorid (Tris HCl). The solution is vortexed for a few seconds and 400µl of Chelat binding Chelex Resin solution is added. After vortexing for another time, the vial is incubated at 100°C for 10 minutes (min). The solution is then centrifugated for 5 seconds (s) with 14.000 relative centrifugal force (rcf) (Eppendorf, Microcentrifuge 5424).

After centrifugation, 100µl of the DNA containing supernatant is transferred in Maximum Recovery Tube. The extracted DNA is conserved at -20°C.

2.4 PHASE IV: PCR, GELELECTROPHORESIS AND 16S RNA GENE SEQUENCING

In 16S rRNA gene sequencing, isolates are confirmed applying bacterial universal forward primer 616F (5'-AGAGTTTGGATCMTGGCTCAG-3') and universal reverse primer Univ1492R (5'-CGGTTACCTTGTTACGACTT-3'). 16S rRNA PCR for 616F are performed according to JURETSCHKO et al.

(1998) and 1492R according to LANE (1991) and subsequently sent to LGC Genomics (Berlin, Germany) for Sanger sequencing. The PCR reaction volume of 50µl consists of 48µl Mastermix and 2µl DNA template (Table 7) For negative control, both the negative control of DNA extraction (Chelex DNA) and diethylpyrocarbonate (DEPC) water as non template control (NTC) are used. The settings of the PCR cycler are described in Table 7. The PCR products are sent for purifying and sequencing to LGC Genomics (Berlin, Germany). The obtained sequence chromatograms were checked for quality (FINCH TV; Geospiza Inc., https://digitalworldbiology.com/FinchTV; accessed on 17-02-2021).

Gelelectrophoresis is used to control and visualize the purity of the extracted amplicon before 16S rRNA gene sequencing. The amplificates are separated into their molecular components according to moleculare size and moleculare charge and similar moleculare groups form gel bands. The 1.5% agarose gel consists of 1.5g Agarose, 100ml 10xTris Borat EDTA (TBE) puffer and 3.5µl PeqGreen. The 10xTBE puffer is prepared with 108g Trishydroxymethylaminmethan, 55g boric acid (H_3BO_3), 9.3g EDTA and 1000ml Millipore water. After curing, the Agarose gel is loaded.

The gel pockets on the first and last position in the gel are loaded with 5µl DNA marker (100 bp), the remaining gel pockets are loaded with a mix of PCR amplificate and 1-2µl sample loading buffer (SLB). After charging the Agarose gel with 120 volt (V) for 30min, the specific gel bands are exposed to UV light and visualized with GelDoc 2000 (Biorad).

Table 7: PCR Mastermix and PCR cycler settings for 16 S rRNA sequencing.

Mastermix	final conc.		stock conc.		number of PCR rounds	volume (µl)
					1x	53
DEPC water					34.10	1807.3
10x PCR buffer	1x				5	265
MgCl2	2	mM	50	mM	2	106
616F	200	nM	5000	nM	2	106
1492R	200	nM	5000	nM	2	106
dNTP`s	250	µM	5000	µM	2.5	132.5
Taq pol (Plat.)	2	U	5	U/µl	0.4	2.2
Mastermix					48	2544
Template					2	
PCR reaction volume					50	
PCR conditions						
Initial denaturation		95 °C	5 min			
Denaturation		94 °C	30 sec	30 cycles		
Annealing		52 °C	30 sec			
Elongation		72 °C	60 sec			
Final elongation		72 °C	7 min			
		4 °C	hold			

3. RESULTS

The bacterial isolates (n=217) collected after sampling event S1-S3 (n=39 samples) were further characterized by 16S rRNA sequencing.

The results of the physicochemical monitoring during sampling event S2 and S3 are summarised in Supplements Table 2.

The bacterial isolates collected during sampling event S1-S3 were associated to more than the half to Gram positive (53.90%; n=117/217) and to a smaller extend to Gram negative bacteria (46.10%; n=100/217). The most abundant bacterial phyla in water were Proteobacteria (Gammaproteobacteria; n=100/217; 46.10%), Actinobacteria (n=65/217; 29.95%), followed by Firmicutes (n=47/217; 21.65%), Bacteroidetes (n=8/217; 3.69%) and Deinococcus-Thermus (n=5/217; 2.30%). The predominant bacterial families were *Moraxellaceae*, *Pseudomonadaceae* (n=50/217; 23.04% and 16/217; 7.37%; both Proteobacteria), *Micrococcaceae, Microbacteriaceae* (n=40/217;

18.43% and n=25/217; 11.52%; both Actinobacteria) and *Bacillaceae, Streptococcaceae* (n=17/217; 7.83% and n=10/217; 4.61%; both Firmicutes) (Figure 7).

The isolates were categorized according to a potential recontamination event (n=108/217; 49.77%) or the category thermoduric organisms (n=109/217; 50.23%). Recontaminants were most gram-negative bacterial organisms and staphylococci detected in this study. Thermoduric bacteria were identified as aerobic spore formers (*B. cereus group*-BCG, *B. subtilis group*-BSG, *B.* spp., *Paenibacillus*) assigned to Firmicutes and to the family *Bacillaceae* and *Paenibacillaceae*. Furthermore, Actinobacteria (family *Micrococcaceae, Microbacteriaceae*), Firmicutes (family *Streptococcaceae, Caryophanales* and *Mycobacteriaceae*) and *Deinococcus Thermus* (*Deincoccaceae*) (Figure 7). The most abundant bacterial genera were *Acinetobacter* (n=50/217; 23.04%), *Kocuria* (n=30/217; 13.82%), *Microbacterium* (n=25/217; 11.52%), *Pseudomonas* (n=16/217; 7.37%), *Micrococcus* and *Lactococcus* (each 10/217; 4.61%) (Figure 8).Further details on differentiation and species confirmation of bacteria by 16 S RNA sequencing is depicted in Supplement Table 3.

The bacterial reservoir in the UHT milk processing line was heterogeneous in composition during the three sampling events. Overall, only sterile tank 2140 was tested negative for recontaminants or thermoduric bacterial organisms in all three sampling events. The residual rinse water in sterile tank 2136 contained the greatest bacterial diversity at all three sampling times. Residual rinse water taken from sterile tank 2135, 2137, 2138, 2142 and sterile pet filling BC2 were contaminated in two of three sampling events. Four, seven and nine of thirteen sampling sites in the processing line of UHT milk processing were tested positive for thermoduric bacteria or/and recontaminants (Figure 9). During sampling event S1 the positive sampling sites (Pasteur, UHT heater, sterile tank 2138) contained a broad spectrum of thermoduric bacteria and recontaminants. In detail, up to five different bacterial species were detected in the latter environmental samples. In sampling event S2 the residual water rinse samples in sterile tank 2135, 2136, 2137, and 2142 contained similar bacterial diversity. Sterile tank 1238 and sterile pet-filling BC2 contained bacterial monocultures of *Microbacterium oxydans* and *Staphylococcus haemolyticus*, respectively.

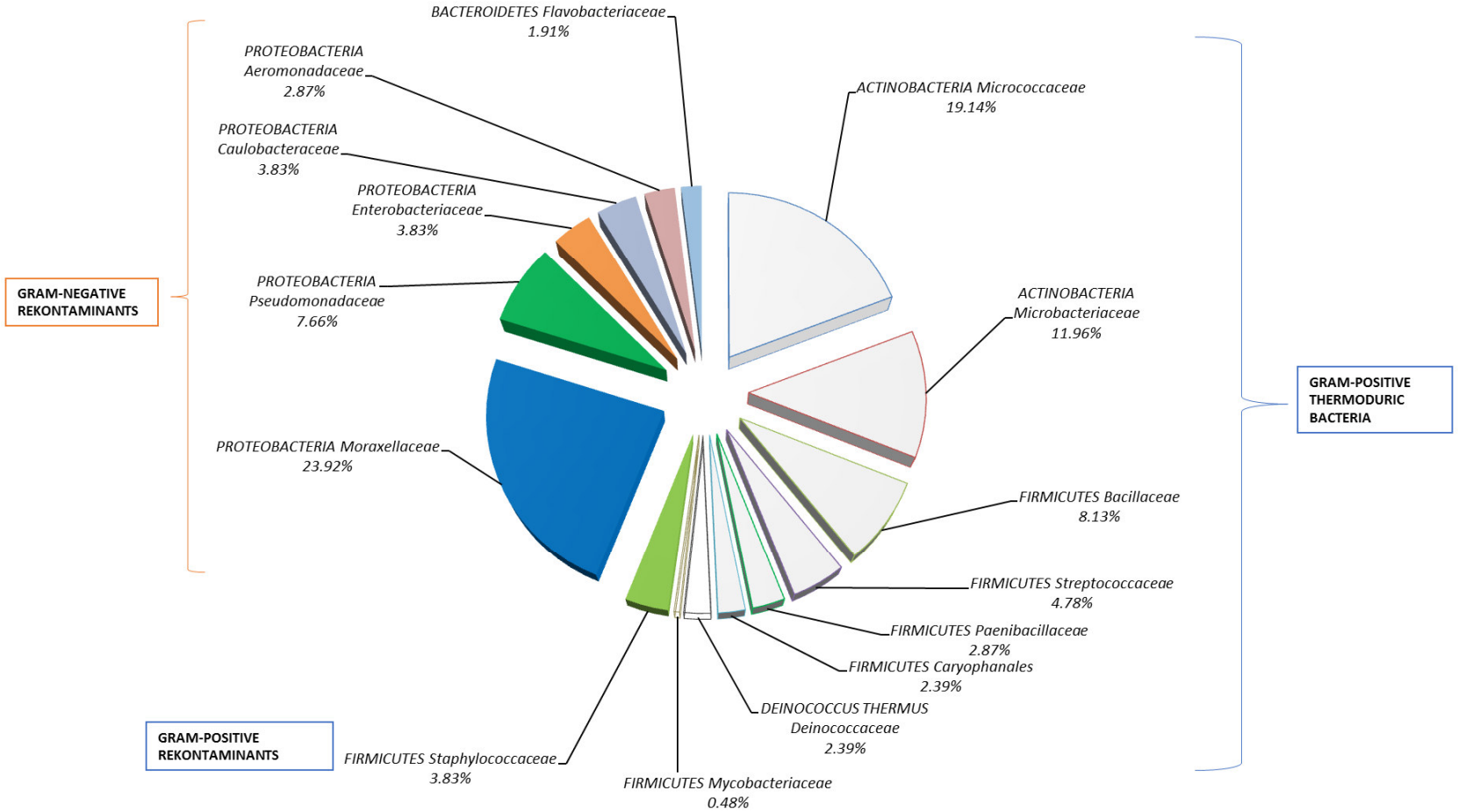


Figure 7: Bacteria isolated from water samples (sampling event 1-3) ordered according to phyla, families and Gram-staining (n=217). The colored segments are for recontamination bacteria, in comparison thermoduric bacteria are illustrated with empty segments.

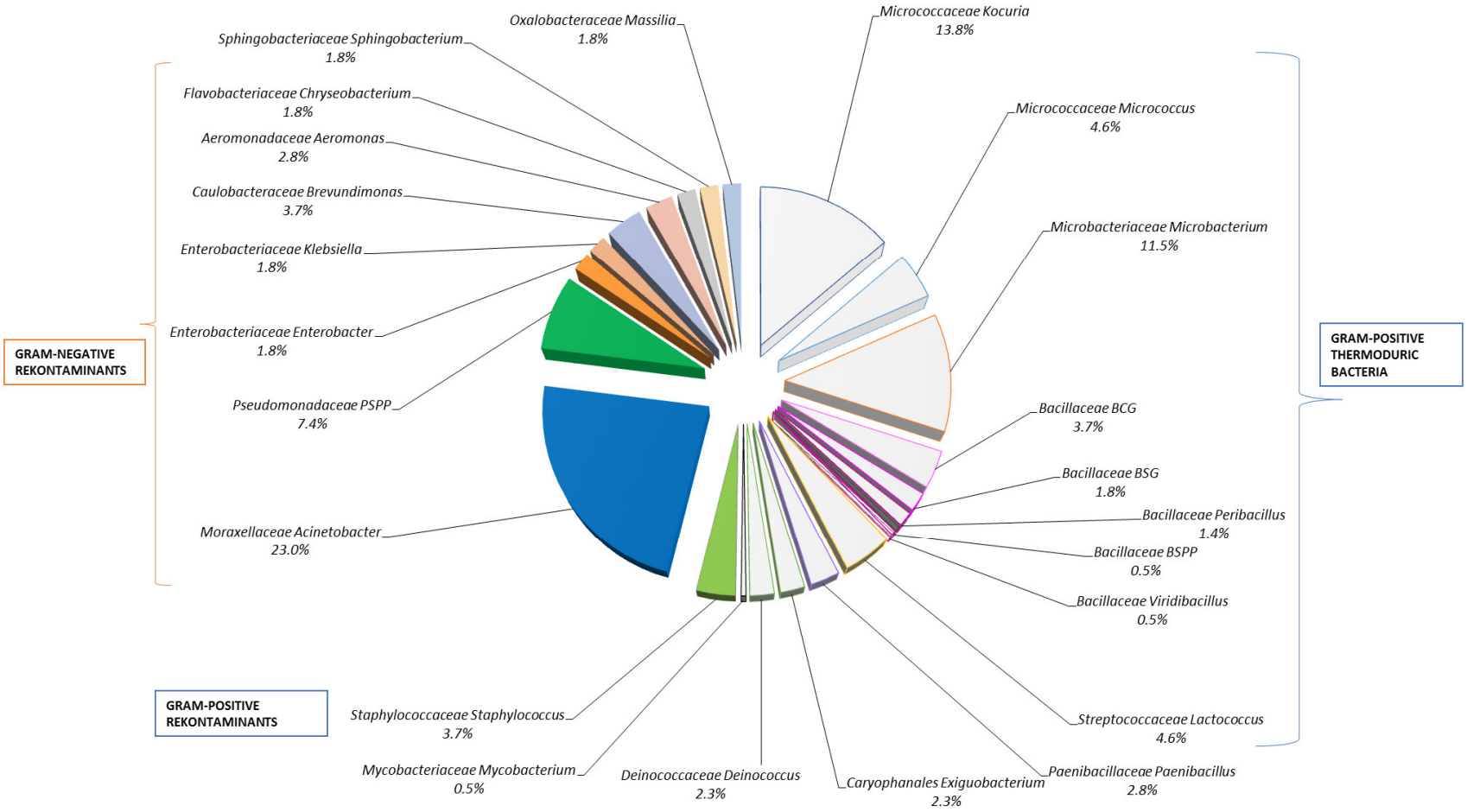


Figure 8: Bacteria isolated from water samples (sampling event 1-3) ordered according to families, genera and Gram-staining (n=217). Explanation for Figure 8: The colored segments are for recontamination bacteria, in comparison thermoduric bacteria are illustrated with empty segments. Abbreviations: BCG*, Bacilllus cereus* group; BSG, *Bacillus subtilis* group; BSPP, *Bacillus* spp. diverse; PSPP, *Pseudomonas* spp.

During the third sampling the bacteria were diversely composed in sterile tank 2142 (thermoduric bacteria and recontaminants), whereas the majority of other positive sampling points contained *Acinetobacter* associated with a second contaminant (BSG, *Massila, Staphylococcus* or *Aeromonas*). In sampling site UHT heater, sterile tank 2137 and 2142 a monoculture of *Mycobacterium diernhofer* and *Kocuria marina* were detected, respectively. The highest amount of bacterial species (n=10) was detected in the fresh water tank, a reservoir for the CIP system, during sampling event S2 (Figure 9).

The most abundant bacterial reservoirs in the pipelines, sterile tanks and pet filling stations of UHT processing were *Acinetobacter.*

Acinetobacter (A.) iwoffi, as a recontaminant during milk processing and risk group 2 organism (Supplement table 3; definition https://bacdive.dsmz.de/, accessed on 25-02-2021), was detected during sampling S2 and S3 in sterile tank 2136; during sampling three in sterile pet filling BC2 and sterile tank 2135 and 2139. *A. johnsonii* (recontaminant, risk group 2) was observed during sampling S3 in sterile tank 2142, 2135 and sterile pet filling H1. *A. radioresistans* was isolated in sampling S2 from sterile tank 2136, 2137 und 2142. The fresh water tank was not identified as a source of an *Acinetobacter* contamination pathway. *Brevundimonas vesicularis* (recontaminant) was primarily isolated from sterile tanks (2136, 2137, 2142; 1 and third sampling.

BCG (*B. cereus* group; thermoduric) was isolated in the first sampling in the Pasteur, sterile tank 2136 and 2138. Furthermore, BCG was persistent during samping S2 in sterile tank 2136, 2142 and fresh water tank.

B. haynesii (*B. subtilis* group; thermoduric) was isolated from sterile tank 2135 and 2142 during S2 and S3. *B. simplex* was present in the Pasteur and sterile tank 2142. *Kocuria marina* and *Kocuria rhizophila* (thermoduric) was present in sterile tank (2135, 2137, 2141; sampling 2 and 3). *Staphylococcus pasteuri* was present during the first sampling in the Pasteur and sterile tank 2142, wheras *Staphylococcus haemolyticus* was present in sterile tank (2135) and sterile PET filling (H1, BC2) (sampling 2 and 3).

The microbiological diversity and niche attribution of bacterial species of all sampling sites in sampling event S1-S3 is summarised in Supplements Table 3.

Generally, *A. johnsonii*, *A. iwoffi* (both risk group 2) and *A. radioresistens* are highly associated to the human niche. *Brevundimonas vesicularis* (risk group 2) is good adapted to diverse niches, therefore can be found ubiquitously. Aerobic spore formers are perfectly adapted to milk processing environment, are thermoduric and opportunistic pathogenes (*B. cereus* group). *Kocuria* (thermoduric) are risk group 1 organisms and are often isolated from environment.

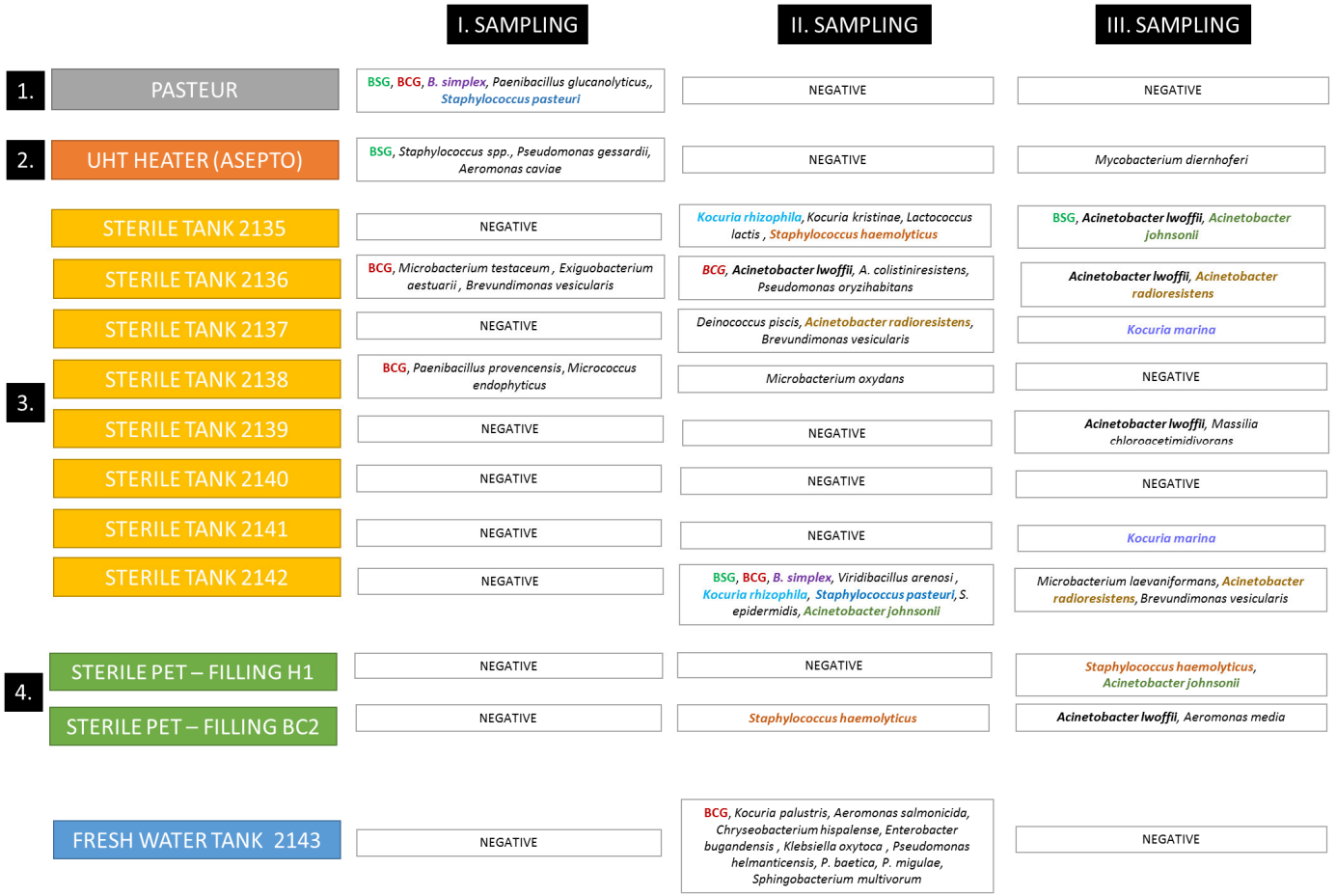


Figure 9: Bacterial reservoirs and bacterial diversity of the sampling sites in sampling event S1-S3 according to species. Bacteria species, which were isolated for at least twice are coloured similary and written bold. Abbreviations: BCG, *Bacilllus cereus* group; BSG, *Bacillus subtilis* group.

4. DISCUSSION AND CONCLUSION

Since the main goal of the CIP process is continuous, effective cleaning and disinfection of dairy equipment, there should be no microbial residues left in milk processing lines after CIP cleaning. Reasons for detection of spoilage-causing bacteria in aseptic milk processing lines after CIP include recontamination of process equipment, ineffective cleaning and disinfection of the CIP cycle, or bacterial heat (thermoduric bacteria) and cleaning resistance (OSTROV et al., 2019; THOMAS and SATHIAN, 2014).

An important group of microbiota associated with the spoilage of UHT processed milk products are thermoduric *Bacillus* and *Bacillus* like species. Most attention in UHT processing from a spoilage point of view are the mesophilic and thermophile *Bacillus* and *Bacillus* like species. In this study, species of the mesophilic *B. subtilis* group (*B. nakamurai*, *B. subtilis*, *B. haynesii*) were isolated from the milk pasteur, the UHT heater and sterile tanks. These mesophilic non pathogenic spore formers produce highly heat resistant spores which survive UHT treatment and have optimum growth temperatures from 20-40°C (VYLETELOVA et al., 2002; PINTO et al. 2018). This wide range of optimum growth temperature enables species of the BSG to cause UHT milk product spoilage at ambient storage and reduce shelf life stability. *B. nakamurai* and *B. haynesii* are originally isolated from soil (DUNLAP et al. 2016, 2017) and *B. subtilis* is isolated in raw milk during summer period (GOPAL et al., 2015), therefore it is possible that these species enter the milk processing route already on dairy farm via contamination of bovine raw milk (EVANOWSKI et al., 2020). Udder cleanliness and and milking hygiene reduce the amount of aerobic spore formers (*Paenibacillus, Bacillus*) effectively and contribute to improved raw milk quality (EVANOWSKI et al., 2020).

B. pacificus (BCG; *B. cereus group*) was isolated from the milk pasteur, three sterile tanks and the fresh water tank 2143. *B. pacificus* is a novel species of the BCG (LIU et al., 2017), a recontamination of milk processing lines with final rinse CIP flushing water is possible as *B. pacificus* is environmental and especially water associated and was isolated in the fresh water tank (VIDIC et al., 2020). Psychrotrophic aerobic spore formers are able to grow at temperatures <7 °C (MEER et al., 1991) although their optimum temperature for growth is 20-30°C (UBONG et al., 2019). This ability is particularly relevant for refrigerated stored milks such as extended shelf life milk (ESL). In general, the heat stability of spores produced from psychrotrophic spore formers is lower than that of mesophilic or thermophile species, but there are exceptions like the spores of *Paenibacillus* (DEETH and LEWIS, 2017). In S1, *Paenibacillus glucanolyticus* was isolated from the milk pasteur and *Paenibacillus provencensis* (both environmental associated; Supplement Table 3) was isolated from

sterile tank 2138. *Paenibacillus* sp. has become very important for dairy industry as it is produces highly heat resistant spores and can growth both at refrigeration and ambient temperatures (MUGADZA et al., 2018), some strains also survive HTST and UHT treatment (IVY et al. 2012; SADIQ et al. 2018; SCHELDEMANN, 2004). Other *Bacillus* and *Bacillus* like species isolated in this study were *B. simplex*, *B. bataviensis* and *Viridibacillus arenosi*, they were all detected in the final CIP rinsing water of sterile tank 2142. *B. simplex* produces a heat labile cereulide toxin, but has not been implicated in food poisoning cases yet (GOPAL et al., 2015). *B. bataviensis* has been isolated in recent studies in human oral cavity (AKINYEMI et al., 2017; MARTELLACCI et al., 2020) and also soil (https://bacdive.dsmz.de/strain/1214; accessed on 04-03-2021). The latter spore formers seems to be adapted to a broad a variety of niches and is also assigned to thermoduric bacteria.The *Bacillus* like species *Viridibacillus arenosi* is associated with the spoilage of HTST milk products (RANIERI 2009b) and according to BacDIVE soil associated (https://bacdive.dsmz.de/search?search=Viridibacillus+arenosi+; accessed on 04-03-2021). Four different species of *Bacillus* and *Bacillus* like species (*B. bataviensis*, *B. haynesii*, *B. simplex*, *Viridibacillus arenosi*) were isolated from the final CIP rinsing water of sterile tank 2142, this suggests that attached biofilms on stainless steel surface decrease the cleaning and disinfection performance of the CIP sequence in sterile tank 2142 (CHMIELEWSKI and FRANK, 2003; LINDSAY et al., 2002).

Another important group of bacteria associated with the spoilage of UHT processed milk products is *Pseudomonas* spp. In particular the UHT heater and the fresh water tank 2143 were identified as sampling sites contaminated with different *Pseudomonas* species. *P. gessardii* was isolated in the final CIP rinsing water of the UHT heater in S1. This species was originally isolated in natural mineral water (VERHILLE et al., 1999), but is also identified as spoilage bacteria with proteolytic activities in raw milk and HTST milk (ZAREI et al., 2020). *P. gessardii* is part of the *P. fluorescens* group. *Pseudomonas* spp. vary considerably in their propensity to produce heat stable enzymes (SHELLEY et al., 1987; CHAMPAGNE et al., 1994; MUIR, 1996) and *P. fluorescens* is named to be one of the most important producers of heat resistant enzymes in milk products (SHELLEY et al., 1987; CHAMPAGNE et al., 1994; MUIR, 1996). The heat resistant proteases of *P. fluorescens* survive UHT treatment and can cause detectable proteolysis in UHT milk products during storage at ambient temperature (BUTTON et al., 2011; DEETH and LEWIS, 2017). The recontamination of milk processing lines of the UHT heater after CIP with *P. gessardii* is possible through recontamination with final rinse flushing water or the presence of attached biofilms on the surface of the heating equipment (BAGGE et al.,

2004; KHAJANCHI et al., 2009; RAJENDRAN et al., 2010; TEH et al., 2014). Cell clumps containing vegetative cells of *Pseudomonas* spp. and their extracellular enzymes disperse from the mature biofilms into milk during storage and processing and increase the risk of product spoilage (TEH et al., 2014). The fresh water tank 2143 was identified as reservoir for *Pseudomonas* spp., as in S2 *P. helmanticensis*, *P. baetica* and *P. migulae* could be isolated from the final CIP rinsing water. These bacteria are associated with soil and water (KUMAR et al., 2019; LÓPEZ et al. 2017; VERHILLE et al., 1999), therefore a recontamination of the aseptic milk processing lines with the flushing water from the fresh water tank 2143 is possible. Furthermore, *P. migulae* is part of the *P. fluorescens* group and its isolation in the cleaning water seems critical because of its propensitiy of heat resistant enzyme production.

The microbial profile of cooled raw milk at dairy level consists mainly of psychrotrophic non spore forming *Pseudomonas* species (MACHADO et al., 2015; ZHANG et al., 2019a). The significance of the SPC as indicator for the risk of contamination through extracellular enzymes is controversial because different psychrotrophic bacteria have different propensities to heat resistant enzyme production (HARYANI et al. 2003), but nevertheless the SPC of stored cooled raw milk can be a reasonable guide to the probability of heat resistant enzymes being present (VELÁZQUEZ-ORDOÑEZ et al., 2019) in the final milk product. It is for sure that raw milk with a good microbiological condition is necessary to ensure the production of a high quality UHT product with a stable shelf life. *Brevundimonas vesicularis* and *P. oryzihabitans* are water and plant associated bacteria with the potential to cause infection in humans (BEILSTEIN and DREISEIKELMANN, 2006; HASSON, 2019; RYAN and PEMBROKE, 2018). For this reason a recontamination of the milk processing lines after CIP through contaminated flushing water or a recontamination of the water sample through aseptic sampling is possible.

In general, the study suggests that microbiological recontamination of the sterile processing equipment after CIP constitutes a critical point. GRIFFITHS (2010) and SARKAR (2015) named post sterilisation contamination to be the major reason for product spoilage after UHT treatment. In the water samples taken from fresh water tank 2143 the Gram negative species *Aeromonas salmonicida*, *Chryseobacterium hispalense, Enterobacter bugandensis, Klebsiella oxytoca* and *Sphingobacterium multivorum* could be isolated. These species seems to be recontaminants as they are not thermotolerant and they are isolated from soil, water or human body (DEL CARMEN MONTERO-CALASANZ et al., 2013; PATI et al., 2018; PERNAS-PARDAVILA et al., 2019; TRIVEDI et al., 2015; VINCENT et al., 2019). *Klebsiella oxytoca* has the ability to form biofilms on dairy processing

equipment (TANG et al., 2009), this seems critical as the final CIP rinsing water from tank 2143 is the last cleaning step of the CIP cycle. The sterile tanks 2135-2139 and 2141-2142 seem to be another source for microbiological recontamination and reservoir of thermoduric bacteria of milk processing lines after CIP. The sterile tank 2140 was the only sampling site in this study with no detected isolates in S1-S3. Bacterial residues attach on the inner surface of milk processing equipment, especially on critical points which are difficult to access for cleaning. If these critical control points are not properly cleaned, the following product passing the bacterial residues gets contaminated (ASTERIADOU et al., 2006). *A. johnsonii*, *A.Iwoffii* and *A. radioresistens* were isolated as the most abundant species from the final CIP rinsing water of sterile tank 2135-2137, 2139 and 2142. These Gram negative bacteria are water and human body associated and cause spoilage in raw milk (HAHNE et al., 2019; JÚNIOR et al., 2018; VAZ-MOREIRA et al., 2017; ZHANG et al., 2019b). *A. colistiniresistens* was isolated from sterile tank 2136 in S2, this bacterium causes human body infections (NEMEC et al., 2017) and a recontamination of the water sample through aseptic sampling is possible. *Aeromonas media* was isolated from the sterile PET filling machine BC2, this bacteria is water associated but is also named as opportunistic pathogen for humans and animals (TALAGRAND-REBOUL et al., 2017).

The Gram positive species of *Staphylococcus (S. epidermidis*, *S. haemolyticus*, *S. pasteuri*) were isolated from sterile tank 2135 and 2142. These *Staphylococcus* species are human body associated (LIU et al., 2020; SAVINI et al., 2009) and a recontamination of the water samples through aseptic sampling is very likely, but the isolation of *S. pasteuri* seems critical as it shows resistance against thermal treatment of 80°C for 20 min and it is an opportunistic pathogen for humans (MONTANARI et al., 2015; NAIDOO and LINDSAY, 2010). Gram positive bacteria isolated from the final CIP rinsing water from sterile tanks with water association are *Kocuria marina* (sterile tank 2137 and 2141), *Microbacterium laevaniformans* (sterile tank 2142), *Deinococcus piscis* (sterile tank 2137) and *Exiguobacterium aestuarii* (sterile tank 2136) (SHASHIDHAR and BANDEKAR, 2009; KIM et al., 2005; ZHU et al., 2020), a recontamination of milk processing lines with these partly extremophiles species through flusing water seems possible. *Exiguobacterium aestuarii* has the ability to form biofilms on dairy processing equipment (WANG et al., 2019) and is strongly associated to water (https://bacdive.dsmz.de/strain/18110 accessed on 04-03-2021). The genus *Kocuria* with the isolated species *Kocuria marina* (sterile tank 2137 and 2141), *Kocuria palustris* (fresh water tank 2143), *Kocuria rhizophila* (sterile tank 2135 and 2142) and *Kocuria kristinae* (sterile tank 2135) are environmental and human body associated bacteria (ALWADEI et al. 2020; BERNSHTEYN et al. 2020;

TAKARADA et al., 2008; VITAL et al., 2019) with *Kocuria kristinae* and *Kocuria rhizophila* being part of the microbial bovine thermoduric raw milk flora (PUKANČÍKOVÁ et al., 2016; RIBEIRO-JÚNIOR et al., 2020). Also *Microbacterium oxydans* (sterile tank 2138) is a Gram positive environment associated bacteria being part of the microbial bovine raw milk flora (VITHANAGE et al., 2016; RIBEIRO-JÚNIOR et al., 2020).

Micrococcus endophyticus is a plant associated Gram positive bacteria isolated from the final CIP rinsing water of sterile tank 2138 (RUSTAMOVA et al., 2020). Recontaminants isolated in sterile tanks with an association to milk processing facility are *Lactococcus lactis* (sterile tank 2135) and *Microbacterium testaceum* (sterile tank 2136) (BRANDL et al., 2014; MÄRTLBAUER and BECKER, 2016).

A high standard in dairy plant hygiene is necessary to produce an UHT milk product with a good microbiological quality, which results in a stable shelf life. CIP ensures a continuable cleaning and disinfection of the milk processing equipment to reduce the risk of bacterial contamination. In this study, spoilage associated bacteria from the *Bacillus* and *Bacillus* like group could be isolated in the final CIP rinsing water of the non sterile milk pasteur, the UHT heater, the sterile tanks and the fresh water tank 2143. This suggests that biofilms of *Bacillus* and *Bacillus* like species attached on the process equipment surface increase the risk of final milk product recontamination and reduce cleaning and disinfection efficiency of the CIP procedure. Also species from the *P. fluorescens* group isolated in the final CIP rinsing water of the UHT heater and the sterile tanks have the ability to form biofilms and produce heat resistant enzymes, which cause spoilage in UHT milk products. Biofilms are more resistant against disinfection than planctonic bacteria (CHMIELEWSKI and FRANK, 2003) and the resistance of bacteria attached in biofilms increases if the biofilm is microbiological diverse (LINDSAY et al., 2002). For this reason, the verification of the CIP process of the sterile equipments including the Asepto (ultra-high temperature) UHT heating system, the aseptic tanks 2135-2142 and the aseptic PET filling machines BC2 and H1 packaging is necessary to detect critical points in the milk processing lines where attached biofilms decrease cleaning efficiency of the CIP procedure. This study suggests that especially the cleaning and disinfection efficiency of the sterile tanks has to be improved as not only spoilage associated bacteria were isolated from the final CIP rinsing water but also diverse bacterial recontaminants. The recontamination of the collected rinse water through aseptic sampling was the reason for the detection of human associated bacteria like *Staphylococcus*

sp., *Brevundimonas* sp., *Acinetobacter* sp. and *Kocuria* sp., an alternative sampling technique within the verficiation process like swabbing is recommended. Swabs are an alternative to detect the presence of specific bacteria and the areas chosen for swabbing may be either a representative sample of pipelines in regular use or specific areas that are suspected not to have been cleaned effectively. As all sterile and unsterile equipments of this study are cleaned by one CIP station comprising three circuits (51 to 53) and the rinsing water for CIP is used from fresh water tank 2143, the microbiological water quality of fresh water tank 2143 has to be observed critical. As Gram negative recontaminants of the genera *Aeromonas*, *Chryseobacterium, Enterobacter, Klebsiella, Pseudomonas* and *Sphingobacterium* could be isolated from the fresh water tank 2143, this study suggests that the fresh water tank is a reservoir for Gram negative recontaminants. The isolation of this genera proposes a repeated sampling and sterile filtration of fresh water tank 2143 with the use of selective agar plates incubated at temperatures corresponding to the optimal temperatures of hygiene indicators and potential pathogenic bacteria. Furthermore the use of appropriate treated wasterwater could be an alternative to potable water for CIP applications (DOGAN et al., 2020) regarding to economical and ecological viewpoints. As raw milk with a good microbiological condition is necessary to ensure the production of a high quality UHT product with a stable shelf life, the use of raw milk classified to S class (SPC ≤50 000/ml, SCC ≤250 000/ml) is an opportunity to reduce the spoilage risk of UHT milk products through contamination with heat resistant enzymes from non-spore forming psychrotrophic bacteria and heat resistant spores being produced from bacteria originated in raw milk. Bactofugation and microfiltration as preheating treatments of raw milk can additionally reduce bacteria population and increase storage stability of UHT milk at room temperature (D'INCECCO et al., 2018; RIBEIRO-JÚNIOR et al., 2020). As an increased international trade of UHT milk products is observed, a critical microbiological observation of UHT processing equipment including cleaning and disinfection management deserve special consideration of dairy manufacturers. Therefore, further studys to evaluate the hygienic status of the UHT processing sampling sites examined in this study are recommended.

5. EXTENDED SUMMARY

In 2014, two thirds of the sold drinking milk products were ultra-high temperature (UHT) milk. Raw milk which is selected for UHT processing has to be in a good microbiological condition, EU Directive No 326/2015 establishes microbiological quality criteria for bovine raw milk used for further milk processing and in EU Regulation No 2073/2005 and No 853/2004 the criteria for bacterial contamination in bovine raw milk aimed for UHT processing are listed. Diaries have to ensure these legal requirements with their HACCP schedule. Due to increased spoilage and reduced shelf life stability of UHT products, raw milk should not contain high counts of heat resistant spores from spore-forming bacteria like *Bacillus* and *Bacillus* like species and heat resistant enzymes from non-spore-forming psychrotrophic bacteria, e.g. *Pseudomonas* spp. In this diploma thesis the bacterial residues in rinse water after cleaning in place (CIP) in aseptic milk processing lines in an Austrian scale dairy were examined in order to evaluate the presence of bacteria producing heat resistant spores and heat resistant enzymes in comparison to bacteria associated with post sterilisation contamination. For 6 months the rinse water of twelve sterile equipments including the Asepto (ultra-high temperature) UHT heating system, eight aseptic tanks, two aseptic packaging machines for milk products, the aseptic fresh water tank and the non-sterile milk pasteur were collected for three times (S1-S3). All sterile and unsterile equipments of this study are cleaned by one CIP station comprising three circuits (51 to 53). The rinse water was collected under sterile conditions and after sterile water filtration, bacteria were isolated using unselective and selective agar plates incubated at temperatures corresponding to the optimal temperatures of hygiene indicators and potential pathogenic bacteria. Single colonies were characterized by colony morphology and classical bacteria differentiation and purified bacteria isolates were confirmed by 16S rRNA gene sequencing in order to allow risk assessment of the isolated bacteria. In S1-S3, an isolation set of 217 bacteria could be collected. The bacterial isolates collected during sampling event S1-S3 were associated to more than the half to Gram positive (53.90%; n=117/217) and to a smaller extend to Gram negative bacteria (46.10%; n=100/217). Recontaminants (n=108/217; 49.77%) were most gram-negative bacterial organisms and staphylococci detected in this study. Thermoduric bacteria (n=109/217; 50.23%) were identified as aerobic spore formers (*Bacillaceae* and *Paenibacillaceae*), *Micrococcaceae, Microbacteriaceae*, *Streptococcaceae, Caryophanales*, *Mycobacteriaceae* and *Deincoccaceae*. The most abundant bacterial genera were *Acinetobacter* (n=50/217; 23.04%), *Kocuria* (n=30/217; 13.82%), *Microbacterium* (n=25/217; 11.52%), *Pseudomonas* (n=16/217; 7.37%), *Micrococcus* and

Lactococcus (each 10/217; 4.61%). Generally, *A. johnsonii*, *A. iwoffi* (both risk group 2) and *A. radioresistens* are highly associated to the human niche. Aerobic spore formers are perfectly adapted to milk processing environment, are thermoduric and opportunistic pathogenes (*B. cereus* group). *Kocuria* (thermoduric) are risk group 1 organisms and are often isolated from environment. The bacterial reservoir in the UHT milk processing line was heterogeneous in composition during the three sampling events. Overall, only sterile tank 2140 was tested negative for recontaminants or thermoduric bacterial organisms in all three sampling events. The residual rinse water in sterile tank 2136 contained the greatest bacterial diversity at all three sampling times. The highest amount of bacterial species (n=10) was detected in the fresh water tank, a reservoir for the CIP system.

For this reason, the verification of the CIP process of the sterile equipments is necessary to detect critical points in the milk processing lines where attached biofilms decrease cleaning efficiency of the CIP procedure. An alternative sampling technique with less recontamination potential within the verification process like swabbing is recommended to confirm the presence of specific bacteria. As all sterile and unsterile equipments of this study are cleaned by one CIP station and the rinsing water for the CIP process is used from fresh water tank 2143, the microbiological water quality of the cleaning water has to be observed critical. The identification of fresh water tank 2143 as a reservoir for diverse Gram-negative recontaminants proposes a repeated sampling and sterile filtration of the cleaning water. The use of selective agar plates incubated at temperatures corresponding to the optimal temperatures of hygiene indicators and potential pathogenic bacteria with a following 16S rRNA gene sequencing in order to allow risk assessment is recommended. Appropriate treated wastewater regarding economical and ecological viewpoints is an alternative to potable water for CIP applications. As raw milk with a good microbiological condition is necessary to ensure the production of a high quality UHT product with a stable shelf life, the use of raw milk classified to S class (SPC ≤50 000/ml, SCC ≤250 000/ml) is an opportunity to reduce the spoilage risk of UHT milk products through contamination with heat resistant enzymes from non-spore forming psychrotrophic bacteria and heat resistant spores being produced from bacteria originated in raw milk.

In general, the critical microbiological observation of UHT processing equipment including cleaning and disinfection management deserve special consideration of dairy manufacturer.

6. ZUSAMMENFASSUNG

Im Jahr 2014 stellten zwei Drittel der weltweit verkauften Trinkmilchprodukte UHT Produkte dar. Rohmilch, die für die UHT Weiterverarbeitung bestimmt ist, muss eine hohe mikrobiologische Qualität nachweisen. Die erforderlichen mikrobiologischen Qualitätskriterien für Kuhmilch sind gesetzlich in den EU Richtlinien Nr. 2073/2005 und Nr. 853/2004 geregelt. Milchverarbeitende Betriebe müssen diese gesetzlichen Anforderungen im Rahmen ihres HACCP Plans erfüllen. Aufgrund einer verminderten Mindesthaltbarkeitsdauer und Produktstabilität bei Raumtemperatur, sollte in Rohmilch, die zu UHT Milch weiterverarbeitet wird, keine hohe Anzahl an hitzeresistenten Sporen sporenbildender Bakterien wie *Bacillus* und *Bacillus* ähnlichen Spezies sowie an hitzeresistenten Enzymen psychrotropher Bakterien wie *Pseudomonas* spp. enthalten sein. Im Rahmen dieser Diplomarbeit wurden die bakteriellen Rückstände nach stationärer CIP Reinigung im Nachspülwasser aseptischer milchverarbeitender Produktionsanlagen in einer österreichischen Molkerei untersucht, um das Verderbnisrisiko durch hitzeresistente, sporenbildendende Bakterien sowie enzymproduzierender Bakterien als potentielle Rekontaminanten zu evaluieren. Für eine Dauer von 6 Monaten wurden die Nachspülwasserproben von zwölf Probenahmestellen jeweils dreimal untersucht. Zu diesen Probenahmestellen zählten ein UHT Erhitzer, acht Steriltanks, zwei aseptische Abfüllanlagen für Trinkmilchprodukte, ein für die CIP Reinigung verwendeter Frischwassertank sowie ein Milchpasteur. Alle sterilen und unsterilen Probenahmestellen wurden von einer CIP Station (Kreislauf 51-53) gereinigt. Das Nachspülwasser wurde unter aseptischen Bedingungen gesammelt und sterilfiltriert. Die Isolierung der Bakterien erfolgte durch selektive und unselektive Nährmedien, die entsprechend den Wachstumsanforderungen der Hygieneindikatoren sowie potenzieller bakterieller Pathogene inkubiert wurden. Die kultivierten Isolate wurden durch die klassische Grobdifferenzierung charakterisiert, aufgereinigt und durch 16S rRNA Genanalyse identifiziert. Es stand ein Isolatset von 217 Keimen zur Verfügung. Die identifizierten Isolate der Probennahmedurchgänge 1-3 setzten sich zu 53,90% aus Gram positiven Keimen (n=117/217) sowie zu 46,10% aus Gram negativen Keimen zusammen. Die Rekontaminationskeime (n=108/217; 49,77%) stellten Gram negative Bakterien sowie *Staphylococci* dar. Als thermodure Bakterien (n=109/217; 50,23%) konnten aerobe Sporenbildner (*Bacillaceae* und *Paenibacillaceae), Micrococcaceae, Microbacteriaceae*, *Streptococcaceae, Caryophanales*, *Mycobacteriaceae* und *Deincoccaceae* identifiziert werden. Die am häufigsten isolierten Bakteriengenera stellten *Acinetobacter* (n=50/217; 23,04%), *Kocuria* (n=30/217; 13,82%), *Microbacterium* (n=25/217; 11,52%), *Pseudomonas* (n=16/217; 7,37%), *Micrococcus* und *Lactococcus* (jeweils 10/217; 4,61%) dar. *A. johnsonii*, *A. iwoffi* (jeweils

Risikogruppe 2) und *A. radioresistens* werden als humanassoziierte Keime beschrieben. Aerobe Sporenbilder sind Kontaminationskeime milchverarbeitender Oberflächen, zeigen thermodures Verhalten und können opportunistisch pathogen (*B. cereus* Gruppe) sein. *Kocuria* ist ein thermodurer umweltassoziierter Keim und zählt zur Risikogruppe 1. Das Keimreservoir der UHT milchverarbeitenden Prozessanlagen stellte sich in den Probennahmedurchgängen 1-3 als heterogen dar. Der Steriltank 2140 war die einzige Probenahmestelle, aus dessen Nachspülwasser keine bakteriellen Rückstände isoliert werden konnten. Das Nachspülwasser von Steriltank 2136 zeigte in allen Probennahmedurchgängen die größte bakterielle Diversität. Die meisten Bakterienspezies (n=10) konnten im Frischwassertank 2143 nachgewiesen werden.

Anhand der Versuchsergebnisse ist die Verifizierung des CIP Regimes der aseptischen milchverarbeitenden Prozessanlagen notwendig, da Biofilme an kritischen Oberflächen die Reinigungseffizienz des CIP Regimes vermindern. Im Rahmen der Verifizierung ist eine alternative Probenahmetechnik (z.B. Tupferprobe) zur Nachspülwasseruntersuchung sinnvoll, um das Rekontaminationsrisiko der Proben durch die Probenahme zu reduzieren. Da alle untersuchten Probenahmestellen vom Spülwasser des Frischwassertanks 2143 gereinigt werden, muss die mikrobiologische Wasserqualität des Spülwassers evaluiert werden. Aufgrund der Identifizierung des Frischwassertanks als Reservoir für Gram negative Rekontaminationskeime, empfiehlt sich eine wiederholte Beprobung und Sterilfiltration des Spülwassers aus Tank 2143. Um eine Risikobewertung der Ergebnisse zu ermöglichen, sollte nach der Keimisolation durch selektive Nährmedien, die entsprechend der Wachstumsanforderungen der Hygieneindikatoren sowie potenzieller bakterieller Pathogene inkubiert werden, eine 16S rRNA Genanalyse folgen. Die Verwendung von aufbereitetem Abwasser der Milchproduktion ist hinsichtlich ökologischer und wirtschaftlicher Faktoren eine Alternative zur Verwendung von Trinkwasser für CIP Reinigungsabläufe. Da Rohmilch mit einer geringen mikrobiologischen Belastung für die Produktion von UHT Milch mit einer stabilen Haltbarkeit notwendig ist, sollte nur Rohmilch der S-Klasse (Keimzahl ≤50 000/ml, Zellzahl ≤250 000/ml) verarbeitet werden, um das Verderbnisrisiko durch hitzeresistente Sporen und Enzymen aus der Rohmilchflora zu minimieren.

Milchverarbeitende Betriebe sollten die Reinigungs-und Desinfektionsmaßnahmen der aseptisch milchverarbeitenden Anlagen im Rahmen ihres HACCP stets kritisch kontrollieren.

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9. SUPPLEMENTS

Supplement Table 1: Equipment and consumables.

Equipment	Manufacturer
Microcentrifuge 5424	*Eppendorf AG, Hamburg, Germany*
Cond 3110 (electric conductivity)	*WTW electronic GmbH, Weilheim, Germany*
CSB reader DR 2800	Hach Lange GmbH, Düsseldorf, Germany
Electrophoresis unit	Bio-Rad Laboratories GmbH, Hercules, USA
Filtration unit	Sartorius AK & Co., Nümbrecht, Germany
Freezer for chelex samples (-20°C)	Liebherr International AG, Bulle, Switzerland
Freezer for cryo samples (-80°C)	Sanyo, Morigushi, Japan
GelDoc2000 UV camera	Bio-Rad Laboratories GmbH, Hercules, USA
HT 200S incubator	Hach Lange GmbH, Düsseldorf, Germany
Incubator 30°C	Memmert GmbH & Co.KG, Schwabach, Germany
Incubator 37°C	Memmert GmbH & Co.KG, Schwabach, Germany
Incubator 55°C	Memmert GmbH & Co.KG, Schwabach, Germany
pH electrode SensoLyt	*WTW electronic GmbH, Weilheim, Germany*
pH meter (pH 323)	*WTW electronic GmbH, Weilheim, Germany*
Pure water	Sartorius AK & Co., Nümbrecht, Germany
Scale	Sartorius AG, Göttingen, Deutschland
T100™ Thermal Cycler	Bio-Rad Laboratories GmbH, Hercules, USA
Vortex V3	VWR International GmbH, Radnor, Pennsylvania
Water bath MBT 250	Kleinfeld Labortechnik GmbH, Gehrden, Germany

Materials	Manufacturer
100bp DNA Ladder	MBI Fermentas, St. Leon-Rot, Germany
10x PCR Rxn Buffer	Thermo Fisher Scientific, Waltham, USA
10x TBE Buffer (Tris-Borat-EDTA-Puffer)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Anaerokult A Mini	Merck GmbH, Darmstadt, Germany
Aqua bidestillata	Milli Q- Integral water treatment system
Chelex®100 Resin	Bio-Rad Laboratories GmbH, Hercules, USA
Cryogenic vials	Biologix Group, Jinan, China
CSB Measuring Cuvette 15-150mg, 150-1000mg	Hach Lange GmbH, Düsseldorf, Germany
DEPC H_2O (Diethylpyrocarbonate)	Sigma-Aldrich Co. LCC, St. Louis, USA
dNTP Mix 20mM	Thermo Fisher Scientific, Waltham, USA
Ethanol 96%	Carl Roth GmbH, Karlsruhe, Germany
Filter disk	GE Healthcare, Buckinghamshire, UK
Glycerol for cryogenic conservation	Sigma-Aldrich Co. LCC, St. Louis, USA
Inoculation loops 10 µl, 100 µl	Sarstedt, Nümbrecht, Germany
Latex gloves	B. Braun Melsungen AG, Melsungen, Germany
Lysozyme	Sigma Aldrich, Vienna, Austria
$MgCl_2$ 50mM	Invitrogen, Lofer, Austria
Mikrozid AF Liquid	Schülke & Mayr GmbH, Norderstedt, Germany
Natriumthiosulfat	Carl Roth GmbH, Karlsruhe, Germany
PCR Primer	Microsynth AG, Balgach, Switzerland

PCR Tubes	Sarstedt, Nümbrecht, Germany
peqGreen	Peqlab, Erlangen, Germany
peqGOLD Universal Agarose	Peqlab, Erlangen, Germany
Petri dishes	Sarstedt, Nümbrecht, Germany
Pipette	Eppendorf AG, Hamburg, Germany
Pipette tips	Sarstedt, Nümbrecht, Germany
Platinum® Taq DNA Polymerase	Thermo Fisher Scientific, Massachusetts, USA
Proteinase K	Roche Diagnostics GmbH, Vienna, Austria
SafeSeal SurPhob pipette tips 1-1250 µl	Biozym Scientific GmbH, Hessich Oldedorf, Germany
Sample loading buffer (SLB)	Institute for Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Vienna, Austria
Sterile vials 1,5 ml; 2,0 ml	Eppendorf AG, Hamburg, Germany
Tris HCl	Sigma-Aldrich Co. LCC, St. Louis, USA

Selective media	**Manufacturer**
Brain heart infusion (BHI) for cryogenic conservation	Oxoid Limited, Hampshire, UK
Glutamat starch phenolred (GSP) agar	Merck GmbH, Darmstadt, Germany
***Lactobacillus* agar acc. to deMan, Rogosa and Sharpe (MRS) agar**	Oxoid Limited, Hampshire, UK
Mannitol yolk polymyxin agar (MYP)	Oxoid Limited, Hampshire, UK
Tryptic soy agar plus 6 % yeast (TSAY)	Biokar Diagnostics, Pantin, France
Violet red bile glucose (VRBG) agar	Merck GmbH, Darmstadt, Germany

Supplement Table 2: Results of the physicochemical monitoring of the final rinse flushing water in sampling 2-3.

Sampling station	Sampling	CIP circle	pH value	Conductivity (µs/cm)	COD (mg/L O_2)
Milk pasteur	2	51	7.63	457	982
	3	51	7.60	482	4.9
UHT heater	2	51	7.67	467	110
	3	51	7.61	422	23.4
H1	2	51	7.52	470	71
	3	53	8.23	450	98.9
BC2	2	52	7.62	464	56.6
	3	53	7.33	544	13.2
2135	2	53	8.10	439	237
	3	53	8.37	450	150
2136	2	53	8.21	452	106.2
	3	53	7.10	480	13.1
2137	2	53	8.05	472	181
	3	53	7.85	459	56.6
2138	2	53	7.81	461	68.3
	3	53	7.21	475	20.8
2139	2	53	8.01	497	27.1
	3	52	7.43	473	13.1
2140	2	53	8.02	466	27.3
	3	52	7.98	460	6.88
2141	2	52	8.03	447	69.2
	3	52	7.09	472	19.1
2142	2	52	7.97	461	78.2
	3	53	7.69	465	12
2143	2	53	8.25	463	89.6
	3	53	7.70	454	5.1

Supplement Table 3: Differentiation and species confirmation of isolated bacteria by 16 S RNA sequencing.

Gram	Phyla	*Family*	*Group*	*Species*	KOH	Cat	Ox	Risk group	Niche	Sampling	Sampling site	Isolates (n)
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter colistiniresistens*	P	P	N	2	H	II	2136	3
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter johnsonii*	P	P	N	2	H	II	2142	3
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter johnsonii*	P	P	N	2	H	III	2135	3
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter johnsonii*	P	P	N	2	H	III	H 1	6
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter lwoffii*	P	P	N	2	H	II	2136	10
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter lwoffii*	P	P	N	2	H	III	2135, 2136, 2139	13
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter lwoffii*	P	P	N	2	H	III	BC 2	3
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter radioresistens*	P	P	N	1	H	II	2137	3
N	PROTEO	*Moraxellaceae*	*Acinetobacter*	*Acinetobacter radioresistens*	P	P	N	1	H	III	2136, 2142	6
N	PROTEO	*Aeromonadaceae*	*Aeromonas*	*Aeromonas caviae*	P	P	P	2	H, A	I	Asepto	2
N	PROTEO	*Aeromonadaceae*	*Aeromonas*	*Aeromonas media*	P	P	P	1	H, A, FP	III	BC 2	2
N	PROTEO	*Aeromonadaceae*	*Aeromonas*	*Aeromonas salmonicida*	P	P	P	1	A	II	2143	2
N	PROTEO	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas vesicularis*	P	P	P	2	UBIQ	I	2136	2
N	PROTEO	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas vesicularis*	P	P	P	2	UBIQ	II	2137	2
N	PROTEO	*Caulobacteraceae*	*Brevundimonas*	*Brevundimonas vesicularis*	P	P	P	2	UBIQ	III	2142	4
N	PROTEO	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter bugandensis*	P	P	N	2	H	II	2143	4
N	PROTEO	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella oxytoca*	P	P	N	2	H, A	II	2143	4
N	PROTEO	*Oxalobacteraceae*	*Massilia*	*Massilia chloroacetimidivorans*	P	N	P	1	E	III	2139	4
N	PROTEO	*Pseudomonadaceae*	*PFLUOR*	*Pseudomonas gessardii*	P	P	P	1	E	I	Asepto	6
N	PROTEO	*Pseudomonadaceae*	*PFLUOR*	*Pseudomonas migulae*	P	P	P	1	E	II	2143	4
N	PROTEO	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas baetica*	P	P	P	1	A	II	2143	2
N	PROTEO	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas helmanticensis*	P	P	P	1	E	II	2143	2
N	PROTEO	*Pseudomonadaceae*	*Pseudomonas putida group*	*Pseudomonas oryzihabitans*	P	P	P	2	E	II	2136	2
N	BACTERO	*Flavobacteriaceae*	*Chryseobacterium*	*Chryseobacterium hispalense*	P	P	P	1	E	II	2143	4
N	BACTERO	*Sphingobacteriaceae*	*Sphingobacterium*	*Sphingobacterium multivorum*	P	P	P	2	E	II	2143	4
P	FIRM	*Bacillaceae*	*BCG*	*Bacillus pacificus*	N	P	P	2	E, FP	I	MP	2
P	FIRM	*Bacillaceae*	*BCG*	*Bacillus pacificus*	N	P	P	2	E, FP	I	2136, 2138	3
P	FIRM	*Bacillaceae*	*BCG*	*Bacillus pacificus*	N	P	P	2	E, FP	II	2136, 2142	2
P	FIRM	*Bacillaceae*	*BCG*	*Bacillus pacificus*	N	P	P	2	E, FP	II	2143	1
P	FIRM	*Bacillaceae*	*BSG*	*Bacillus subtilis*	N	P	V	1	E, FP	I	Asepto	1
P	FIRM	*Bacillaceae*	*BSG*	*Bacillus haynesii*	N	P	V	1	E	III	2135	1
P	FIRM	*Bacillaceae*	*BSG*	*Bacillus haynesii*	N	P	V	1	E, FP	II	2142	1
P	FIRM	*Bacillaceae*	*BSG*	*Bacillus nakamurai*	N	P	V	1	E	I	MP	1

P	FIRM	*Bacillaceae*	*BSPP*	*Bacillus bataviensis*	N	P	V	1	E, FP	II	2142	1
P	FIRM	*Bacillaceae*	*BSPP*	*Bacillus simplex*	N	P	V	1	FP	I	MP	1
P	FIRM	*Bacillaceae*	*BSPP*	*Bacillus simplex*	N	P	V	1	FP	II	2142	2
P	FIRM	*Bacillaceae*	*BSPP*	*Viridibacillus arenosi*	N	P	V	1	E	II	2142	1
P	FIRM	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus glucanolyticus*	N	P	V	1	E	I	MP	1
P	FIRM	*Paenibacillaceae*	*Paenibacillus*	*Paenibacillus provencensis*	N	P	V	1	H	I	2138	5
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus epidermidis*	N	P	N	2	H, A, FP	II	2142	1
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus haemolyticus*	N	P	N	2	H, A	II	2135	1
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus haemolyticus*	N	P	N	2	H, A	II	BC 2	1
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus haemolyticus*	N	P	N	2	H, A	III	H 1	1
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus pasteuri*	N	P	N	2	H	I	MP	1
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus pasteuri*	N	P	N	2	H	II	2142	1
P	FIRM	*Staphylococcaceae*	*Staphylococcus*	*Staphylococcus sp.*	N	P	N	no	H, A, E	I	Asepto	2
P	FIRM	*Mycobacteriaceae*	*Mycobacterium*	*Mycobacterium diernhoferi*	N	N	P	1	E	III	Asepto	1
P	FIRM	*Caryophanales*	*Exiguobacterium*	*Exiguobacterium aestuarii*	N	P	N	1	UBIQ	I	2136	5
P	FIRM	*Streptococcaceae*	*Lactococcus lactis*	*Lactococcus lactis*	N	N	N	1	FP	II	2135	10
P	DEINO	*Deinococcaceae*	*Deinococcus*	*Deinococcus piscis*	N	P	P	1	E	II	2137	5
P	ACTINO	*Micrococcaceae*	*Kocuria*	*Kocuria kristinae*	N	P	P	1	H	II	2135	5
P	ACTINO	*Micrococcaceae*	*Kocuria*	*Kocuria marina*	N	P	P	1	E	III	2137, 2141	10
P	ACTINO	*Micrococcaceae*	*Kocuria*	*Kocuria palustris*	N	P	P	1	E	II	2143	5
P	ACTINO	*Micrococcaceae*	*Kocuria*	*Kocuria rhizophila*	N	P	P	1	E	II	2135, 2142	10
P	ACTINO	*Micrococcaceae*	*Micrococcus endophyticus*	*Micrococcus endophyticus*	N	P	P	1	E	I	2138	10
P	ACTINO	*Microbacteriaceae*	*Microbacterium*	*Microbacterium laevaniformans*	N	P	N	1	FP	III	2142	5
P	ACTINO	*Microbacteriaceae*	*Microbacterium*	*Microbacterium oxydans*	N	P	N	1	E	II	2138	10
P	ACTINO	*Microbacteriaceae*	*Microbacterium*	*Microbacterium testaceum*	N	P	N	1	E	I	2136	10

Abbreviations: A, Animals; ACTINO, Actinobacteria; BACTERO, Bacteriodetes; BCG, *Bacillus cereus* group; BSG, *Bacillus subtilis* group; BSPP, *Bacillus* spp. diverse; DEINO, *Deinococcus* Thermus; E, Environment; FIRM, Firmicutes; FP, Food Processing; H, Human; N, Negative; P; Positive; PFLUOR, *Pseudomonas fluorescens group*; PROTEO, Proteobacteria; UBIQU, Ubiquitous; V, Gram-variable.