Sandra Freudenthaler-Holzer, BSc MSc

Microbial investigation of milk product quality in different operating levels

DISSERTATION

zur Erlangung des akademischen Grades
Doktorin der Naturwissenschaften

eingereicht an der

Technischen Universität Graz

Betreuer

Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. Michael Murkovic

Institut für Biochemie

Graz, Mai 2020

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Das in TUGRAZonline hochgeladene Textdokument ist mit der vorliegenden Dissertation identisch.

Datum, Unterschrift

Danksagungen

In erster Linie gilt mein Dank Professor Michael Murkovic, der es mir ermöglicht hat, diese Arbeit auf den Weg zu bringen. Seine Ideen, seine Unterstützung bei der Ausführung und nicht zuletzt die Korrektur der Arbeit haben mir unbeschreiblich geholfen. Vor allem die unkomplizierte Kommunikation hat mich sehr beeindruckt und dafür möchte ich mich sehr herzlich bedanken.

Großen Dank schulde ich DI Judith Königswieser und dem Team von Biomerx Laboratorium, da ich mit deren Hilfe erst diese große Menge an Proben und Daten sammeln konnte. Das ganze Team hat mich wahnsinnig unterstützt und ich danke jedem von euch für die Zeit, die ihr für meine Arbeit geopfert habt.

Zuletzt, und doch am Wichtigsten, war jedoch die Unterstützung meiner Familie. Meinen Eltern habe ich zu verdanken soweit gekommen zu sein, um diese Arbeit zu schreiben. Ihr seid mir mein ganzes Leben mit Rat und Hilfe zur Seite gestanden und habt mich immer unterstützt. Vielen Dank für alles.
Bei meinem Ehemann Philipp möchte ich mich für seine Geduld, Nachsicht und Hilfe bedanken. Ohne dich wäre es nicht möglich gewesen, dieses Projekt zu verwirklichen. Du bist mir immer eine Stütze und es ist von unschätzbarem Wert, solch einen Menschen bei sich zu haben.
Unserer kleinen Tochter Christiane gebührt natürlich auch noch ein Dankeschön für ihre vielen Schlafenszeiten, die ich für diese Arbeit nutzen konnte.

Zusammenfassung

In dieser umfassenden Studie wurden Vollmilch, Halbfettmilch und Protein-Molkekonzentrate (Whey Protein Concentrate), mit unterschiedlicher Proteinkonzentration, während des gesamten Produktionsverlaufes untersucht. Zu diesem Zweck wurden Proben an spezifischen Produktionsschritten entnommen und die mikrobielle Kontamination detektiert. Die Probenahme startete mit der Entnahme der Rohmilchprodukte und endete mit dem finalen Produkt nach der Sprühtrocknung.
Die hier verwendeten Methoden entsprechen alle der EN ISO/IEC 17025 und alle untersuchten Proben wurden in einem akkreditierten Labor bearbeitet. Einer der vielen Vorteile eines akkreditierten Labors ist die Vergleichbarkeit der Ergebnisse mit anderen akkreditierten Labors, deren Hygienestandards sowie die korrekte Durchführung der Methoden in regelmäßigen Intervallen durch außenstehende Personen auditiert werden. Generell konnte hier festegellt werden, dass die Rohmilchprodukte am Produktionsbeginn eine durchwegs schlechtere Qualität aufweisen als die Endprodukte. Ganz im Speziellen die Rohprodukte, die für die Herstellung der Proteinkonzentrate verwendet werden. Hier konnte außerdem eine relativ hohe Anzahl an *Cronobacter sakazakii* positiven Proben nachgewiesen werden. Weiters war die Belastung mit *Enterobacterien, Coliformen* und daraus resultierend auch die Gesamtkeimzahl sehr hoch in den Rohprodukten. Nach einigen Erhitzungsschritten während der Produktion sank die Anzahl an nicht-hitzestabilen Bakterien in großem Maße, jedoch konnte dann eine Steigerung der thermophilen Bakterien und Sporen festgetellt werden. *Cronobacter sakazakii* konnte in einem der Endprodukte nachgewiesen werden.
Die große Standardabweichung, die in dieser Studie auffallend ist, kann mit den großen Qualitätsunterschieden der Rohprodukte in Zusammenhang gebracht werden und hat demnach auch großen Einfluss auf die Qualität der Endprodukte. In diesem Sinne ist es von großer Bedeutung für die Lebensmittelhersteller, die Qualität der Rohmaterialien gewissenhaft zu überprüfen um mikrobiell einwandfreie Endprodukte herstellen zu können.
Zusammenfassend kann festgehalten werden, dass die wichtigsten Punkte bei der Produktion von Milchpulver, eine strikte Hygienevorschrift, steriles Arbeiten und gute Rohmilchqualität sind um bakterielle Kontaminationen zu verhindern. Dies führt in weiterer Folge zur Verbesserung der Produktqualität und zur Verhinderung von kostspieligen Verunreinigungen der Produktionsstätte.

Abstract

In this comprehensive study, milk products such as whey protein concentrate (WPC), unskimmed milk and low fat milk were investigated during its whole production process. For this purpose samples were taken at specific production steps and microbial contamination was recorded. Also raw-material quality and the final products were proved, considering the different seasons at delivery.
The methods used for this study are EN ISO/IEC 17025 standard methods and all sample investigations were conducted in an accredited laboratory. One of the several advantages of an accredited laboratory is the comparability of the results to other accredited labs. Quality of the results, hygiene instructions, methods and the correct mode of operation are frequently audited.
In general raw-material quality is much lower than final product-quality, in particular WPC raw-materials. In this case, also a relatively high percentage was tested *Cronobacter sakazakii* positive. Furthermore, the total bacteria count, *Enterobacteria* and *Coliform* bacteria were present in a high number in all raw-material samples. After two or even three heating steps, the number of not-heat-resistant bacteria decreased. *Cronobacter sakazakii* was detected just in one of the final products. In contrast, the number of thermophilic bacteria and thermophilic spores increased in the final product samples in a huge way.
The high standard deviation at all results show a wide range of raw material quality, which also influences the resulting final product. If the raw material quality could be improved, the food producer would have less trouble to produce contamination free final product. The drying process eliminates about 99 % of bacterial contamination, but the point is how high the counts are at the beginning.
All in all, a proper hygienic monitoring, cleaning and sterile handling prevent unnecessary contaminations at the factory and support the production of high quality dryed milk products.

Contents

1 Introduction 15

1.1 Cronobacter sakazakii 16

1.1.1 General facts about Cronobacter 16

1.1.2 Taxonomy 17

1.1.3 Infectious dose and virulence factors 18

1.1.4 Clinical picture and sources 19

1.1.5 Transmission 20

1.1.6 Food safety and public health 20

1.2 Coliform bacteria and Enterobacteriaceae 22

1.2.1 Coliform bacteria 22

1.2.2 Enterobacteriaceae 23

1.3 Enterococcus 26

1.3.1 Introduction 26

1.3.2 Food contamination and environmental sources of *Enterococci* 27

1.3.3 Bacteriocins in *Enterococci* 28

1.3.4 Antibiotic resistance of *Enterococci* 29

1.3.5 Gene transfer 29

1.3.6 Virulence factors 30

1.3.7 Pathology 32

1.3.8 Enterococci in food 33

1.4 Bacillus cereus 36

1.4.1 Introduction 36

1.4.2 Transmission 37

1.4.3 Diseases 37

1.4.4 Public Health Risks 38

1.4.5 *Bacillus cereus* in Foods 38

1.4.6 Prevention 39

1.5 Mold and Yeast ... *41*
1.5.1 Mold ... 41
1.5.2 Yeast ... 45

1.6 Milk Products ... *48*
1.6.1 Raw Milk ... 48
1.6.2 Milk Powder ... 50
1.6.3 Whey Protein ... 51

2 Material and Methods ... 54

2.1 Material ... *54*
2.1.1 Buffered Peptone Water (Biokar diagnostics) ... 54
2.1.2 Modified Lauryl Sulphate (Oxoid) ... 55
2.1.3 Chromocult® Enterobacter Sakazakii Agar (Merck) ... 55
2.1.4 Trypto-Casein Soy Agar (Biokar Diagnostics) ... 56
2.1.5 API ID32E (bioMérieux) ... 57
2.1.6 Violet Red Bile Agar (Biokar Diagnostics) ... 59
2.1.7 Violet Red Bile Glucose Agar (Biokar Diagnostics) ... 60
2.1.8 Slanetz & Bartley Medium (Oxoid) ... 60
2.1.9 *Bacillus Cereus* Agar acc.to Mossel (Biokar Diagnostics) ... 61
2.1.10 API 50 + CHB Medium (BioMérieux) ... 63
2.1.11 Yeast Extract Glucose Chloramphenicol Agar (Merck) ... 66
2.1.12 Dextrose Tryptone Agar (Oxoid) ... 66
2.1.13 Plate Count Agar (Biokar Diagnostics) ... 67

2.2 Methods ... *67*
2.2.1 *Cronobacter sakazakii Identification* ... 67
2.2.2 Identification *Coliform* Bacteria ... 69
2.2.3 Identification of *Enterobacter* Species ... 70
2.2.4 Identification of *Enterococci* ... 71
2.2.5 Identification of *Bacillus cereus* ... 72

2.2.6 Identification of Mold and Yeast ... 73
2.2.7 Identification of Spores ... 75
2.2.8 Identification of Bioburden and Thermophilic Bacteria ... 76

2.3 Spray Drying ... 77
2.3.1 Introduction ... 77

2.4 Procedure ... 80

3 Results ... 82

3.1 Unskimmed Milk Results ... 82
3.1.1 Contamination in Different Production Steps ... 83
3.1.2 Raw Material and Final Products of Uskimmed Milk ... 86

3.2 Low-Fat Milk Powder Results ... 89
3.2.1 Contamination during the Production Steps ... 89
3.2.2 Comparison of Raw Material and Final Low-Fat Milk Powder Product ... 92

3.3 Results of WPC 35 ... 95
3.3.1 Comparison of Raw Material and WPC 35 Final Product ... 99

3.4 Results of WPC 60 ... 102
3.4.1 Contamination of Production Levels of WPC 60 ... 102
3.4.2 Comparison Raw Material and Final WPC 60 Product ... 106

3.5 Results of WPC 80 ... 110
3.5.1 Contamination of the Production Levels during WPC 80 Production ... 111

3.5.2 Comparison of Raw Material and Final Product of WPC 80 Production ... 113

4 Discussion ... 117

4.1 Cronobacter sakazakii in Milk Products ... 117

4.2 Raw Material Quality ... 118

4.3 Production Steps ... 119

4.3.1 Production Levels for Unskimmed and Low-Fat Milk ... 120

4.3.2 Production Steps for WPC ... 121

4.4 Comparison of Different Seasons ... 123

4.5 Final Products ... 124

List of references ... 126

Lebenslauf ... Error! Bookmark not defined.

Acronyms and Glossary

WPC: Whey Protein Concentrate

IMF: Infant Milk Formula

API: Analytical Profile Index

TSA: Tryptone Soy Agar

VRBGA: Violet Red Bile Glucose Agar

PIF: Powdered Infant Formula

FDA: U.S. Food and Drug Administration

MDR: Multidrug Resistance

ESBL: Extended Spectrum β-Lactamase

LAB: Lactic Acid Bacteria

LMO: Listeria Monocytogenes

VRE: Vancomycin-Resistant *Enterococci*

AS: Aggregation Substance

UTI: Urinary Tract Infection

GHP: Good-Hygiene-Practice

GMP: Good-Manufacture-Practice

BPW: Buffered Peptone Water

mLAB: modified Lauryl Sulphate Broth

TSA: Trypto-Casein Soy Agar

VRB: Violet Red Bile Agar

BC-agar: *Bacillus Cereus* agar

YGC: Yeast Extract Glucose Chloramphenicol Agar

DTA: Dextrose Tryptone Agar

PC: Plate Count Agar

Table of Figures

Fig.1: Electron microscopic image of *Cronobacter sakazakii*
Fig. 2: Electron microscopic image of *Enterococcus faecalis*
Fig. 3: Enterocin type II with antimicrobial activity
Fig. 4: Electron microscopic image of *Bacillus cereus*
Fig. 5: Aflatoxin M_1
Fig. 6: Lactose
Fig. 7: *Cronobacter sakzakii* on Chromocult® agar (Merck)
Fig. 8: API ID32E Biomerieux Industry
Fig. 9: API 50 CH Biomerieux Industry
Fig.10: *Enterobacter faecalis* on Violet Red Bile Agar (Biokar Diagnostics)
Fig.11: *Enterococcus faecalis* on S&B Agar (Oxoid)
Fig. 12: *Bacillus cereus* on BC agar (Biokar Diagnostics)
Fig.13: *Penicillium chrysogenum* on YGC Agar (Merck)
Fig.14: *Saccharomyces cerevisiae* on YGC Agar (Merck)
Fig. 15: *Geobacillus stearothermophilus* on DTA Agar (Oxoid)
Fig. 16: Schematic diagram of a single stage spray dryer
Fig.17: Comparison of raw material and final product contamination of unskimmed milk
Fig. 18: Comparison of final product contamination at different seasons
Fig. 19: Bacterial contamination of unskimmed milk in operating level 3, 5 and 7
Fig. 20: Bacterial contamination of unskimmed milk in operating level 5a and 7a
Fig. 21: Contamination profile at production steps 3, 5 and 7 at low fat milk powder production
Fig. 22: Bacterial contamination at operation level 5a and 7a at low fat milk production
Fig. 23: Comparison of contamination (average + standard deviation) of raw product and final product of low fat milk powder
Fig. 24: Comparison of final product results in summer and winter
Fig. 25: Comparison of contamination profiles of level 3,5 and 7 of WPC 35
Fig. 26: Comparison of contamination profiles of level 1 start and 1 end of WPC 35
Fig .27: Contamination profile of operation level 5a and 7a at WPC 35 production
Fig. 28: Comparison of raw material and final product of WPC 35
Fig. 29: Comparison of bacterial profile of WPC 35 final products at different seasons

Fig. 30: Contamination profile of production level 3, 5 and 7 of WPC 60
Fig. 31: Contamination profile of production level 5a and 7a during WPC 60 production
Fig. 32: Contamination profile of production level 1 start and level 1 end at WPC 60 producion
Fig. 33: Comparison of the bacterial profile of WPC 60 raw material and final product
Fig. 34: Bacterial profile of WPC 60 final product at different seasons
Fig. 35: Bacterial profile of WPC 80 at operation levels 3, 5 and 7
Fig. 36: Bacteria contamination at levels 1 start and 1 end at WPC 80 production
Fig. 37: Bacterial profile of WPC 80 raw material and final product
Fig. 38: Bacterial contamination of WPC 80 final products at different seasons
Fig. 39: *Enterobacter spp.* on VRBG Agar (Biokar Diagnostics)

List of Tables

Tab. 1: Tests included in API ID32E test kit
Tab. 2: Tests included in API 50CHB test kit
Tab. 3: Heating temperatures after receiving department
Tab. 4: Heating temperatures before storage at the cooling tower
Tab. 5: Specific sample taking points during the production
Tab. 6: Distribution of the samples during unskimmed milk production
Tab. 7: Bacterial counts (average in cfu) and standard deviation (cfu) in raw material/final products of unskimmed milk and their reduction/increase in percent
Tab. 8: Bacterial counts (average cfu) and standard deviation (cfu) of production steps 3, 5 and 7 (Unskimmed milk)
Tab. 9: Bacterial counts (average in cfu) and standard deviation (cfu) of production steps 5a and 7a (Unskimmed milk)
Tab. 10: Distribution of low fat-milk samples during production
Tab. 11: Bacterial counts (average) and standard deviation in production steps 3, 5 and 7of low-fat milk
Tab. 12: Tab.12: Comparison of bacterial contamination production level 5a and 7a at low fat-milk production (average and standard deviation in cfu)
Tab. 13: Bacterial counts (average) and standard deviation in raw material/final products of low fat-milk and their reduction/increase in percent
Tab. 14: Comparison of final product results in summer and winter
Tab. 15: Distribution of WPC 35 samples during production
Tab. 16: Bacterial counts (average in cfu) and standard deviation (cfu) at production steps 3, 5 and 7of WPC 35
Tab. 17: Bacterial counts (average in cfu) and standard deviation (cfu) at production steps 1 start and 1 end of WPC 35
Tab. 18: Bacterial counts (average in cfu) and standard deviation (cfu) at production level 5a and 7a of WPC 35
Tab. 19: : Bacterial counts (average in cfu) and standard deviation (cfu) of raw material and final product of WPC 35 and their reduction or increase
Tab. 20: Bacterial profile of WPC 35 at different seasons (average + standard deviation of final products in cfu)

Tab. 21: Distribution of WPC 60 samples
Tab. 22: Bacterial count (average in cfu) and standard deviation (cfu) of production levels 3, 5 and 7 of WPC 60 production
Tab. 23: Bacterial count (average + standard deviation in cfu) of production levels 5a and 7a of WPC 60
Tab. 24: Bacterial count (average and standard deviation in cfu) of production level 1 start and 1 end of WPC 60 production
Tab. 25: Bacterial counts (average in cfu) and standard deviation (cfu) in raw material/final products of WPC 60 and their reduction/increase in percent
Tab. 27: Distribution of samples of WPC 80
Tab. 28: Bacterial counts (average and standard deviation in cfu) of operating levels 3, 5 and 7 of WPC 80
Tab. 29: Bacterial count (average and standard aviation in cfu) of production levels 1 start and 1 end at WPC 80 production
Tab. 30: Comparison of bacterial contamination (average and standard deviation in cfu) of raw material and final product at WPC 80 prodcution
Tab. 31: Comparison of bacterial contamination of WPC 80 final product at different seasons (average and standard deviation in cfu)
Tab. 32: Comparison of bacterial contamination of unskimmed milk final product at different seasons (average and standard deviation in cfu)

1 Introduction

In the last few years healthy nutrition has become more and more a major theme in people`s lives. Subsequently the interest in potential food contaminations also rises. Food poisoning caused by bacterial contamination is the most common reportable disease in Austria. Food poisoning is defined as an inflammation of the gastrointestinal tract, usually caused by bacteria, viruses, or parasites and leads to diarrhea.
Pathogens are toxic or release toxins. In 2012 6700 cases of food poisoning were reported in Austria, it is consequently the most common illness in this country. 5-72 hours after ingestion of an affected food, first symptoms like sickness, stomach ache or diarrhea occur. In case of a bacterial food poisoning the causative organism assign directly on the patient`s intestinal mucosa. (1)
Milk and milk powder are in general hygienic save food products. Less than 10% of food poisoning leads back to dairy products. (1) On the one hand pasteurization-steps lead to such good results; on the other hand, a severe hygienic monitoring is necessary. This is much more important when milk powder is used for infant milk formula (IMF). However, milk powder is not just used for IMF, but also for sports food (Whey protein concentrate), for cheese-, chocolate- or yoghurt-production, for long-life products and many more. (2)
The production process from raw milk to final milk powder contains pasteurization and divers heating steps.
In this study raw milk runs through a spray drying process with different heating steps to receive milk powder in high quality. To ensure this high-quality final product, it is necessary to supervise every production step upon its microbial contamination. A very important part of supervising microbial contamination is to use high quality raw milk. Requirement of high quality raw milk is healthy dairy cattle, hygienic milking and also an effective cooling system from recovery to milk powder production. Normally raw milk contains a multitude of different microbes like *Staphylococcus aureus, bacillus*, *coliform bacteria* or *pseudomonas.* (3) Consequently, fewer microbes in high quality raw products make it easier to guarantee contamination free final products. Therefore, hygienic screening must start at the cattle-farm and not at the point of starting production.

1.1 Cronobacter sakazakii

1.1.1 General facts about Cronobacter

Cronobacter sakazakii is a gram-negative, motile opportunistic food borne pathogen (Fig1). [4, 5]

It is a member of the family *Enterobacteriaceae*, facultative anaerobic and generally peritrichous. This microorganism is generally indole, malonate and dulcitol negative, but methyl-α-D-glucopyranoside positive. [18] As a selective marker in differential chromogenic agar the activity of α-glucosidase has been implemented. [19] The optimum growth temperature is 39 °C, but some species of the genus can grow at temperatures between 6-47 °C. [14] Incubation temperature also influences pigment production greatly and colony size on TSA (tryptone soy agar). After 24 h incubation at 25 °C yellow pigment production is more distinct than at 36 °C. [20] Continuative two different stains have been detected when samples were streaked on TSA (tryptone soy agar). TypeA is dry or mucoid and matt, typeB is glossy, smooth and often shows little pigment production. [20, 14] Subculturing these two types shows that matt colonies may change to glossy colonies and it is common to find both types in cultures. [20] Also observed were differences between clinical and environmental strains. [21] On VRBGA (violet red bile glucose agar) the clinical strain grew mucoidal, the environmental strain produced crinkled, matt colonies. Furthermore, a reported heteropolysaccharide capsule may enable survival of *Cronobacter* throughout the long shelf-life of powdered infant formula (PIF), as well as attachment and creation of biofilms, supporting resistance to disinfectants and cleaning agents. [14, 22, 23]

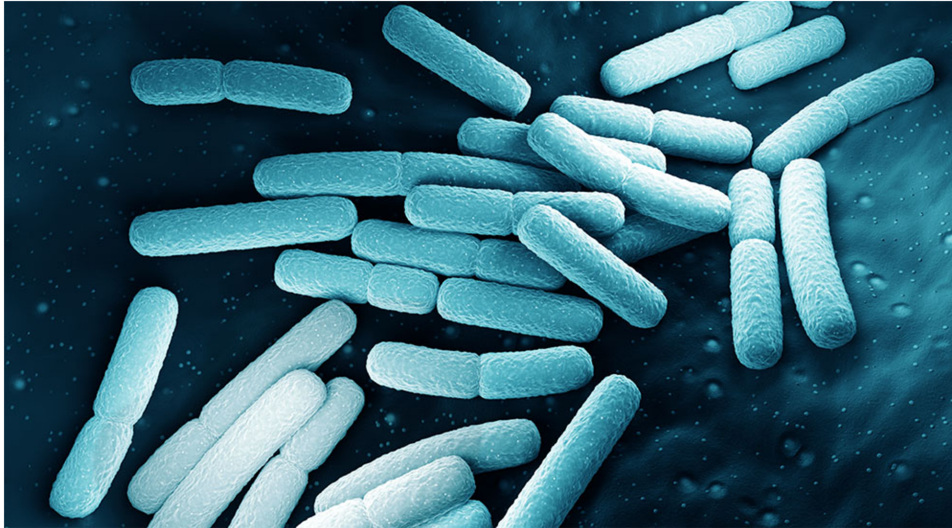


Fig.1 Electron microscopic image of *Cronobacter sakazakii* (Environmental Isolate Case File: Cronobacter sakazakii) [120]

1.1.2 **Taxonomy**

Cronobacter sakazakii was previously described as "yellow pigmented E. cloacae" and was defined as a new species in 1980. Also 15 biogroups were defined based on biochemical characterization. [20] Members of this species were deemed to be relatively phenotypically and genotypically heterogeneous, a 16th biogroup has been found and the existence of diverse genetic groups has been reported based on 16S rRNA gene sequence analysis. [24, 25] In the last years, a definite species has been identified and researchers named it after the Japanese bacteriologist Riichi Sakazaki, who greatly participated in understanding Enterobacteriacae. [20] In 2008 E. sakazakii isolates were reclassified in a distinct genus, Cronobacter. Now there are five proper species, namely Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter turicensis, Cronobacter dublinensis and Cronobacter muytjensii. [18] Of all the other Cronobacter species, the closest related to Cronobacter sakazakii is Cronobacter malonaticus and it is characterized by utilization of malonate. [26] Cronobacter malonaticus is indole-negative and dulcitol-negative. Cronobacter turicensis arose in biogroup 16. [25] The only Cronobacter species which is negative for the utilization of 1-O-methyl-α-D-glucopyranoside is Cronobacter muytjensii, but it is also positive for indole, dulcitol, malonate utilization and derived from biogroup 15. [20] Cronobacter dublinensis is subdivided in three species, namely C. dublinensis subsp. dublinens, C. dublinensis subsp. lausannensis and C. dublinensis subsp. lactaridi.

Cronobacter dublinensis subsp. dublinensis was isolated from environmental samples in a milk processing facility. [18]

1.1.3 Infectious dose and virulence factors

Health Canada is working on a dose-response relationship, but the infectious dose has not been determined yet. [27] The infectious dose will be influenced by the immune system of the patient, the state of the bacteria and by the environment in which *Cronobacter* grew before infection. The supposed infectious dose value is 1000 cfu.g^{-1}, but researchers found that 10,000 cfu per mouse was the lowest dose to be lethal in an infant mouse assay. [28] However, it will take about 9 days at 8 °C in reconstituted IMF for the bacteria to reach 1000 cfu $g^{-1,}$ but only about 18 h at room temperature with a contamination level of 0.36 cfu 100 g^{-1}. In this case it is very unlikely that normal contamination levels would lead to infections. It is much more likely that temperature abuse and/or contamination from preparation utensils leads to clinical symptoms. [14]
The factors involved in the pathogenesis of *Cronobacter* at the molecular level are still not completely clarified. All strains in this genus show differences in pathogenicity and may also have different virulence factors. [28, 29, 30] The only species which have been isolated from neonatal meningitis-cases are *Cronobacter sakazakii, Cronobacter turicensis* and *Cronobacter malonaticus.* Interestingly, a strain belonging to *C. muytjensii* has been isolated from a human bone marrow; normally it would be sterile. [31] About the different virulence factors or the mechanism of infection very little is known. In mammalian cultures, *Cronobacter* can attach to intestinal cells and survive inside macrophages. [32] The specific adhesins and the host cells receptors involved in these processes are still unknown. Some strains of *Cronobacter* produce capsular material, but the process how this material contributes to macrophage evasion is unknown. [33] This capsule may also protect the pathogen and ensure its survival in inhospitable environments. *Cronobacter sakazakii* is also able to attach plastics and silicon rubbers by growing in biofilms. [34] These biofilms may also be important for altered susceptibility to antimicrobials. [23, 33]
One of the virulence factors of *Cronobacter* is the O-antigen with its variable polysaccharide side chains, which are responsible for serological diversity among bacteria. Two serotypes of the *rfb* locus were identified in *Cronobacter* strains, these serotypes are

also involved in the synthesis of the O-antigen. In gram-negative bacteria the O-antigen is a major surface antigen and it has important consequences for the virulence of *Cronobacter.*[35] The O-polysaccharide produced by this strain can differ in structure, for example in size according to sugar composition or complexity of the structure, when compared to O-polysaccharide structures of other *Cronobacter sakazakii* strains. These differences lead to variety between serotypes and may show that this pathogen is serologically heterogeneous relating to the O-antigens. [30]
The second virulence factor of *Cronobacter* species is the production of proteolytic enzymes. The action of various proteases causes cell deformation, particularly "rounding" the cells. [36] A zinc-containing metalloprotease was found in *Cronobacter* cells which caused rounding of Chinese hamster ovary cells. The reason why the pathogen can cross the blood-brain barrier or can cause such bad cell damage found in neonates with necrotizing enterocolitis may be, that this enzyme has a collagenolytic activity. The *zpx* gene, which codes for the proteolytic enzymes, was found in all tested strains. [37]
Furthermore, *Cronobacter* species is also able to produce an enterotoxin. [28] This enterotoxin shows a molecular mass of 66 kDa and it is most active at pH 6, it is also proofed to be very stable;- after incubation at 70 °C for 30 min it was unaffected and after incubation at 90 °C for 30 min it just decreased in activity. [38]

1.1.4 **Clinical picture and sources**

It is associated with infections in infants and neonates.[5] Symptoms of *Cronobacter* infections include meningitis (complicated by brain abscess, cerebral infarction, ventriculitis and cyst formation) , septicemia and necrotizing enterocolitis.[6] Mortality rates vary from 40%-80% among infected infants.[7] A contamination level of 1 $cfu.100g^{-1}$ of *Cronobacter sakazakii* can lead to severe impact on health, only a fast detection and correct identification of these dangerous pathogens are important for food safety.[8] Many of the children that survive a *Cronobacter* – associated meningitis (94%) develop irreversible sequelae like neurological complications, which are often attributed to cerebral infarcts.[6] Only three *Cronobacter* species are associated with neonatal infections, namely *C. sakazakii, C. malonaticus* and *C. turicensis.* They have the genes encoding for a cation efflux system, which enables bacteria to enter brain micro vascular

endothelial cells. Since that it is still unknown whether all of the species are virulent, the whole genus is now classified as pathogenic. However, IFM (Infant formula milk) is the only source that has been linked to epidemiologically disease outbreaks caused by *Cronobacter.* [8, 9, 10, 11] It has also been reported that regularly used disinfectants to kill *Cronobacter* cells are insufficient when the cells are imbedded in biofilms.[12] The original reservoir of this pathogen is still unknown, but there are signs that *Cronobacter* might be of plant origin.
The strains of *Cronobacter* have been isolated from a wide range of food products, such as meat, milk, mixed salad and cheese. [13, 14, 15, 16] Other food than IFM has been rarely investigated, nevertheless these bacteria could be isolated from a wide range of food and food ingredients. [17]

1.1.5 **Transmission**

The ways of transmission and the sources of *Cronobacter sakazakii* are not completely clear. The bacterium has been found in multiple food sources, but only in PIF a strong association has been detected. The contaminations were differed in intrinsic and extrinsic, in which intrinsic means the introduction of the organism to the PIF at some manufacturing process step and extrinsic contamination is caused fusing of contaminated utensils, like spoons or blenders, when preparing PIF. [39]

1.1.6 **Food safety and public health**

Children and especially infants are very fragile to food borne infections. For this reason, the microbiological safety of infant and follow up formula is extremely important. In hospital neonatal units care givers should be careful with PIF, because it is not a sterile product and the use of hygienic steps during preparation are essential. PIF has been used to feed millions of children and it is the most used infant formula worldwide, because this product imitates the nutritional profile of human breast milk. [40] Bovine milk is the major ingredient of PIF and of course a potential source of bacteria, so PIF is a perfect medium to support bacterial growth, also bacteria that are pathogenic to infants. A wide range of bacterial pathogens have been found in PIF, including *Citrobacter, Enterobacter,*

Klebsiella, Staphylococcus, Yersinia and *Streptococcus.* The presence of *Cronobacter sakazakii* in PIF can lead to infections. [41] A link between an unopened PIF package and an outbreak of infection also has been reported. [40] After the death of an infant caused by a *Cronobacter sakazakii* meningitis in New Zealand in 2004, this kind of infections have recently been added to the list of notifiable diseases. [42] For this reason, The World Health Organization recommends exclusively breast-feeding for the first six months, if that is not possible infants should be nourished with an appropriate breast milk substitute corresponding to the Codex Alimentarius Commission standards. Therefore, producers of PIF have to implement strategies to reduce the risk of product contamination. Controlling the initial population of the pathogen during all production steps and avoiding post processing contamination will have positive effects on product quality. [8, 43, 44] However, the real frequency of contamination is not known, so it is difficult to quantify the level of risk to infants. Also, the role of other infant food chain and dairy cattle and their environment as source of contamination has not been investigated. [45]

1.2 Coliform bacteria and Enterobacteriaceae

1.2.1 Coliform bacteria

Some of the *Coliform bacteria* are opportunistic pathogens; others are members of the normal intestinal flora. *Escherichia, Klebsiella, Enterobacteria, Seratia and Citrobacter* are members of the so-called *Coliform* bacilli.They can cause a wide range of infections. (46) Phenotypic characteristics that define *Coliform bacteria* are their ability to ferment lactose and produce gas and acids after 48 h at a temperature of 35°C. (47) This fact differantiates *Coliform bacteria* from other lactose non-fermenters, when they are plated on selective or differential *Coliform* media. (48)
The United States has used *Coliform bacteria* as an indicator for microbiological quality and clarity of drinking water since 1914 (US Treasury Department, 1914). In the last 100 years this group of bacteria has become more and more an indicator organism for standard hygienic quality testing in food and beverage production. *Coliform bacteria* include over 20 genera of gram-negative, non-spore-forming organisms which lack the ability to survive typical milk heat treatment, pasteurization, and therefore they can be used as an indicator of post-pasteurization contamination. (49)
The U.S. FDA determined *Coliform* and total bacterial limits to ensure quality standards, which are also used to observe hygienic quality of dairy foods. (48) In raw milk bulks almost always *Coliforms* are found, but with clean methods of production the count of these organisms can be kept very low. (50) A high number of *Coliform bacteria* are a sign of unsanitary production and/or improper handling of milk or milk utensils. (51)
According to College of Agriculture and Life Science (2001) the presence of *Coliforms* is associated with fecal and environmental contamination. Therefore, a count of less than 100 cells per mL is acceptable, but a number less than 10 cells/mL are desirable. (50) Counts above 500 cells/mL are an indicator of poor hygienic conditions during equipment cleaning, milking or maybe also with common contaminants from bedding, water, soil or manure. (52) Aside from the hygienic status of dairy products and processing quality, a high number of *Coliform bacteria* also seems to influence quality of milk products. Studies show that some selected strains of these bacteria are also able to grow at refrigerator temperatures and can produce proteolytic and lipolytic enzymes. (53) These enzymes may

have an influence on the taste of dairy products, as contaminated milk samples are associated with a decrease in sensory scores in comparison with uncontaminated products. [54] In this context it is important for dairying to avoid contamination with *Coliform bacteria* although it is not an obligatory health risk.

1.2.2 **Enterobacteriaceae**

Another group of indicators used in Europe are bacteria within the taxonomic family *Enterobacteriaceae.* This is a group of gram-negative, heat-labile, glucose fermenters and representatives of a wide range of dairy related genera.In addition, these organisms also can indicate post-pasteurization contamination. *Enterobacteriaceae* are able to colonize the intestine of mammals and birds and can infect the mammary glands after milking. Reasons for such infections may be contact of the udder with infected water, milking utensils, cattle shed or bedding. [55] The result of infected glands is called mastitis, a disease which affects the milk production and normally cows do not regain full production levels after recovery. Mastitis cause high treatment costs and has a negative effect on milk quality. [56]

The family of *Enterobacter* has more than 200 species and about 50 genera. In special *Klebsiella, Escherichia, Enterobacter, Serratia* and *Proteus* are genera often isolated from dairy environments. *E. coli* is a natural member of the feces from warm blooded animals, *Enterobacter, Klebsiella, Serratia* are geobiotic species or inhabit grains or water. *Proteus spp.* contaminate hose water, used for washing the udders before milking. [57, 58] For this reason it is important to understand the circulation of *Enterobacteriaceae* in the dairy environment to avoid contamination.

1.2.2.1 Antibiotic resistance

Antibiotics are often used in cattle-breeding with the target of prevention and treatment of diseases and for better growing and development. [59, 60, 61] As a result this routine affects many aspects of food production, since antibiotic-resistant bacteria from cattle can be transferred in various food products. [62] Such gene transfers also occur in the lumen of animal and human intestines and may create new pathogenic strains. [63] Milk also seem to be a perfect medium for gene transfer by conjugation, some studies reported an efficiency

10 times higher than in laboratory specific media. [64] Studies have shown that members of the *Enterobacteriaceae* family, isolated from milk products, generated multidrug resistance (MDR). In special, *Klebsiella pneumonia* isolated from raw milk samples manifested MDR. [65] Surprisingly, in milk powder samples also MDR isolates from the genera *Citrobacter, Klebsiella* and *Enterobacteriaceae* were found. [66] An alarming fact is that scientist found out that some representatives of *Enterobacteriaceae* can produce extended spectrum β-lactamase (ESBL). [67, 68] Inhibitors of ESBL are often used as a therapy for infections with gram-negative bacteria. For this reason, ESBL production can also lead to resistances to other antibiotic-classes and the *Enterobacteriaceae* family is the greatest producer of ESBL. [69, 70, 71]
Nevertheless, different studies have shown very controversial results. Some of them have indicated that milk is a bad disseminator of ESBL-producing organisms; others have come to the result that milk can support the dissemination of these bacteria. Studies also have found out that there are differences between the regions the samples were taken. In developing countries, like Brazil or India, ESBL-producing organisms were present in milk, in developed countries, like Switzerland, the results contrasted. [65, 72, 73]

1.2.2.2 Biofilm production

In the dairy industry biofilm formation appears within hours after processing. [74] Milk as the major part of dairy products may benefit biofilm production, because of its composition rich in lipids, proteins and divalent cations, like calcium, which promotes biofilm formation. [75, 76] In milk processing plants the presence of *Enterobacteriaceae* biofilm producers have been reported in various studies. Distinct gram-negative bacteria, like *Serratia marcescens, Klebsiella pneumoniae* and *Enterobacter spp,* have been found attached to stainless steel surfaces of dairy production plants. [77] As a result of biofilm formation these bacteria can be resistant to cleaning products. Malek et al. (2002) collected samples from dairy farms producing skimmed milk powder and pasteurized milk and described that the production line, which was to be cleaned with ammonia- and peracetic acid based products, still was contaminated with *Enterobacter spp.*[78]

1.2.2.3 Lipolytic and proteolytic activity

Proteolytic and lipolytic enzymes produced by *Enterobacteriaceae* are responsible for the degradation of milk and milk products, which is a serious quality problem within dairy industry. (79, 80) One of these problems is that the enzymes can destabilize casein micelles and modify or prevent the coagulation of milk. In the cheese production these destabilized micelles can affect the formation of the product. (81) Another problem is that these bacteria can cause a change of the product flavor or can affect the color or texture of foods. (81, 82) Such flavor variations may have a direct effect on the acceptance or rejection of the foods by the consumer. (69) An example for such flavor changes is the so-called hydrolytic rancidity caused by lipolysis. Hydrolytic rancidity leads to sour taste and unpleasant odor of the product. (83, 84)

1.3 Enterococcus

1.3.1 **Introduction**

The genus of *Enterococcus* was defined by Thiercelin and Jouhaud (1903) as gram-positive diplococci of intestinal origin and was first described as a group in 1899 by Thiercelin. [91, 92] The classical taxonomy of *Enterococcus* is very broad, because the lack of phenotypic characteristics that clearly differentiate them from other gram-positive, coccus-shaped, catalase-negative microorganisms. [93] However most of the *Enterococci* groups can be differentiated from other gram-positive bacteria because of their ability to grow from 10 to 45 °C, in 6.5 % NaCl, at pH 9.6 and they are able to survive heating at 60 °C for 30 min. [94, 95] Correct identification of species is of great importance for food microbiologists, for instance to select a starter strain and labeling the product to which the starter strain is attached. The development of more advanced starter cultures and the rapid changes in the taxonomy of LAB (lactic acid bacteria) is getting more and more important for microbiologists. They must be aware of the correct nomenclature. [96] The fact that *Enterococci* are regular denizens of the intestinal tract makes these microorganisms to indicators of fecal contamination and important for food and public health microbiology. [97, 98]

Enterococci, in special *Enterococcus faecium* and *Enterococcus faecalis*, are ubiquitous in the environment, human and animal gastrointestinal tracts, also in fermented foods and are also common in milk. [85, 86] Some strains of *Enterococcus* are used as a starter culture or as probiotics. In other cases *Enterococcus* strains are known as pathogenic bacteria or spoilage bacteria. [86] Some antibiotic-resistant strains, in special vancomycin-resistant *Enterococci*, are supposed to cause nosocomial infections. [87] *Enterococci* can survive inappropriate conditions, like high temperatures or high salinity and they are able to grow on many different substrates. [88] Some strains of those bacteria are in contrast helpful in various fermentation processes, including dairy or vegetable products, seemingly in processes like proteolysis, exopolysaccharide production, lipolysis or citrate breakdown. [89, 90]

1.3.2 **Food contamination and environmental sources of *Enterococci***

Enterococci are widespread in the mammalian gastrointestinal tract, particularly *Enterococcus faecalis* (Fig.2) is the predominating *Enterococcus* spp. in humans, though in some countries and individuals *Enterococcus faecium* excels the number of *Enterococcus faecalis*.[99,100] In particular the fact that just *Enterococcus faecalis* and no *Enterococcus faecium* occurs in neonates is very interesting. [101,102]
Devriese et al. (1992) found that the distribution of *Enterococcus* strains in animals is very different between cattle, pigs or poultry. [103] *E. faecalis* is common in pre-ruminant calves, also in young cattle and dairy cows, in contrary *E.faecium* was isolated from pre-ruminating young calves, but not from milk cows or ruminating cattle. [104]
Enterococci are not only found in warm blooded animals but also in soil, surface waters and on plants or vegetables. [105,106,107,108] In raw milk *E.faecium* is one of the predominant microorganisms, which has an important effect on dairy industry. [100]

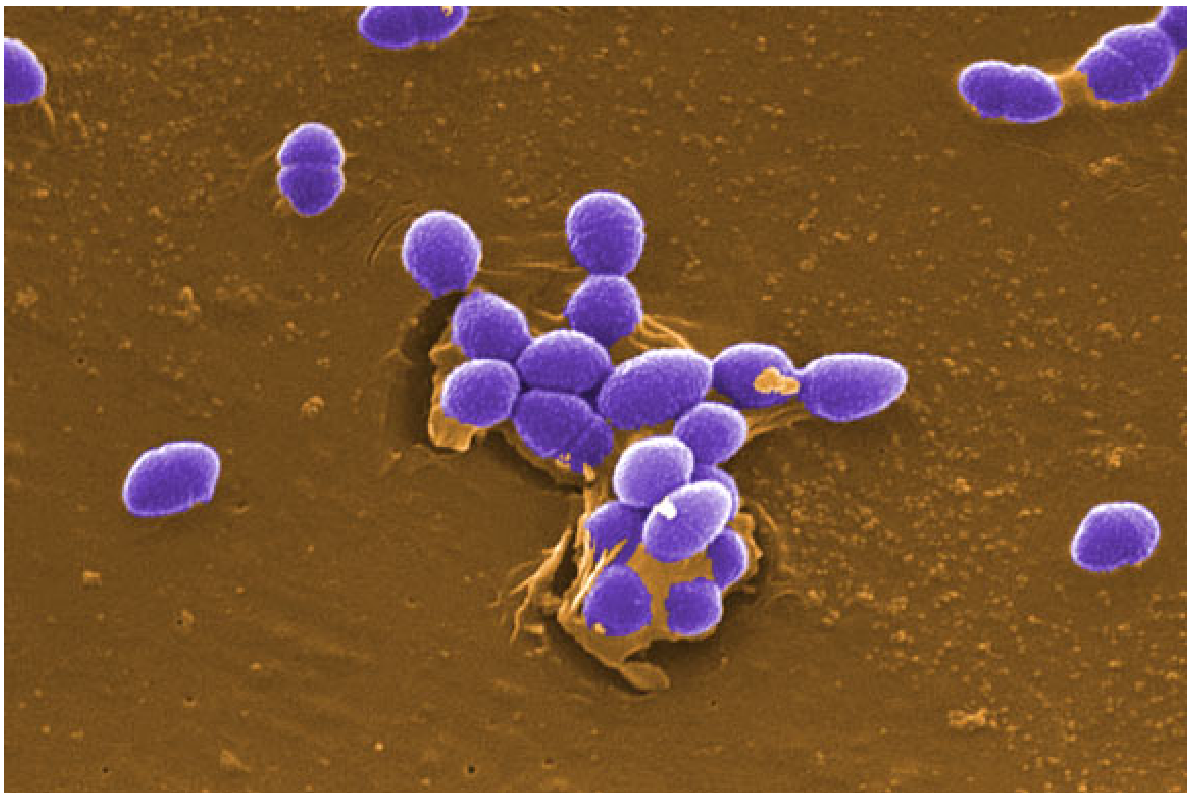


Fig.2 - Electron microscopic image of *Enterococcus faecalis*. Image credit: Pete Wardell / CDC. [119]

1.3.3 **Bacteriocins in *Enterococci***

Bacteriocins are membrane-active peptides, microbially produced, with antimicrobial activity against closely related bacteria-strains. [109] Gram-positive and gram-negative bacteria including all genera of LAB seem to be able to produce bacteriocins. [110] Three major classes of bacteriocins have been described; [109] Class I are ribosomally-synthesized lantibiotics which undergo post-translational modification. These peptides contain the amino-acids lanthionine and β-methyllanthionine. [111] Class II bacteriocins are heat-stable and small non-lantibiotics, which indeed are ribosomally synthesized but without post-translational modification, with the sole exception for cleavage of the leader peptide. [112] Class III bacteriocins are large and heat labile. Also, a fourth class has been reported as a protein conjugated with lipid or carbohydrate parts. These proteins are not well investigated and so it is not clear at all if the nonprotein chemical parts are important for their activity or not. [109,112] *E.faecium* and *E.faecalis* can produce bacteriocins, which are called enterocins (Fig.3) and in general they belong to class II bacteriocins. The best characterized enterocins are type A, a pediocin-like enterocin and type B, which is heat stabile and has an anti-*Listeria* activity. [113,114] The last well described enterocin is type P, which also belongs to class II and its secretion occurs by *sec*-pathway. [115] All of these enterocins have an antimicrobial activity, but together they show synergistic activity. [116] In general enterocins are active against all other *Enterococci* and *LMO (Listeria monocytogenes).*[117]

Listeriae and *Enterococci* have a close phylogenetically affinity, therefore the anti-*Listeria* activity of enterocins could be explained. [100]

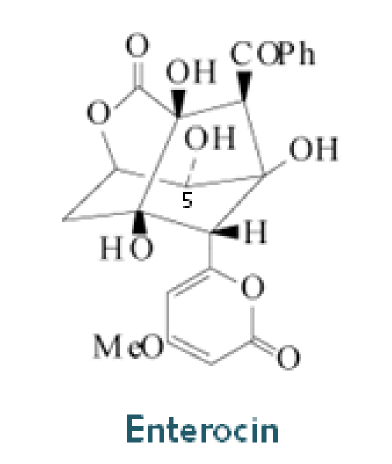


Fig.3- Enterocin type II with antimicrobial activity [118]

1.3.4 **Antibiotic resistance of *Enterococci***

The group of *Enterococcus* has become more and more interesting for hospitals because of their increasing resistance to antibiotics. Nosocomial infections are the result of these resistances because they allow microorganisms to grow in the hospital environment, where antibiotics are used. (101) Intrinsic resistances include β-lactams, cephalosporins, sulfonamides, and low levels of aminoglycosides and clindamycin. (101, 95,121,122) The treatment of *Enterococci,* which such many antibiotic resistances, could be very difficult. Though, studies show that a combination of cell-wall-active antibiotics with aminoglycosides act synergistically and the treatment has been successful against enterococcal infections. (101,122,123) Another anxiety is the appearance of vancomycin-resistant *Enterococci (VRE).* For *E. faecium* and *E .faecalis* different phenotypes with vancomycin-resistance have been found. VanA and VanB are phenotypes which are transferable by conjugation. (124)
The difference between VanA and VanB types is that VanA-type confers inducible and high-level resistance to both vancomycin and teicoplanin, while VanB –types have variable levels of inducible resistance just to vancomycin. There is also a VanC-type which shows a low-level resistance to vancomycin and seems to be an intrinsic property of the species *E.casseliflavus, E. flavescens* and *E. gallinarium.* (125,126,127) In the past vancomycin was used for therapy of enterococcal infections with strains which show a high level of β-lactam resistance. By mischance, a lot of VRE are also highly resistant to all standard anti-enterococcal pharmaceuticals, also including penicillin-aminoglycoside combinations. The result is that just a few drugs remain for successful therapy. This fact makes VRE in the present to a serious risk group among bacterial nosocomial pathogens. (128.129)

1.3.5 **Gene transfer**

In enterococci several gene transfer mechanisms have been reported. The mechanisms include both conjugative and nonconjugative plasmids as well as conjugative transposons, which seem to carry antibiotic resistance genes. Conjugative plasmids transfer at a low frequency in broth, but have a wide bacterial host range, or they have a narrow host range,

like the plasmids of *E. faecalis,* and transfer at a high frequency in broth and are also able to respond to sex pheromones. [130,131]
Sex pheromone response is a highly efficient system for exchange of genetic material in *E.faecalis*.[132,133,134] Chromosomally encoded pheromones, consisting of seven to eight amino acid hydrophobic peptides, were produced by a plasmidless recipient strain. These pheromones induce genes on the plasmid of the donor strain to produce an aggregation substance (AS). The aggregation substance affords binding to recipient cells by a complimentary receptor on these cells, called "enterococcal binding substance". [132] A sort of mating channel enables the transfer of plasmid DNA from donor to recipient cell. A plasmid encoded surface exclusion protein protects the new recipient cell from responding to its own pheromone. [134,133,132] For *E. faecalis* strains it is not uncommon to accommodate two or three sex pheromone plasmids. [134]
These sex pheromone plasmids may be able to encode haemolysin/bacteriocin production or may be carrier of one or more antibiotic resistance genes, e.g. penicillin, tetracycline, gentamycin, streptomycin or kanamycin. [134, 130] The haemolysin/bacteriocin protein lyses human, rabbit and horse erythrocytes. It has also the ability of antibacterial activity and inhibits a wide range of gram-positive bacteria. [130] This so called cytolysin system is defined by two peptides ($CylL_L$ and $CylL_S$) which seem to be activated by proteolytic cleavage involving a serine protease activator component (CylA). The two peptides are post-translationally modified after transcription and include lanthionine and β-methyllanthionine. [135]
Antibiotic resistance may help to understand the establishment of enterococci as nosocomial bacteria, but virulence factors must be taken into consideration to explain pathogenicity of enterococci. [136]

1.3.6 **Virulence factors**

For many years enterococci were presumed to be harmless commensals. [137,122,121] This seems to be true compared with other gram-positive bacteria like *L. monocytogenes, S.aureus* or streptococci, but the view is changing because enterococci have an increasing role in nosocomial infections. [122, 121, 138]

Enterococci can just cause infections when they colonized host tissue, survive host specific and unspecific defense mechanisms and in the end produce pathological changes. [139,140]

1.3.6.1 Colonization

Enterococci are normal inhabitants of human`s intestine and so they are able to colonize the gastrointestinal tract. Enterococcal infections also originate always from gastrointestinal or genitourinary tracts. [138, 140, 101] Another way infection spread seems to be patient-to-patient and hospital staff seems to carry strains from outbreaks and excretes them in their feces. [138,140] Colonization as such does not generate virulent factors, but it is possible that it amplifies potential pathogenicity of an enterococcal strain in combination with other virulence factors. [136]

1.3.6.2 Adherence

Crucial for infection is the adherence of pathogens to the extracellular matrix of host tissues. Two systems may be involved in binding to host cell matrix, specific adhesin-ligand and hydrophobic interactions. [141] *E. faecium* and *E. faecalis* were shown to bind extracellular matrix proteins, particularly lactoferrin, vitronectin and thrombospondin. The components which are responsible for binding on the cell surface were still not isolated. [141] Enterococci are efficient colonizers of special host cells, as their role in urinary tract infections and endocarditis shows. The tendency for pathogens to infect cells is often analog to their capability to bind to the respective tissue in vitro. [142] For Example, *E.faecalis* isolated after a urinary tract infection had a greater ability to bind to urinary tract cells or human embryo kidney cells than strains isolated from endocarditis patients. The other way around it is the same result; bacteria isolated from endocarditis adhere more likely to Girardi heart cells than to urinary tract epithelial cells or human embryo kidney cells. [142]

Persistence in blood has shown a change in surface antigen expression. The cells seem to get more resistant to phagocytosis and adhere more likely to cardiac cells. [142] Afterwards it was shown that *E. faecalis* isolates, extracted from cases of UTI (Urinary Tract

Infection) and endocarditis, which were bred in broth, adherence was communicated by surface adhesins as D-glucose and D-mannose. [143]
AS (Aggregation Substance) seems to be produced in response to sex pheromones mediates adhesion of *E.faecalis* to in vitro cultured renal tubular cells. An Arg-Gly-Asp-Ser-amino acid motif was found in the AS and it is involved in binding to eukaryotic cells. This motif is also found in fibronectin and provides the binding to eukaryotic cells by receptors called integrins. In serum AS expression can be induced by an unknown factor, so that the cell is enabled to "adapt" the eukaryotic environment and lead to synthesis of AS adhesin. [144,134] Another suggestion was that AS plays an important role in invasion of cultured cells. AS expressing *E.faecalis* cells were taken by enterocytes more likely than non-expressing mutant strains. It was shown that AS can interact with the surface of the enterocyte microvillus and that enterococci were found in membrane-bound vacuoles located in the cytoplasm of the enterocyte. This leads to the assumption that AS is an important virulence factor in enterococci. [145]

1.3.6.3 Translocation

High rates of enterococcal infections are thought to have their origin in the intestinal tract. The translocation model describes that intraepithelial leucocytes or intestinal epithelial cells phagocytose bacteria cells adhering to them at the lumen side. Then the bacteria cells migrate in phagocytes to mesenteric lymph nodes, there they proliferate and move to distant places. [139] A mouse-experiment showed that under specific conditions of intestinal overgrowth with antibiotic-resistant *E.faecalis*, the bacterial cells could translocate across an intact epithelium and cause a systemic infection. [146]

1.3.7 Pathology

Acute inflammation is one of the most common symptoms associated with enterococci infection. [140] Involved in these inflammatory responses seem to be sex pheromones and surface exclusion proteins. In vitro studies showed that these constituents are chemotactic for humans and for rat polymorphonuclear leucocytes. Furthermore, they induce superoxide production and lead to secretion of lysosomal enzymes. [140, 147,148] Bacterial

endocarditis also cause platelet activation and accumulation, in particular *E. faecalis, E. faecium* and *E. avium* induce these platelet aggregations in vitro leading to serotonin release as a concomitant. (140, 149) Some of the enterococci strains can produce cytolysin, which is thought to increase virulence in animal models, like rabbit endophthalmitis or murine peritonitis. (150, 151) In the rabbit model, cytolysin concurred to virulence only in association with AS, in the endophthalmitis model cytolysin is associated with tissue damage. (152,153) Enterococci hemolysin production is also associated with lethality in mice and is observed with dermonecrosis in rabbit skin. (154) A study in Japan had shown hat 60% of clinical strains, which caused an infection, showed a hemolytic phenotype, isolates from feces of healthy patients only showed in 17% of cases a hemolytic phenotype. (155) It is certain that cytolysis is involved virulence generation in enterococci, but there are also nonhemolytic strains causing infections. (140) Another enzyme considered to cause pathogenicity in enterococci strains is gelatinase. This protease hydrolyses gelatin, hemoglobin, collagen and other bioactive peptides. (156) In Germany 63.7% of the isolated *E. faecalis* strains from intensive care units produced this kind of protease. (157)
Most of the clinical isolates of *E. faecalis* manifested at least one of the three described virulence factors. In contrast, more than 45% of isolates from endocarditis cases lacked gelatinase, AS or hemolysin. Subsequently it is obvious that other properties must be involved in the pathogenesis of *E. faecalis* endocarditis. (156) Furthermore none of the non-*E.faecalis* (*E. faecium, E. gallinarium, E. raffinosus, E. casseliflavus* and many other species) strains show any of these virulence factors, so there must be some unknown virulence factors causing the pathogenesis of enterococci. (156)

1.3.8 **Enterococci in food**

Enterococci, including *E.faecium* and *E.faecalis,* are opportunistic pathogens. In most cases infections with enterococci are nosocomially, related to persons who have other diseases or received medical treatment. In the last years these infections became more and more serious, because of the multiple antibiotic resistances of enterococci strains and the difficulties of treating these infections. Based on these reasons, enterococci are among the most important emerging human pathogens. (136)

Human infections seem to have different sources like the patient`s endogenous microflora, but also a person-to person transmission is thought to be a possible way, as well as stool carriage of strains. (137,158,159) Now research focused on whether these bacteria can be transmitted by food and cause infections, especially because of VRE (Vancomycin-Resistant Enterococci). VRE were thought to originate in hospital environment and then spread out to the community but it may also be the opposite. (160-163) A possible origin of VRE is farm animals because of the use of avoparcin, which is a glycopeptide antibiotic. (162,163) VRE have been found in many different farm animals,which could be an important source of VRE. It is possible that contaminated food is transmitted to the hospital environment and causes infections. (162, 164) VRE also has been isolate from chicken, pork and beef samples, bought at different markets in the UK. So, it seems to be possible that *van*A resistance genes are transmitted to the community via the food chain. (165)

The most important detail is that different blood and urine samples from hospital patients showed the same ribotyping pattern as porcine isolates. Therefore, it is obvious that food transmission occurred, so that two European countries (Germany and Denmark) prohibited the use of avoparcin, followed by an EU-wide ban. (166,167)

In the USA the situation is different from Europe; avoparcin has never been licensed for use. (167) A study, to isolate VRE from healthy persons without any contact to hospital environment or other environmental sources or probiotic preparations failed. (168) Consequently, in the USA transmission of VRE from the community to hospital environment is very unlikely. In addition food has not been linked to VRE transmission,- in contrast to Europe. Another thesis for VRE transmission is that low levels transmit undetected to the community or the bacteria acquired vancomycin resistant genes from unknown gastrointestinal bacteria. (166, 169)

Using molecular methods, it has been found, that meat products and cheeses showed the same resistance determinants for enterococci as found in corresponding determinants in clinical samples. (170) It is not clear whether and how frequent VRE strains are transferred via the food chain. So, it is important to investigate for the presence of other virulence factors, like AS or enzymes in food samples. (136)

Enterococci strains isolated from dairy products are not able to produce hemolysin and it was recommended to use starter strains for dairy use without hemolytic activity. (117,171) The absence of hemolytic activity in enterococci does not mean that the bacteria are always pathogenic. Also, antibiotic-resistant enterococci have been found in raw milk cheeses, raw

meats or in sausages. [172-175] Studies revealed that Salami and Landjäger often show streptomycin- and linomycin-resistances. This is in contrast to isolates from Emmental and Appenzeller-cheeses, which frequently show a high resistance to gentamycin, vancomycin/tetracycline and erythromycin. [170] The pathogenic potential of enterococci in food based on their ability of horizontal gene transfer, which cause virulence or antibiotic resistances. [156] Enterococci used in food production are often *E. faecium* strains; also, many of the bacteriocin-producing enterococci, used as starter cultures, are *E. faecium* strains. The pathogenic risk is higher in *E. faecalis* strains than in *E. faecium,* because more than 80% of enterococci infections are caused by *E. faecalis.* [139] Plasmid transfer in association with sex pheromones seems to be special for *E. faecalis.* This special way for gene transfer is highly effective and thought to be connected to virulence-factors. In contrast, vancomycin-resistance is special for *E. faecium* strains, which is an important factor in nosocomial diseases. [159,166]

In this connection, food is an important source of enterococci strains, which bear plasmids for antibiotic resistances and virulence factors, so it would be prescient of food producers to keep track of these organisms and monitor the presence and colonization in food products. [136]

1.4 Bacillus cereus

1.4.1 Introduction

Until now more than 250 foodborne indispositions have been described. The illnesses can appear sporadic or also in epidemic form and cause high morbidity and mortality in many different countries. One of these foodborne pathogens is *Bacillus cereus*, reported from many nations in the world. (176-179)
Bacillus organisms are gram-positive, mesophilic, aerobic heterotrophs and live ubiquitous. Moreover, bacillus species are characterized by their capability to form heat resistance endospores with a wide range growth range between 10 °C to 48 °C. Their optimal growth temperature is between 28 °C and 35 °C. (180) Only two, *Bacillus anthracis* and *Bacillus cereus,* are known as origin of human illnesses.
The first appearance of *Bacillus cereus* was in a cowshed in 1887, isolated from the air by Frankland and Frankland. In the last 70 years many outbreaks in Europe caused by *Bacillus* in different foods like vegetable soups, meat, milk fish and ice cream were described. (182) There are two different types of enterotoxin, which cause food poisoning, the thermostable emetic toxin and the thermosensitive diarrhegenic one. (183)
Bacillus cereus is widespread in the food industry, because as a soil bacterium it can spread in almost every type of food. It is proposed that this organism is responsible for 25% of foodborne intoxications caused by its emetic- and enterotoxins. (184) Food poisoning is caused by the fact that meals are left without cooling for hours before serving. The main problems are the spores from contaminated food remaining after cooking. They grow very well after cooling down. (185) Transmission of infection is not only the result of contaminated food, but also of improper handling, storage or cooling of food. The infection with *Bacillus cereus* can hit everyone, because diseases are transferred by ingestion of contaminated foodstuffs. However, children, old persons, or immuno compromised patients are more endangered to get more serious side effects. (181)

1.4.2 **Transmission**

The most common way of transmission of *Bacillus cereus* (Fig.4) is through ingestion of contaminated food. There are two types of illnesses, the emetic type is described after consumption of starchy food, such as pasta, potatoes or rice, the diarrheal type is more likely transmitted by milk products, fish, vegetables, meat or casseroles. The main problem are the spores, they can spread very easy. [186] Another way of transmission in hospitals is via contaminated linen. [187]

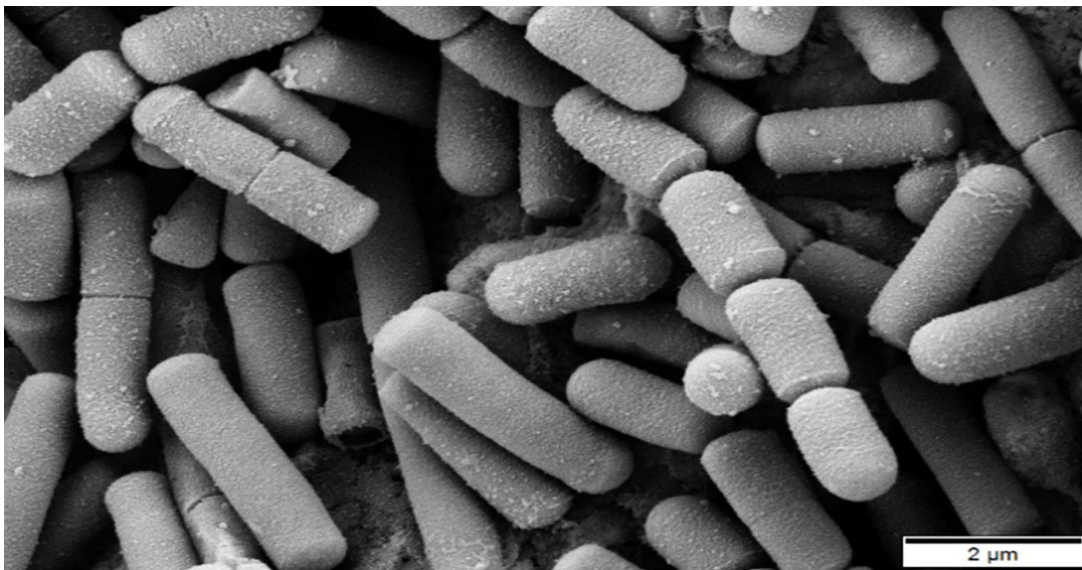


Fig.4: Electron microscopic image of *Bacillus cereus* [188]

1.4.3 **Diseases**

There are two types of foodborne illnesses in humans caused by *Bacillus cereus,* diarrheal syndrome and emetic illness. The prevalent disease is a diarrheal illness caused by ingestion of *Bacillus cereus* contaminated food. Symptoms of the infection are abdominal pain, nausea, watery diarrhea; rectal tenesmus and vomiting which occur within 5-16 hours and will last for about 24 hours. The syndrome is like *Clostridium perfringens* infections and normally rather mild. [182] The dosage of *Bacillus cereus* contaminated food ingestion to cause diarrheal syndrome is between 10^5 and 10^7 per g food, to cause emetic illness the dosage is 10^5-10^8 per g food. [185] Symptoms of the emetic syndrome is nausea and

vomiting, caused by a heat stable toxin. Additional possible symptoms are abdominal cramps and/or diarrhea, with an incubation period of 1-5 hours. The illness is similar to an infection with *Staphylococcus aureus*. (182)

The most probable way of diarrheal poisoning is the ingestion of *Bacillus cereus* cells with contaminated food. *Bacillus* spores are the common way of food contamination, but whenever the conditions for spore germination and growth are not ideal, *Bacillus cereus* could also be ingested as spores. When the environmental conditions do not allow spore germination and growth, *Bacillus cereus* also can be ingested as vegetative cells. (189)

1.4.4 Public Health Risks

Bacillus cereus, known as a ubiquitous pathogen, causes food poisoning and infections. The bacterium is not just known to cause foodborne intoxications but has also been reported to be responsible for local and systemic infections. Especially at risk are immunocompromised patients, newborns and patients right after a surgery. The pathogen can cause a wide range of symptoms like ocular infections; it can produce gangrene, bovine mastitis, cellulites, infant death, lung abscess, periodontal diseases and endocarditis. (187)

1.4.5 *Bacillus cereus* in Foods

This pathogen is found in about 25% of food products tested and detected in many different products, including cream, dry milk, meat, rice, vegetables, fish and many more. Food mixtures like soups, puddings, sauces, salads and pastries frequently led to food poisoning outbreaks. (186) Major factors for these outbreaks caused by processed foods are heat resistance, spore germination and outgrowth. Especially dried milk products and infant formula are frequently tested positive for *Bacillus cereus*. (190)

As a primary source for *Bacillus cereus* contaminations in food products are soil and air obvious. *Bacillus cereus* is a soil resident and a natural member of the microbiota of plant raw material. The plant material is used for fresh for human consumption or for food production and for animal feed or animal feed production. When cows get contaminated feed, they excrete spores and the spores could contaminate raw milk. This fact could lead

to problems with milk safety or shelf-life reduction. A main problem for food safety and food quality is the direct or indirect soil route of contamination or disposal. It is the start of events or instances in the agriculture-food chain which may lead to these problems. *Bacillus cereus* contaminations could be curtailed by pasteurizing or by using dried products in food processing factories. (179,186)

In convenience foods *B. cereus* is introduced by fruits and vegetables, also possible by herbs and spices. Spores constituted by these bacteria could, apart from plants, also be introduced in the food production chain via milk or dried milk/milk powder. Because of the hydrophobic character and the presence of appendages on the surface, exosporium can adhere on different equipment surfaces such as pipelines. The biofilms produced by *Bacillus cereus* could form in half filled pipelines or storage-systems and may disperse when be induced to food production systems. Biofilms also protect the spores against hygienic measures like cleaning with disinfectants. (191)

In food production, distribution and at home in the kitchen it is necessary to use refrigeration to keep processed food fresh. The problem with *Bacillus cereus* strains is its special feature to be psychrotolerant, which means that this pathogen is able to grow at temperatures lower than 7 °C. Especially food-environment strains and diarrheal strains have this important feature, but amazingly none of the emetic strains. However, the majority of *Bacillus cereus* strains have the ability to start growing at the temperature of 10 °C. (192) In raw foods or in possessed foods before storage there is a low number of *Bacillus cereus* (<100 spores/g or mL), which is constitutes no health problem. The storage of possessed food and the use of contaminated condiments may lead to germination and outgrowth of spores to a health risk level for consumers. (193)

In pasteurized milk the presence of *Bacillus cereus* is the main limiting factor for its shelf life. (194) If high levels of *Bacillus cereus* are present in milk products before the end of its shelf common structural defects known as bitty cream or sweet curdling can occur. (195)

Diarrheal outbreaks caused by *Bacillus cereus* are very rare, although it is commonly isolated from milk products. (194)

1.4.6 **Prevention**

The number of spores must be kept as low as possible in all processes of food production. A complete destruction of *Bacillus cereus* spores is ensured by heat treatments used for canning of foods. In other processes the number of spores must be kept low by proper cleaning, disinfection of equipment and rapid cooling can prevent the germination and growth of spores. Moreover, *Bacillus cereus* is not able to grow at a pH below 4.5 and the storage of foods at temperatures below 4 °C prevents growth of all types of these pathogens, also psychrotrophic strains. Vegetative cells can be destroyed by roasting, frying, grilling and steaming under pressure. The germination of spores can be prevented by rapid cooling the foods. The diarrheal toxin in food products can be inactivated by heating it for 5 minutes at 56 °C. In contrast, food contaminated with the emetic toxin must be heated at 126 °C for more than 90 minutes. In meat processing facilities it is very important to use good manufacturing practices (GMP) to prevent contamination or toxin formation. In slaughterhouses and processing units it is necessary to use proper cleaning and disinfection with hypochlorite to get sure to remove all fecal bacteria from food contact surfaces. The prevention of spore formation makes it necessary to keep hot foods at a temperature over 60 °C and cold foods below 4 °C. Workers are obliged to wash hands and food contact surfaces after touching meat, before meat preparation. Especially after using the bathroom the hands have to be cleaned with hot soapy water. To prevent food intoxication caused by *Bacillus cereus* cooking of beef and beef products thoroughly and after cooking,cooling the leftovers is necessary. [(196)]

1.5 Mold and Yeast

1.5.1 Mold

Molds are responsible for a lot of contaminations and losses in the food production chain. Some of species, including the genera *Aspergillus, Fusarium* and *Penicillium* and can produce mycotoxins. (197) These toxins can be acute toxic or carcinogenic and so they are potentially dangerous for animal and human health. The main problem is that these toxins cannot be destroyed by heat. So, it is necessary to find ways to avoid mold in production chains.

Pasteurization of milk for 15 min at 72 °C can inactivate molds and their spores, but some of the dairy-borne molds produce heat-resistant spores. As an example, *Byssochlamys fulva* spores can survive a heat treatment of 90 °C for 5 min. (198,199) Strains of these species were found in milk used for cheese production. So, it is possible that mycotoxins are produced in the resulting cheese. *B. fulva* canproduce a variety of different toxins, including byssotoxin A, byssochlamic acid, patulin, fischerin, verruculogen and more. (200)

There is no documented outbreak known during which dairy products were involved. However, the presence in milk products makes it possible to be implicated in chronic or sub chronic intoxications. (201)

1.5.1.1 Mycotoxins in Dairy Products

Mold is accepted or even necessary in some dairy products, in cheese production mold is widely used, such as in Camembert or Roquefort. There are also some cheeses made from raw milk, in this cases mold is involved in the maturation process. The mold originates from the microbiota of raw milk, from the environment where the cheese is stored, or it is added to the process milk as some kind of starter culture for cheese production. (201)

The mold used for white- or blue-mould cheese is *Penicillium camemberti* or *Penicillium roqueforti*, which can produce higher levels of mycotoxins in laboratory studies.(202) In reality, the available mould strains usually do not produce mycotoxins and when they do, the toxicity is very low, so they do not have any dangerous effect on human`s health.

[203,204] Nevertheless, mycotoxins have been detected in some cheeses (white- and blue-molded), also in raised levels. [205,206,207,208,209] The same mycotoxins produced by *P.camemberti* and *P.roqueforti* could also originate from other mold strains, which frequently contaminate dairy products. [201] Therefore, contamination of cheese is not only caused by the added mold, but also could arise from the complex mycobiota in the cheese factory environment. It also could be contaminated while processing or during the storage. [210, 201]

1.5.1.2 Contamination sources

The arise of mycotoxins in dairy products is either caused by direct or indirect contamination. When the contamination is caused because of in situ production of mycotoxins by toxigenic moulds used for dairy production, it describes the direct way of contamination. This route of contamination is wide spread at the cheese production, where mold is part of the normal microbiota. In general, there are three different origins of the mycotoxin producing molds. [201]

- The mycotoxin producing mold is a contaminant of the raw milk and survives pasteurization process (*B.fulva)* or the contamination happens after pasteurization. The molds could produce their toxins in later production steps or while storage. [210, 211,212]
- Wilde strains growth is stimulated during the production steps of raw milk cheese. In this case it is dangerous to contain higher levels of toxins because of the diversity and unknown identities of the different mold strains or species, which are involved in the whole process and storage. This could cause a high level of toxin. [213]
- The last way for direct contamination is the use of commercial strains added as starter cultures, which could be able to produce toxins in some special situations. [214,215]

The indirect contamination is caused by contaminated feeds. In case of aflatoxins (AFM_1 and AFM_2) the toxins were excreted by lactating animals which were fed with

contaminated feed. [216] Furthermore, there is a linear correlation between the concentration of ingested aflatoxin and the concentration in the milk which the intoxicated cow produced. [218] In this case it is likely that aflatoxin can be found in any dairy product, which is produced with contaminated raw milk, even after pasteurization, because the toxins are heat stable. [218, 219,220] Particularly cheese made from contaminated raw milk seems to have a higher concentration of AFM_1 (Fig.5) than the original milk. [221,222] This is because mycotoxin AFM_1 binds to the casein in raw milk. [223,224] Another notable fact is that dried milk or evaporated milk contain a significantly higher level of AFM_1 than raw milk. [219] The reason therefore seems to be a combination of different effects, like the heat resistance of the toxin and the evaporation of water during the process, which causes a higher mycotoxin concentration in the products. [224,225,226]
Other mycotoxins like AFB_1, CPA, FB_1, OTA or trichothecenes and patulin were reported to be transmitted to dairy products by a similar way, but the concentration levels were a lot lower. [219, 227,228,229] However, it is possible to transmit so called *Fusarium* mycotoxins to milk or dairy products with contaminated feed. [227,229,230] For this reason it is necessary to investigate possible health risks for humans by ingestion contaminated dairy products. [231]

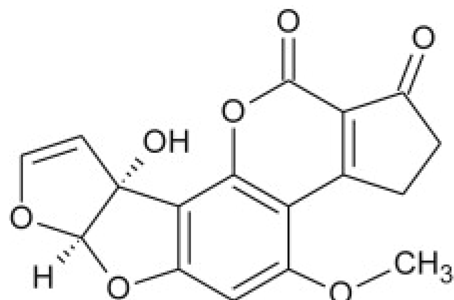


Fig. 5 Aflatoxin M_1 [234]

1.5.1.3 Mycotoxin Control Strategies

The best strategy is to prevent in situ toxin production or if it is already present to remove or inactivate it. A first step for prevention is to eliminate or reduce the count of mycotoxin producing mold, which means to reduce the count of mold in general. A far-reaching problem therefore is the production of toxins during process steps and storage. Mycotoxin producing mold growth could be prevented or reduced by executing a good-hygiene

practice (GHP) pre-and post-harvest, during milking and while the whole production process. Additional very important points are to ensure a proper staff hygiene, clean equipment in the whole factory and the reduction of the natural molds from the environment of the production plant. Concurrent it is necessary to hold the mold-contamination in feed as low as possible to avoid any carryover phenomenon from the lactating animals to raw milk. (232,212) Apart from all the hygienic points, the first step to minimize the mold-rate and subsequently also the mycotoxin-level is to use pasteurized milk for all continuative products. (233)

1.5.1.4 Inactivation and Conclusion

Aside from preventive methods to avoid mold, it is also possible to inactivate or remove mycotoxins from food with several physical, chemical and biological procedures. Pasteurization is, as mentioned above, a strategy to prevent mold in milk, but it also can be used as an inactivating method. Heat treatments were shown to be a semi-effective method to reduce the mycotoxin AFM_1 concentration to a lower level in milk. (233) These surveillances contrast with other studies mentioning the high heat stability of these toxins. (235) The general tendency is that pasteurization as well as sterilization do not lead to an adequate reduction of AFM_1 concentration in dairy products. (232)
Another described method to reduce toxins, in this case just AFB_1, in foods is the use of UV, X-rays or γ-rays, but the consumers of the irradiated products perceived them as less palatable. (236)
Increased interest caused the biological detoxification, which promised a better result in controlling the occurrence of mycotoxins in dairy products. The method based on live or dead microbial cells, which adsorb mycotoxins to their cell wall and decrease their bioaccessibility. (237,238,239, 208) More precisely, microbial strains used as starter cultures in dairy industry, including *Lactobacillus, Streptococcus, Lactococcus* and *Propionibacterium* have been shown to bind AFB_1 in vitro and in vivo. (238,239) The exact way how the cells bind the toxin to their cell-wall is not yet described in detail, but until now, the bindings are specific but non-covalent. (238) Live cells are also able to inactivate mycotoxins or at least reduce the toxicity by a total or partial degradation of special enzymes. (240) Microorganisms like yeast or also mold species such as *Flavobacterium*

aurantiacum, Candida lipolitica, Aspergillus niger, Candida tropicalis, Saccharomyces cerevisiae and many more, can degrade mycotoxins enzymatically. [232, 241] The best way seems to be the use of these mycotoxin-detoxifying microorganisms in dairy production, but it is not always possible because of regulatory or technical restrictions. An alternative way would be the use the special purified enzymes instead of the whole cells, but enzymes extracted from genetically modified organisms (GMOs) to use this benefit is facing a strong public refusal. [242]
As chemical methods for mycotoxin decontamination or inactivation using ammoniation, acidification, ozonation, alkalinisation, chlorine treatment and deamination have been described. A lot of them are restricted to specific foods or feed. Another problem is the high cost and after some of these methods are detoxification processes necessary. [236, 232]
An advantage of fermented dairy products is the fact that decontamination happens naturally caused by the acidity of the LAB of the starter cultures. [243] In summary, the best method is to avoid, as well as possible, all species of mold by observing the hygienic guidelines and control the level of mycotoxin concentration in raw milk and the final dairy products. [201]

1.5.2 Yeast

In many foods yeast is responsible for spoilage or used for desirable fermentations. [244] Infections caused by yeast, in most cases caused by *Candida albicans* or *Cryptococcus neoformans*, are not transmitted by food. Therefore, the public health risks are minimal, so food producer and health authorities spend less attention to yeast contaminations. However, studies in Canada show that yeast is responsible for some cases of foodborne poisoning. [245] Also, allergic reactions after consuming foods or their contaminants may be caused by yeast strains, so health authorities are forced to pay attention to these diseases. [246] In dairy products the focus is still on bacteria and data for yeast occurrences are very rare. [247,248]

1.5.2.1 Probiotic yeast

In general, probiotic organisms are adjuncts in food to help support health, exactly the microbial gastrointestinal balance. [249] Usually the probiotic organisms incorporated in dairy products are bacteria, mostly *Lactobacilli* and *Bifidobacteria*. The use of yeast as probiotic ingredient for human food is restricted, although yeast is a natural part of many dairy related products. [250] To find potential probiotic yeast species for dairy industry, it is necessary to search for new starter cultures for fermented dairy products. [251] In former times *Saccharomyces* species had been used for livestock feed and in the 1950s a non-pathogenic yeast, isolated from lychee fruits, was used for diarrheal diseases treatment. [251, 252] There also have been shown antagonistic interactions between *Saccharomyces cerevisiae* and pathogen bacteria like *E. coli* or *Salmonella*. Therefore, it seems to be possible that yeast can pass through the intestinal tract and could therefore be used as a probiotic agent. [253]

In general, yeasts are more involved in the spoilage of final dairy products than in fermentation of yogurt. Normally, populations of 10^3 cells/g in yogurt products can be found; under certain circumstances populations up to 10^7 cells/ g are possible. [254] The fact that yeasts appear in many dairy related products, is a hint that yeast can survive and maybe metabolize milk components. It might also be possible that yeasts create a secondary flora in milk after bacterial growth. Studies showed that the high concentration of galactose in some milk products, such as yogurt, induce the growth of galactose positive, non-fermenting yeasts. [255]

Examples for milk including yeast as a starter culture are acidophilus-yeast milk, kefir or laban. [256] The fermentation is caused by yeast in concurrence with lactic acid bacteria and other bacteria. Amazingly the growth of *L.acidophilus* was raised by the application of yeast, bacterial growth such as *Bacillus cereus* and *E.coli* was inhibited in acidophilus-yeast milk. [257,258]

1.5.2.2 Fungi and Mastitis

In general fungi are eukaryotic, osmotrophic, aerobic, gram-positive, non-acid, heterophilic organisms and can reproduce sexually or asexually by spores. [259] In cases of acute or chronic mastitis a plurality of fungi, such as *Aspergillus flavus, A.niger, A.terreus, Candida albicans, C.kefyr, C. tropicalis*, *Cryptococcus neoformans* and many more have been

found. [260, 261] The most common fungi in conjunction with mycotic mastitis are *Candida* species. [262]

The causes for a mastitis formation could be tiny injuries of the mammary gland, probably caused by milking machines, or irritation of one or more teats. Through these injuries fungi can invade the udder and induce a mastitis infection. [259] Other reasons for a mastitis infection are antibiotic infusions, given into the gland, which are contaminated by fungi or unsanitary conditions in livestock breeding. When the udder gets dirty or dusty, this also could be a source of fungal infections. [259, 263]

Infected animals suffer from swelling, induration and enlargement of the udder and enlarged lymph nodes. Furthermore, mild temperature, decreased milk yield and reduced appetite could be noticed by infected cattle. [264,265]

Mycotic mastitis represents a major economic issue for dairy industry, and some infections also carry public health significance. [259] Beside sporadic outbreaks of this acute or chronic illness also outbreaks involving a high number of lactating cattle are reported. [259, 266] Another reason for fungal infection of the mammary gland seems to be the extensive use of antibiotics or corticosteroids for bacterial mastitis treatment. The use of high dose broad antibiotics may cause a vitamin A reduction, which could implicate injury to the epithelium of the udder and damage the microflora of the mammary gland. The microflora normally acts as a natural protection against infections and fungal invasion. [267] Other factors such as unsanitary conditions in stock farming, contaminations of the teats by environmental fungi, mold contaminated feed, high humidity or temperature, inaccurate cleaning of dairy facilities and poor hygiene practice could lead to intramammary infections as well. [259, 266]

1.6 Milk Products

1.6.1 Raw Milk

Milk is defined as a whitish liquid and contains milk proteins, fat, lactose vitamins and minerals. In Europe the monks, like the Benedictines, were the main producers of cheese. Therefore, milk had also an importance as human food before the industrial revolution in the nineteenth century, when techniques for fermenting milk or butter and cheese became more available. (268) About 8000 years ago breeding of dairy cattle began and had become an important part of nutrition, especially in infant feeding. The human consumption of milk is very specific, which means that milk must come from healthy lactating livestock and always should have a temperature of +4° C during the whole operating process and the delivery to the consumers. (269) Milk quality is affected by contaminations and so proper handling is obligatory, particularly because milk is a perishable material. (270)
In 1909 human milk consumption was defined in France as "milk is the product of total, full and uninterrupted milking of a dairy female in good health, also nourished and not overworked. It must be collected properly and not contain colostrums." (270, 271)
The composition of milk varies among species and breeds of the same species, also from dairy to dairy and depends on diet and period of lactating. (268) In general milk contains several groups of nutrients, such as water, carbohydrates, proteins, lipids and functional micronutrients like vitamins, minerals, enzymes and calcium, magnesium, potassium and sodium. Furthermore, dissolved gases (about 5% by vol.), including carbondioxide, nitrogen and oxygen. (273) The main ingredient in milk is water comprising 88.6%, which is limited by the amount of lactose produced by the mammary gland. (270) As the main carbohydrate, lactose is formed by combining D-galactose and D-glucose (Fig.6). Lactose has a limited sweet taste and the lactose concentration varies from 4.5-5.2 g/100 g. Unlike fat, the lactose concentration cannot be modified by feed. (274) Milk proteins have a great nutritional quality, because they contain all essential amino acids. They have similarly the same formation as egg proteins; just the amounts of methionine and cystine are lower. The whole complex of milk proteins, including the caseins, contains a nearly perfect proportion of all amino acids for growth and health promotion. (268)

Soluble proteins in the whey after precipitation of casein at pH 4.6 and 20 °C are indicated as whey proteins. (275)

The main source of energy in milk is of course fat. Bovine milk has a low amount of polyunsaturated fatty acids, which would be very important for human nutrition. (276) Fat in milk occurs as a form of an emulsion of fat globules; the fat concentration varies depending on the race, the feeding of the animals and the duration of lactation. The lipids in milk include two major groups, the simple lipids (triglycerides) and the complex ones (phospholipids). (277) Although animal fat has a few disadvantages on our health, the risks just appear at excessive consumption. Moreover, milk fat is full of essential vitamins to human body; vitamin A and D all above. Vitamin A is important for the epithelia and, therefore has a role in reproduction and vision. Vitamin D is important for the uptake f calcium and bone growth. (278) Due to the fact that vitamin A,D, and E are fat-soluble, they can be found in fat, so they are lost during skimming. Other vitamins are water-soluble and can be found in the milk serum. Vitamin C is present in low amounts in fresh milk, but it gets destroyed at air-contact, also during pasteurization. (279)

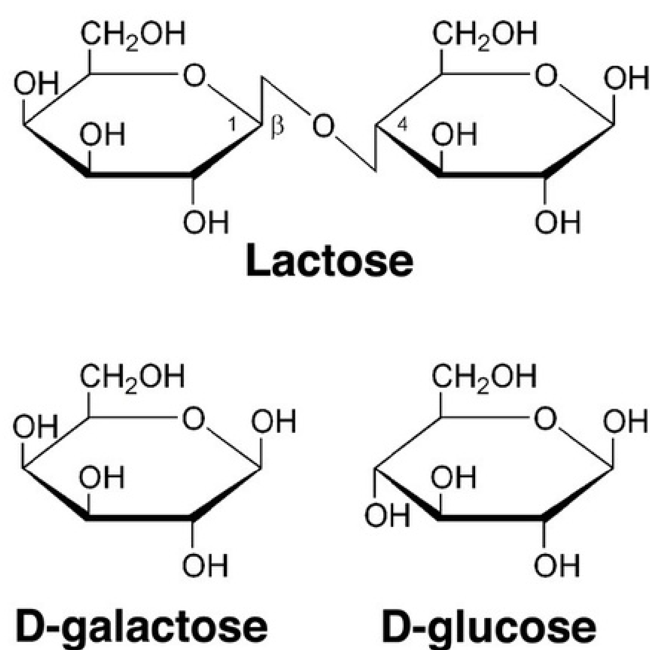


Fig.6: Structural formula of lactose (280)

1.6.2 **Milk Powder**

Producing milk powder means removing water from fresh milk. This is necessary to prevent milk from bacterial growth. The dried milk is used for several food products, such as cheese, ice cream, yoghurt or whey powder. The advantages of milk powder over fresh milk are also an important point for industry, for example quality keeping is easier, less storage place is needed which results in lower transport costs. [281] Since antiquity drying milk was used for food preservation. Marco Polo reported after his travels to Asia that the Mongolians dried milk in the sun for milk powder production. Nowadays milk powder is produced at huge scales in modern dairy factories. The maximum shelf life of skim milk powder is about 3 years; in contrast, the shelf life of whole milk powder is approximately about 6 months. [282]

Milk powder is not only used for reconstitution or recombination, but also as food ingredient, to use its functional characteristics, in bakery or meat production. The advantages of powdered food ingredients are their properties to be stable and convenient for storage. Bacterial growth is widely inhibited, and the quality of powdered milk is mostly high. [283] An exception are thermophilic bacteria-; they have significant consequences when the critical values aresurpassed, because of the downgrading of the product. These thermophilic bacteria can generate extremely heat-stable spores and that is an important factor for pasteurization. [284] There are several quality parameters for milk powder, of course the microbiological safety, but also sensory qualities, physical and chemical properties, such as moisture level, fat, total protein, lactose, acidity, ash, and the content of other nutrients like calcium. [285]

The water content should be very low, at about 5%, to protect the dried milk from bacterial growth, both vegetative cells and spores, when they survived the drying process. There are several important factors which affect the microflora in milk powder, such as the rate of microorganisms in the used raw milk, also the type of present bacteria, preheating temperatures, the operating conditions and the hygiene in the whole factory. High numbers of bacteria in raw milk also may lead to high numbers of bacteria in the final milk powder. The decline of the bacterial contamination of raw milk after several heating steps may also be offset by the removal of water during the powder production. [286] Bacteria and other microbes like fungi are everywhere in our environment and so could easily contaminate

our food. These contaminations could be caused by improper cleaning or sanitation of dairy facilities and may lead to food poisoning or food spoilage. [286,287] Also post-processing contaminations are a problem in milk powder production. During the process vegetative cells of pathogens are eliminated by lethal temperatures, however, milk-powder outbreaks in the past show that contaminations also could happen after processing in zones which are difficult to clean, such as the drying tower. Other reasons could be the presence of water that enables microorganisms to grow or abuse of reconstructed milk powder. [288] Bacteria such as *Bacillus cereus, Staphylococcus aureus* or *Cronobacter* species are of major concern, because they may start to grow after reconstructing the milk powder, also after a long period of time. [289] It is also possible that milk in contaminated by infected cows having diseases like tuberculosis, mastitis or brucellosis. Subsequently also humans may transport infection which may contaminate milk, such as typhoid fever, diphtheria or scarlet fever. Dairy cows and their cowsheds are also home of pathogenic bacteria like *Listeria, Salmonella* and *E.coli* strains, which may be found in the final milk powder. [288] The Codex Alimentarius Commission advises to pasteurize all liquids and milk products before drying. An inadequate pasteurization may also lead to the survival of pathogen microorganisms in dried milk. Therefore, it is necessary to monitor every step of production, beginning with the raw milk handling until the final product is finished. [290]

1.6.3 **Whey Protein**

Whey protein is used as food ingredient because of its functional properties such as viscosity, solubility, gelation, adhesion, foaming, water-holding capacity and emulsification. [291] Another reason for using whey protein in food industry is not only their functional advantages but also their high nutritive value. [292] There are a few properties of whey proteins, one of them is their hydration property, based on protein-water interactions, which is important for their performance on wet ability, swelling, solubility, viscosity, water absorption, water holding and many more characteristics. Surface tension, emulsification and foaming qualities are interfacial properties. Properties based on protein-protein interactions are specific aggregation and gelation characteristics. [292,293,294]

The functional properties are a result of their structure and particularly of their β-lactoglobulin, which is the greatest part of proteins and is able to adsorb water-oil and water-air interfaces. [295] The conformation, aggregation and so the functionality of pure β-lactoglobulin was notable affected by high pressure treatments. [296] Therefore new technologies for gentle food treatments are getting more and more interesting for food producers. That means treatments without using chemicals or high pressure, the food could be processed with minimal loss of natural color, taste, flavor and has still the same texture and no vitamins get lost. [297, 298]
After coagulation of milk, whey is the left-over part. It consists of various components such as several proteins, peptides and many others;

- Cysteine: its thiol group acts as reducing agent and tests tissue damages and oxidation [299]

- α-Lacto-albumin: It is an immunstimulator; it could reduce oxidative stress and studies showed that in rats it is able to protect against ethanol and stress induced injury; starts ulcer preventing activity [300,301]
- Leucine: Role in initiating the transcription and important for tissue growth and repair [299]

- Lactoferrin: Important by inducing apoptosis, suppressing angiogenesis and plays a role by modulating carcinogen metabolizing enzymes; and it is a iron scavenger [302,303]

- Immunglobulins: Prophylaction against rotavirus and *Helicobacter pylori* [304]

- Bovine serum albumin: About 9 essential amino acids are present [303]

- β-Lacto-globulin: High number of branched chain amino acids- functions in keeping muscles and brain working; inhibitor of allergies [303]

- Glycomacropeptide: It is responsible for the stimulation and release of cholecystokinine in the intestine to help in digestion process [305]

Milk has a many different chemical components, which can be used to produce several novel milk products. For example, casein is a soluble protein and used for the formation of cheese. For the cheese production the enzyme chymosin is added which causes the clotting of casein; paracasein is formed. Paracasein in cooperation with calcium ions transforms into curd and as supernatant whey is left behind. As described, whey is a by-product of cheese production. The yield is very high; 9 liters of whey could be obtained from 10 liters of milk. [306]

2 Material and Methods

The used methods are EN ISO/IEC 17025 standard methods and all investigations were conducted in an accredited laboratory. One of the several advantages of an accredited laboratory is the comparability of the results to other accredited labs. Quality of the results, hygiene instructions, methods and the correct mode of operation are frequently audited. This ensures constant high quality of all areas in the test laboratory and the test certificates can be labeled with a special approval mark.

2.1 Material

2.1.1 Buffered Peptone Water (Biokar diagnostics)

Buffered Peptone Water is generally used for dilutions, sample preparation and preparation of stock suspensions. This medium also can be used for pre-enrichments for stressed organisms after sublethal treatments like spray drying, pasteurization, osmotic pressure or conservation treatments.
The general principle is to recreate the osmotic balance in samples caused by sodium chloride. For this purpose, the peptone water is buffered with sodium and potassium phosphates.

One liter of 25.5 g media BK018 contains:

- 10.00 g peptone
- 5.00 g sodium chloride
- 9.00 g disodium phosphate, dodecahydrated
- 1.50 g monopotassium phosphate, anhydrous

The pH-value for the ready-to-use medium is at 25 °C 7± 0.2

For 1 liter of buffered peptone water dissolve 25.5 g of the powdered media in 1 liter of distilled water. After complete dissolution dispense in vials or tubes. Autoclave the media at 121 °C for 15 minutes and cool it down to room temperature before use. [(312]

2.1.2 **Modified Lauryl Sulphate (Oxoid)**

The modified lauryl sulphate media is for selective enrichment of *Cronobater spp.* in milk samples. MLST was formerly described by Guillaume-Gentil as a selective enrichment broth for *Cronobacter.*[(313)] The growth of other members of the *Enterobacter* family is restricted by a high salt concentration (34 g/L). The combination of modified lauryl sulphate and vancomycin inhibits the growth of gram-positive bacteria and at once ensures the survival of *Cronobacter* in this media.

One liter of this media contains;

- 34.00 g sodium chloride
- 20.00 g enzymatic digest of animal and plant tissue
- 5.00 g lactose
- 2.75 g potassium dihydrogen phosphate
- 2.75 g dipotassium hydrogen phosphate
- 0.10 g sodium lauryl sulphate

The pH-value of this media should be at 25 °C 6.8 ± 0.2
For 500 mL of modified lauryl sulphate 32.3 g is dissolved in distilled water and sterilized in an autoclave at 121 °C for 15 minutes. After cooling down to approximately 50 °C aseptically 1 vial of Vancomycin Supplement (5 mg) is added. It is reconstructed as directed. [(314)]

2.1.3 **Chromocult® Enterobacter Sakazakii Agar (Merck)**

The selective agar Chromocult® Enterobacter Sakazakii Agar is used for *Cronobacter spp.* identification. On this agar *Cronobacter* grows with an intensive color-reaction of

Cronobacter colonies. A special supplement is used to inhibit the growth of other gram-positiv and gram-negative organisms. Furthermore, the incubation temperature of 44 °C also inhibits the growth of unrequested bacteria.
5-Bromo-4-chloro-3-indolyl-α-D-glucopyranoside is used as a supplement to ensure the differentiation of α-D-glucosidase-positive and negative microorganisms. *Cronobacter sakazakii* is α-D-glucosidase-positive and appears on the agar as blue/green- colored colony.

One liter of this selective media contains;

- 6.00 g peptone
- 5.00 g sodium chloride
- 1.50 g bile salt mix
- 0.10 g 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside
- 12.00 g agar-agar

The pH- value of this media should be 7 ± 0.2 at 25 °C.
For one liter of Chromocult® Enterobacter Sakazakii Agar 24.6 g of the dried medium must be suspended in 1 L boiling deionised water until the powder is completely dissolved. After autoclaving at 121 °C for 15 minutes the medium cooled in a water bath to approximately 45-50 °C. Then pour 15 mL of the medium, after carefully mixing, in sterile Petri dishes for use. [315]

2.1.4 Trypto-Casein Soy Agar (Biokar Diagnostics)

Trypto-Casein Soy Agar (TSA) is used as a universal nutrient medium with a wide range of organism growth. Due to its excellent nutritive value, TSA can be used for the isolation and growth of aerobic and anaerobic organisms and also for the development of microorganisms with special demands. It can be poured in Petri dishes or on stripes for investigation of contaminated surfaces. The typical composition of TSA accords to the specifications defined in the US and EU Pharmacopeia.

One liter of the medium contains;

- 15.0 g tryptone
- 5.0 g papaic digest of soybean meal
- 5.0 g sodium chloride
- 15.0 g bacteriological agar

The pH-value of TSA should be 7.3 ± 0.2 at a temperature of 25 °C.
To prepare one liter of the medium dissolve 40.0 g dehydrated medium in 1 L of distilled or demineralized water. Bring it slowly to boil while constant stirring until the medium is completely dissolved. Then dispense it into tubes or flasks and sterilize it in an autoclave at 121 °C for 15 min. After sterilization cool it down to 44-47 °C and pour it in sterile Petri dishes. Let the medium solidify and dry the plates in an incubator before streaking out samples. The typical bacterial response appears after incubation at 30-35 °C for 48 h. (324)

2.1.5 API ID32E (bioMérieux)

There are different tests included in the whole Api ID32E (Fig.8) test kit (Tab.1). After a colony is streaked out for isolation on TSA (incubation for 18-24 h at 37 °C, to be sure that it is pure and fresh) the new colonies are used for identification. Colonies must be taken from TSA and emulsified in 2 mL of sterile 0.85% saline until a turbidity equivalent to 0.5 McFarland was reached. Then each well of the test kit must be filled with about 55 µL of the colony-suspension. The first seven test wells have to be overlaid with mineral oil to ensure an anaerobic condition. Then the test stripes were incubated at 37 °C for 18-24 h. Before test interpretation one drop of Kovac´s Reagent (2 % *para*-dimethylaminobenzaldehyde in 20%[ESP] HCl, Merck) must be filled in the indole production test well. The interpretation of the test wells was performed as described in the instructions manual by the manufacturer. The identification of the organism was ascertained using the APIWeb database (https://apiweb.biomerieux.com) for ID32E. (316, 317)

Carbohydrate utilization
Adonitol
L-Arabinose
D-Arabitol
L-Arabitol
D-Cellobiose
Galacturonate
D-Glucose
Inositol
Malonate
D-Maltose
D-Mannitol
Palatinose
L-Rhamnose
D-Sorbitol
D-Sucrose
D-Trehalose
5-Ketogluconate

Tab.1: Tests included in API ID32E test kit [316,317]

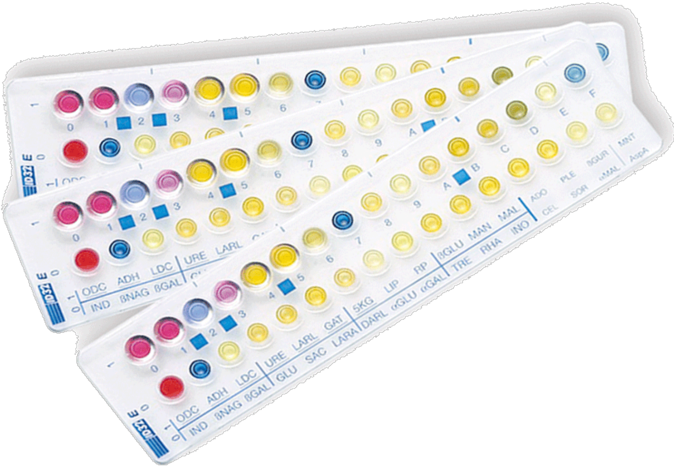


Fig.8: API ID32E Biomerieux Industry [318]

2.1.6 **Violet Red Bile Agar (Biokar Diagnostics)**

Violet Red Bile Agar (VRB) is used for the detection and enumeration of *coliform* bacteria in various food products. It is a selective medium and is has been tested the first time in 1932 from MacCrady for the Committee of Standard Methods of Milk Analysis of the American Public Health Association. It is perfectly appropriate for identification of *coliform* bacteria in raw and pasteurized milk within 24 h of incubation.
The medium contains both crystal violet and bile salt to inhibit the growth of gram-positive organisms. The colonies are surrounded by a precipitate of bile acids and the pH indicator is red colored (neutral red) caused by the lactose fermentation and the resulting acidification of the medium.

One liter of Violet Red Bile Agar contains;

- 7.0 g peptic digest of meat
- 3.0 g yeast extract
- 10.0 g lactose
- 1.5 g bile salt
- 5.0 g sodium chloride
- 30.0 mg neutral red
- 2.0 g crystal violet

12.0 g bacteriological agar

The pH-value of this medium should be 7.4 ± 0.2 at a temperature of 25 °C.
To prepare one liter of VRB suspend 38.5 g of the dehydrated medium in 1 L distilled or deionized water. While bringing it slowly to boil, stir it until it is completely dissolved. After cooling the medium down to a temperature of 44-47 °C transfer 1 mL of the sample in a Petri dish, pour 12 mL of the medium in the plate and homogenize it by swirling. Let it solidify and overlay it with 4 mL of medium to create an anaerobic environment. For detection of *coliform* bacteria incubate it at 30 °C for 24 h. [325]

2.1.7 **Violet Red Bile Glucose Agar (Biokar Diagnostics)**

Violet Red Bile Glucose Agar (VRBG) is used for the detection and enumeration of *Enterobacter* species in milk, milk products and a wide range of other food products. It is also used in the Pharmacopoeia for the detection of gram-negative organisms, which are resistant to bile salts. The presence of bile salt and crystal violet in the same medium inhibits the growth of gram-negative bacteria. The red color of the pH indicator, which is neutral red, is caused by the degradation of glucose to acid.

One liter of VRBG Agar contains;

- 7.0 g enzymatic digest of animal tissues
- 3.0 g yeast extract
- 10.0 g glucose
- 1.5 g bile salts
- 5.0 g sodium chloride
- 30.0 mg neutral red
- 2.0 mg crystal violet
- 13.0 g bacteriological agar

The pH value of this medium should be 7.4 ± 0.2 at a temperature of 25 °C.
To provide one liter of Violet Red Bile Glucose Agar suspend 39.5 g of the dehydrated medium in 1 L of distilled or deionized water. Then slowly bring it to boil while stirring with constant agitation until the medium is completely dissolved. It is important to avoid overheating. It should not be autoclaved. [326]

2.1.8 **Slanetz & Bartley Medium (Oxoid)**

The Slanetz & Bartley medium is used for identification and enumeration of *Enterococcus* species. It is possible to use the technique of membrane filtration or to use the medium as a direct plating medium. [319,320] The medium is selective for *Enterococci* and if it is

incubated at a temperature between 44-45 °C, all red and maroon colored colonies may be described as presumptive *Enterococcus* species. (321,322)

A liter of Slanetz & Bartley contains;

- 20.0 g tryptose
- 5.0 g yeast extract
- 2.0 g glucose
- 4.0 g di-potassium hydrogen phosphate
- 0.4 g sodium azide
    - g tetrazolium chloride
- 10.0 g agar

The pH-value of this medium should be 7.2 ± 0.2 at a temperature of 25 °C.
To prepare one liter of the medium suspend 42 g in 1 L of distilled water and bring to boil to get sure the medium is completely dissolved. Then cool it down to a hand-hot temperature and dispense it into Petri dishes. The agar should not be remelted and excessive heating must be avoided during the production process. (323)

2.1.9 ***Bacillus Cereus*** **Agar acc. to Mossel (Biokar Diagnostics)**

Bacillus Cereus Agar (BC-agar) is used for the detection and enumeration of spores and vegetative cells of *Bacillus cereus* in various food products, including milk and milk products.
The principle of this medium is the fact that *Bacillus cereus* is not able to ferment mannitol and that in most of the tested strains is the enzyme lecithinase present. Tryptone and meat extract favor the growth of the organism searched for. The sterile egg yolk emulsion is used for the detection of the enzyme lecithinase and the insoluble precipitate of the egg yolk emulsion accumulates around the colonies and causes a whitish halo. Mannitol is used to differentiate *Bacillus cereus,* which is not able to ferment mannitol from other bacteria contaminating the samples. The fermentation of mannitol causes a color change of the phenol red to yellow. Polymyxin is used for the inhibition of accessory organisms, when the investigated samples are contaminated.

One liter of BC-agar contains;

- 10.0 g tryptone
- 1.0 g meat extract
- 10.0 g D-mannitol
- 10.0 g sodium chloride
- 25.0 mg phenol red
- 100.0 mL sterile egg yolk emulsion
- $1x10^5$ IU Polymyxin B
- 13.5 g bacteriological agar

The pH-value of this medium should be 7.2 ± 0.2 at a temperature of 25 °C.
To provide one liter of BC-agar suspend 44.5 g of the dehydrated medium in 0.9 L of distilled or deionized water. While stirring the medium constantly bring it slowly to boil. Then dispense it in flasks and sterilize it in an autoclave at 121 °C for 15 minutes. Let the medium cool down to a temperature of 44-47 °C than add 100 mL (900 mL medium + 100 mL supplement) of Sterile Egg Yolk Emulsion with Polymyxin B (Biokar Diagnostics). Mix it rapidly and pour the medium into sterile Petri dishes. After solidification the plates are ready for use. Typical culture response appears after incubation at 30 °C for 48 h. (327,328)

2.1.10 **API 50 + CHB Medium (BioMérieux)**

There are various tests (Tab.2) included at the API 50 test kit (Fig.9). The test is used for the identification *Bacillus* and related species and gram-negative rods, which are members of the *Enterobacter* or *Vibrionaceae* families.
After a colony was streaked out for isolation on TSA (incubation for 18-24 h at 37 °C, to be sure that it is pure and fresh) the new colonies are used for identification. For the identification the CHB Medium is used for making bacteria suspension of the microorganism to be tested and filled in each tube of the test kit. While incubation, the bacteria ferments the carbohydrates to acids, which leads to a decreased pH and a color change in the test tubes. As a result, the special biochemical profile, shown by the different

color changes, is used and for identification the profile should be entered in the identification software (https://apiweb.biomerieux.com).

One vial of 10 mL of CHB Medium contains following composition;

- 2.0 g ammonium sulfate
- 0.5 g yeast extract
- 1.0 g tryptone
- 3.22 g disodium phosphate
- 0.12 g monopotassium phosphate
- 10 mL trace elements
- 0.17 g phenol red
- 1000 mL demineralized water pH 7.4-7.8 at 20-25 °C

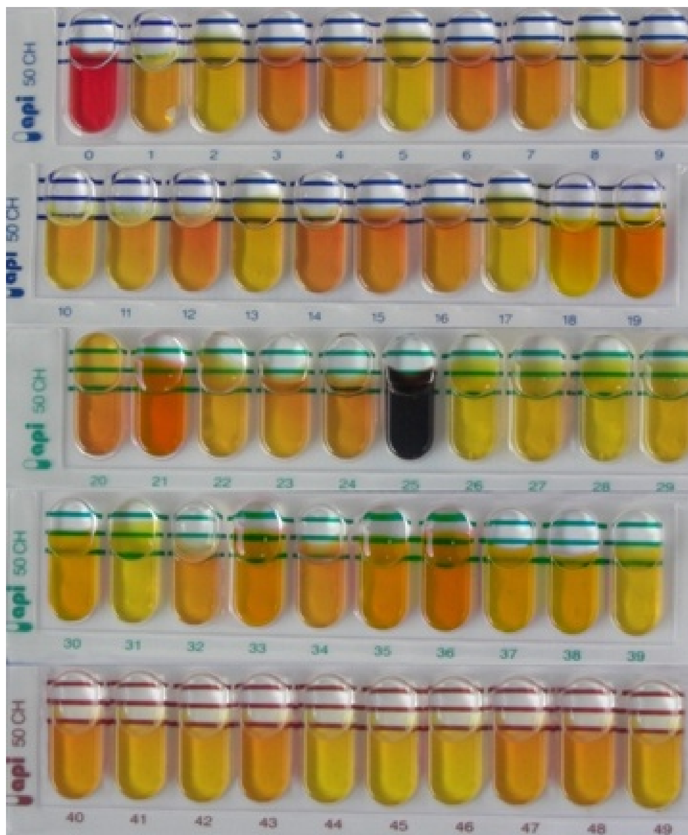


Fig.9: API 50 CH (Biomerieux) [329]

Carbohydrate utilization strip 0-9	Carbohydrate utilization strip 20-29
Control	Methyl-αD-Mannopyranoside
Glycerol	Methyl-αD-Glucopyranoside
Erythritol	N-Acetyl-glucosamine
D-Arabionose	Amygdalin
L-Arabinose	Arbutin
D-Ribose	Esculin ferric citrate
D-Xylose	Salicin
L-Xylose	D-Cellobiose
D-Adonitol	D-Maltose
Methyl-β-D-Xylopyranosid	D-Lactose (bovine origin)
Strip 10-19	**Strip 30-39**
D-Galactose	D-Melibiose
D-Glucose	D-Saccharose
D-Fructose	D-Trehalose
D-Mannose	Inulin
L-Sorbose	D-Melezitose
L-Rhamnose	D-Raffinose
Dulcitol	Amidon (starch)
Inositol	Glycogen
D-Mannitol	Xylitol
D-Sorbitol	Gentiobiose
Strip 40-49	
D-Turanose	
D-Lyxose	
D-Tagatose	
D-Fucose	
L-Fucose	
D-Arabitol	
L-Arabitol	
Potassium gluconate	
Potassium 2-keto-gluconate	
Potassium 5-keto-gluconate	

Tab.2: Tests included in API 50CHB test kit [330]

2.1.11 **Yeast Extract Glucose Chloramphenicol Agar (Merck)**

The Yeast Extract Glucose Chloramphenicol Agar (YGC) is a selective agar for isolation and enumeration of yeasts and molds in milk and milk products.
The medium contains the antibiotic chloramphenicol to inhibit the growth of accompanying bacterial flora and enables the yeasts and molds to regenerate after cell stress and supports their growth.

One liter of YGC contains;

- 5.0 g yeast extract
- 20.0 g D(+)glucose
- 0.1 g chloramphenicol
- 14.9 g agar

The pH-value of this medium should be 6.6 ± 0.2 at a temperature of 25 °C.
To prepare one liter of YGC suspend 40 g of the dehydrated medium in 1 L water and sterilize it in an autoclave at 121 °C for 15 minutes. [(331)]

2.1.12 **Dextrose Tryptone Agar (Oxoid)**

The Dextrose Tryptone Agar (DTA) is a non-selective medium for the detection and enumeration of thermophilic and mesophilic bacteria in food and food products. In this study it is used to detect thermophilic spores.

One liter of this medium contains;

- 10.0 g tryptone
- 5.0 g dextrose
- 0.04 g bromocresol purple
- 12.0 g agar

The pH-value of this medium should be 6.9 ± 0.2 at a temperature of 25 °C. For the preparation of one liter of DTA medium 27 g of the dehydrated medium must be

completely suspended in 1 L of distilled boiling water. Then sterilize the medium in an autoclave at a temperature of 121 °C for 15 minutes. [332]

2.1.13 Plate Count Agar (Biokar Diagnostics)

Plate Count Agar (PCA) contains glucose and yeast extract and is used for enumeration of aerobic bacteria in food, especially in milk and milk products, as well as pharmaceuticals, cosmetics and their raw products. The nutrients tryptone, vitamins of yeast extract and glucose are used as an energy source, which is favored by most of the bacteria. The transparency of the medium and the pleasant size of the colonies formed enable an uncomplicated enumeration of the bacteria.

One liter of PCA contains;

- 5.0 g tryptone
- 2.5 g yeast extract
- 1.0 g glucose
- 12.0 g bacteriological agar

The pH-value of this medium should be 7.0 ± 0.2 at the temperature of 25 °C.
To prepare one liter of PCA suspend 20.5 g of the dehydrated medium in 1 L of distilled or deionized water. Then bring it slowly to boil until the medium is completely dissolved and decant it in flasks or tubes. For sterilization put the medium in an autoclave at 121 °C for 15 minutes. [336]

2.2 Methods

2.2.1 *Cronobacter sakazakii Identification*

For the identification of *Cronobacter sakazakii* an ISO standard method is used, which was first published in 2006 with the International Dairy Federation. The used method is composed of two enrichment steps. The first step, so called pre-enrichment, is to put the

samples in buffered peptone water, which enables the microorganisms to rehydrate and recover from stress of spray drying and storage. [307,308] The second enrichment step, the selective enrichment, is carried out in modified lauryl sulphate broth. [307,308, 309,310] These two different enrichment steps and the increased incubation temperatures lead to the inhibition of other bacteria in the sample and result in better recovery of *Cronobacter*.
Protocol;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Incubation for 18 ± 2 h at 37 °C
- Monitor if pH is between 4.5-9
- Transfer 0.1 mL to 10 mL mLSB
- Incubation for 24 ± 2 h at 45 °C
- Streak on ESIA (Chromocult® *Enterobacter sakazakii* agar Fig.7)
- Incubation for 24 ± 2 h at 44 °C
- Streak suspect green/blue colonies on TSA for identification
- Incubation at 37 °C for 18-24 h
- Pick up 5 suspect yellow colonies from plate and identify it with ID32E test kit
- Incubation at 37 °C for 24 ± 2 h
- Evaluate as described in the test kit

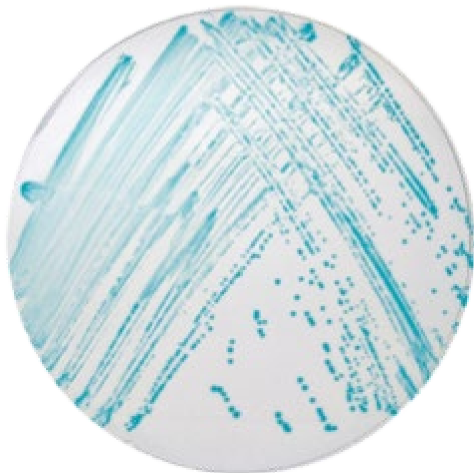


Fig. 7: *Cronobacter sakzakii* on Chromocult® agar (Merck) [311]

2.2.2 **Identification *Coliform* Bacteria**

For the identification and enumeration of *Coliform* bacteria VRB agar is used. In this medium crystal violet and bile salts inhibit the growth of gram-negative bacteria, so it is easier to identify the bacteria searched for. The colonies are surrounded by a precipitate of bile acid and they are red colored because of the fermentation of lactose, which is caused by pH indicator (neutral red).

To identify *Coliform* bacteria the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of VRB agar
- Mix the sample by swirling
- Let the medium solidify
- Overlay the solidified VRB medium with 4 mL of medium
- Let it solidify
- Incubation at 30 °C for 24 h
- Count the violet colonies which have a diameter equal or greater than 0.5 mm
- Lactose negative bacteria are colorless [325]

Fig. 10: *Enterobacter faecalis* on Violet Red Bile Agar (Biokar Diagnostics) [333]

2.2.3 **Identification of *Enterobacter* Species**

The identification and enumeration of *Enterobacter* species is conducted with Violet Red Bile Glucose Agar (VRBG). The advantage of using VRBG is the presence of both crystal violet and bile salts to inhibit the growth of gram-positive bacteria. The fermentation of glucose is shown by a pH indicator, neutral red, which leads to a red color change. The colonies appear violet red with or without a halo of precipitated bile salt.
To identify *Enterobacter* species the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of VRBG agar
- Mix the sample by swirling
- Let the medium solidify
- Overlay the solidified VRBG medium with 4 mL of medium
- Let it solidify
- Incubation at 37 °C for 24 h
- Count the violet colonies [326]

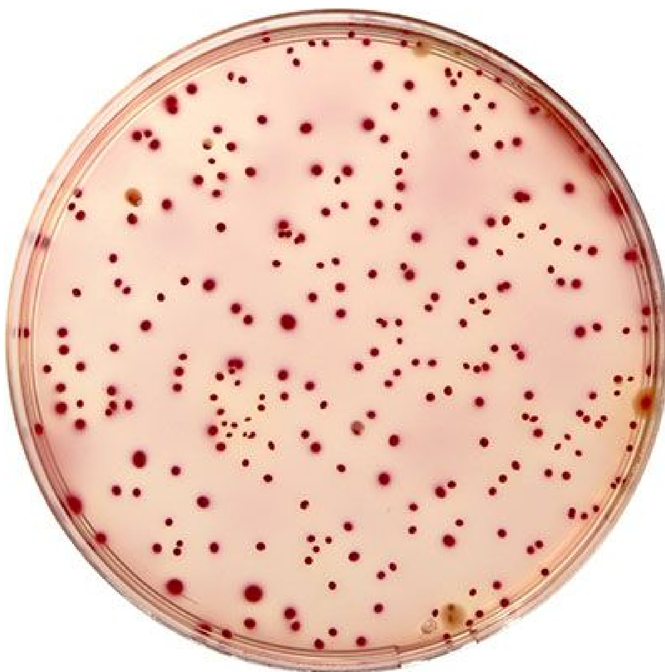


Fig. 39: *Enterobacter spp.* on VRBG Agar (Biokar Diagnostics) [334]

2.2.4 **Identification of *Enterococci***

For the identification and enumeration of *Enterococci* Slanetz and Bartely (S&B) medium is used. The *Enterococcus* colonies appear red, maroon or pink, but not all species reduce tetrazolium chloride (TTC) and therefore also the pale colonies should be observed. TCC is a redox dye which changes the color from pale to red when it gets reduced.

To identify *Entercoccus* species the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of S&B agar
- Mix the sample by swirling
- Let the medium solidify
- Incubation at 35 °C for 48 h
- Count the typical red/maroon colonies [323]

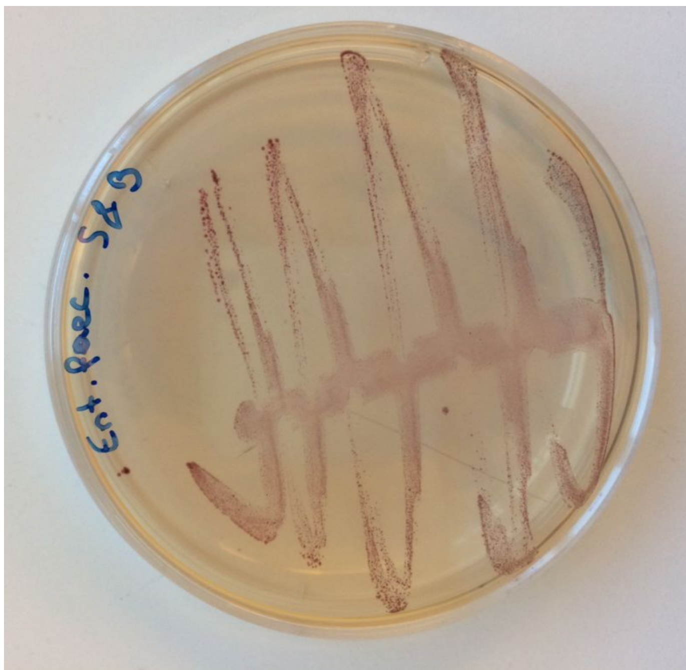


Fig. 11: *Enterococcus faecalis* on S&B Agar (Oxoid) [335]

2.2.5 **Identification of *Bacillus cereus***

The identification and enumeration of *Bacillus cereus* is carried out with *Bacillus Cereus* Agar (acc. to Mossel). The principle of the medium based on two factors; on the one hand the lack of mannitol fermentation of these bacteria and on the other hand the presence of lecithinase in majority of the tested strains. To test the appearance of lecithinase in the strains sterile egg yolk emulsion is used and is shown as insoluble precipitate of the egg yolk surrounding the colonies by forming a whitish color. The turn from red to yellow color of the medium is induced by fermentation of mannitol, which is always caused by contaminating bacteria and enables their identification. The last advantage of BC agar is the presence of Polymyxin B, an antibiotic which inhibits the growth of accompanying microflora.

To identify *Bacillus cereus* the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Prepare two Petri dishes filled with BC agar for each sample
- Transfer 0.5 mL from the same sample in each of the two Petri dishes
- Spread the inoculums on the surface of the medium with a triangle
- Incubation at 30 °C for 48 h
- Count the typical red colonies surrounded by a whitish halo
- Identify doubtful colonies with API 50 and CHB medium [327,328]

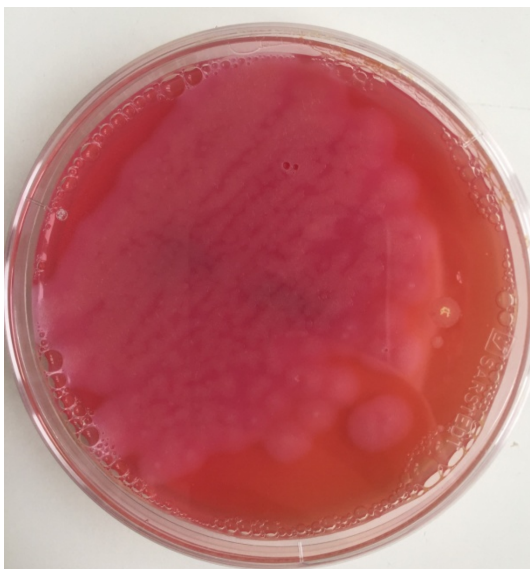


Fig. 12: *Bacillus cereus* on BC-Agar (Biokar Diagnostics) [335]

2.2.6 Identification of Mold and Yeast

The identification of mold (Fig.13) and yeast (Fig.14) is possible on the same medium, the Yeast Extract Glucose Chloramphenicol Agar (YGC). The addition of the antibiotic chloramphenicol ensures the inhibition of bacteriological accompanying flora. The prepared medium is stabile for a long period of time, at least for 4 months.
To identify mold and yeast the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of YGC agar
- Mix the sample by swirling
- Let the medium solidify
- Incubation at 25 °C for 4 days
- Count all mold and yeast colonies

Fig.13: *Penicillium chrysogenum* on YGC Agar (Merck) (335)

Fig.14: *Saccharomyces cerevisiae* on YGC Agar (Merck) (3

2.2.7 **Identification of Spores**

For the identification and enumeration of thermophilic spores Dextrose Tryptone Agar (DTA) is used. Acid producing bacteria such as “flat-sour” thermophiles form yellow colonies which are surrounded by a yellow zone (Fig.15).

To identify thermophilic spores the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 10 mL in a test tube
- To eliminate all vegetative cells, incubate for 10 min an 80 °C water bath
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of DTA agar
- Mix the sample by swirling
- Let the medium solidify
- Incubation at 55 °C for 3 days
- Count all colonies on the medium [332]

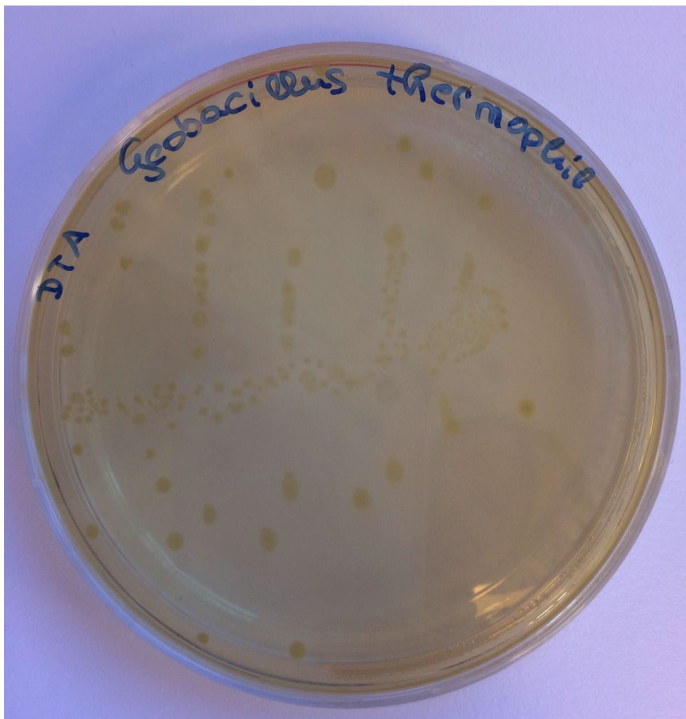


Fig. 15: *Geobacillus stearothermophilus* on DTA Agar (Oxoid) [335]

2.2.8 **Identification of Bioburden and Thermophilic Bacteria**

For bioburden and thermophilic bacteria growth and enumeration Plate Count Agar (PCA) is used. PCA contains glucose and yeast extract for good bacterial growth and the transparency of the medium and the relative size of the colonies formed enable an uncomplicated analysis.

To identify thermophilic bacteria the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of PC agar
- Mix the sample by swirling
- Let the medium solidify
- Incubation at 55 °C for 3 days
- Count all apparent colonies

To identify bioburden the following steps must be conducted;

- Inoculation of 10 g sample in 90 g buffered peptone water
- Homogenize by mixing for 1 minute
- Transfer 1 mL of the sample in a Petri dish
- Pour in 12 mL of PC agar
- Mix the sample by swirling
- Let the medium solidify
- Incubation at 30 °C for 3 days
- Count all apparent colonies [336]

2.3 Spray Drying

2.3.1 **Introduction**

One of the oldest food preservation methods is drying. It describes the removal of water to a lower the moisture content which prevents the food from bacterial growth and moisture mediated deterioration. The most important advantage for drying food is to lower the costs of storage, transport and packaging and that it is possible to store the food products at ambient conditions. (337) In this context several different drying methods have been developed in during the time and the efficiency of these processes has been improved to improve the product quality and uniformity. (338)
Spray drying was first used to produce milk powder from liquid milk in 1901. (339) The nozzle for atomization, which advances the process of spray drying, was developed in 1913. (340) In the food industry spray drying has been established since the 1950s to embed flavor and oils and protect them during further handling, storage or production. The process of spray drying has become one of the most convenient methods of drying, particularly when drying heat-sensitive materials like enzymes or other special proteins for pharmaceutical use. (341,342)

Working principle

During the process of spray drying an effectual volume of hot air is exposed to finely disperse liquid droplets to accomplish rapid evaporation of the solvent and collection of the solid particles. The inert gas or hot air, loaded with moisture, is then able to escape the drying chamber (Fig.16). Atomization and the modulation between the flow of the droplets or particles and the drying medium, which is inside the drying chamber, is very important to minimize the thermal and dehydration stress of the products. It would be optimal to hold the outlet temperature low and that the drying process itself is conducted as quickly as possible. (340)

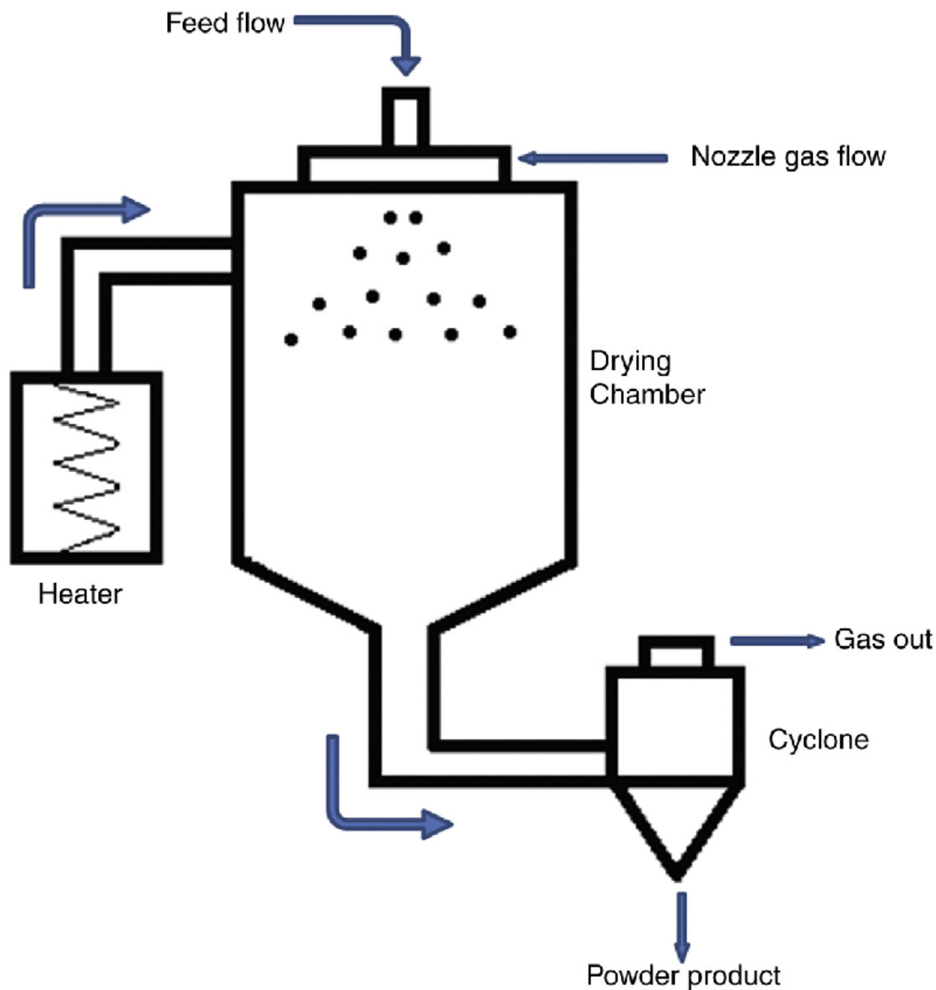


Fig. 16: Schematic diagram of a single stage spray dryer. [343]

There are four fundamental steps in spray drying process;

- The atomization of a liquid into diminutive droplets
- Contact between hot air and droplets
- Evaporation of the water droplets
- Collection of the produced powder [344]

The atomization is the first step and describes the atomizing. It disperses the feed into fine droplets to increase the surface area. It is also an important step for heat transfer and mass increasing. [345] As a basic principle, the higher the energy expended to perform the atomization, the smaller the created droplets. [346] Atomization is very critical because of its influence on the operational economy and the product quality most. The dispersion size of

the droplets and the dispersion size of the produced powder depend on the used atomizers, the rotation speed, the flow rate and the pressure drop. [347]

The next step in the drying process is the mixing of the atomized droplets with the heated inert gas (air) stream. The contact between the hot air and droplets causes the evaporation of the water and results in dried powder. The final product should be adequatly dried when contacting the chamber wall to enable the collection of as much product as possible. There are two ways to bring the droplets in contact with the hot air. The first and more sensitive way is to move the feed and the drying medium within the chamber in the same direction. In this way, the wet product contacts the driest medium while the more dried product meets the cooler drying air. This method is used for heat sensitive final products. [346]
The other method is to move the feed and the drying medium within the chamber in opposite directions. In this way, the hottest medium contacts the driest particle, which could lead to unacceptable heat damage in the final product. [340]

After the contact between the hot air and the droplets the evaporation of water occurs, and the dried powder is produced. The temperature and the vapor pressure gradients are created in a special way that the heat transfer runs from the hot air (inert gas) to the water droplets and the mass transfer the other way round. When the droplets and the drying medium get in contact with each other the heat induces an increase of the water droplet temperature until a constant value is reached and most of the water droplets evaporate. Another important point is the air inlet and outlet temperature in the whole drying process. The higher the inlet air temperature, the faster the water is evaporated and the dried particles are formed. When the difference between the vapor pressure gradient and the temperature gradient is higher, the formation of particles and the evaporation gets faster.[348]

The final step of the spray drying process is the collection the dried powder. This includes the separation of the powder and the drying medium, which is often executed by using a cyclone and a filter bag outside the chamber. The density, size and settling velocity of the dried particles are decisive for the ease or difficulty of the separation; larger particles are found on the base of the chamber and the smaller particles are collected in the cyclone and the bag filter.

2.4 Procedure

The target of this study was to define the different microbial contaminations in the whole milk powder and whey protein concentrate (WPC) production process. After the investigation the results were evaluated and the food producers got feedback for optimization of the current hygienic control system and to find the parameters which may influence the product quality. The operating steps include different heating steps with temperatures between 57 °C and 82 °C.

The milk powder and the WPC were produced in a spray drying process but with different temperature steps. After each temperature step a sample has been taken and investigated. The investigated products comprised unskimmed milk, low fat milk, WPC 35, WPC 60 and WPC 80. The differences of the microbial contaminations in the various production steps and the differences between the various produced products were a point of interest. The production steps also differ from one to another product, depending on the perfect drying temperature of the final product, but the first sample was always taken from the raw product and the last one is always of the final product.

The raw products were delivered from different creameries and after the material passed the receiving department the first heating step at the plate heat exchanger was performed (Tab.3).

Plate Heat Exchanger		
Product	Temperature	Time
Low fat milk	82 °C	90 s
Unskimmed milk	74 °C	90 s
WPC 35	70 °C	90 s
WPC 60	68 °C	90 s
WPC 80	66 °C	90 s

Tab.3: Heating temperatures after receiving department

After the receiving department the raw products are stored in cooling towers until the next production step is induced. Before the material is stored for an undefined period of time it is preheated to ensure the raw material quality during the storage (Tab.4).

Preheating before Storage		
Product	Temperature	Time
Low fat milk	74 °C	4 min
Unskimmed milk	60 °C	4 min
WPC 35	66 °C	4 min
WPC 60	66 °C	4 min
WPC 80	66 °C	4 min

Tab. 4: Heating temperatures before storage at the cooling tower

After storage, the raw material runs through a spray drying process with different heating steps. The samples were taken at specific points (Tab.5) during the production and afterwards investigated in a microbiological laboratory.

Sampling Points		
Production steps	Place	Instant of Time
1 start	After Delivery by a truck	Production start
1 end	Preheating at Plate Heat Exchange	After preheating
3	Process Vessel	After evaporation
5	Feed Balance Tank	Production start
5a	Sampling Valve after Heat Holding Tube	Production start
7	Feed Balance Tank	Production end
7a	Sampling Valve after Heat Holding Tube	Production end

Tab.5: Specific sample taking points during the production

In production step 3 the WPC-raw material is heated until a temperature of 57 °C must be reached and for unskimmed and low fat-milk a temperature of 72 °C is necessary. Production step 5 and 7 are the same heating step, after feeding; in step 5 the samples were taken at the batch beginning and samples of step 7 were taken at batch end. The heating temperature is 66 °C for WPC 60/80, 72 °C for WPC 35 for at least 30 minutes and 74 °C for 15 minutes for milk products.

3 Results

3.1 Unskimmed Milk Results

In this study 128 samples of unskimmed milk were taken. These samples include all operating levels beginning at the raw material and ending with the final products. For the production of final unskimmed milk powder different raw material was used and each of these different materials was investigated. The outcome is, that the number of raw material samples was much higher than all the other operating levels (Tab. 6). In general, samples of the other operating levels were taken just once during a whole production. Before the final product was packed in storage packs the last sample has been taken and investigated.

Distribution of samples		
Operating Level	Quantity (n)	Percentage
Raw material	63	49.2 %
3	9	7.0 %
5	10	7.8 %
5a	10	7.8 %
7	10	7.8 %
7a	10	7.8 %
Final product	16	12.5 %

Tab.6: Distribution of the samples during unskimmed milk production

In case of unskimmed milk none of the samples showed a *Cronobacter sakazakii* contamination.

3.1.1 **Contamination in Different Production Steps**

During the production process the contamination level of the different microorganisms fluctuate (Tab.8). The production steps (operating levels) 3, 5, 5a, 7 and 7a (Tab.5) are part of the investigation and the samples have been taken after each of these production steps.

Bacteria	Step 3	Step 5	Step 7
Total Bac. Count	$1.4 \times 10^4 \pm 1.2 \times 10^4$	$1.7 \times 10^4 \pm 2.2 \times 10^4$	$1.0 \times 10^6 \pm 1.4 \times 10^6$
Enterobacter	44.4 ± 72.7	$710.0 \pm 1.9 \times 10^3$	$2.5 \times 10^4 \pm 4.8 \times 10^4$
Colioform Bacteria	89 ± 270	$660.0 \pm 1.6 \times 10^3$	$4.4 \times 10^4 \pm 9.8 \times 10^4$
Enterococcus	40.0 ± 44.1	416.7 ± 531.8	$3.2 \times 10^4 \pm 4.1 \times 10^4$
Bacillus Cereus	278.9 ± 690.7	$1.3 \times 10^5 \pm 4.1 \times 10^5$	$6.1 \times 10^5 \pm 1.3 \times 10^6$
Yeast	0.0 ± 0.0	0.0 ± 0.0	$3.9 \times 10^3 \pm 1.2 \times 10^4$
Therm.Spores	$1.5 \times 10^3 \pm 3.6 \times 10^3$	$1.6 \times 10^3 \pm 4.5 \times 10^3$	$2.8 \times 10^4 \pm 5.3 \times 10^4$
Therm. Bacteria	$2.4 \times 10^3 \pm 5.5 \times 10^3$	$3.4 \times 10^3 \pm 9.2 \times 10^3$	$2.9 \times 10^4 \pm 6.6 \times 10^4$

Tab.8: Bacterial counts (average in cfu) and standard deviation (cfu) of production steps 3, 5 and 7 (Unskimmed milk)

As shown in Fig. 19, production level 3 and 5 show nearly the same number of total bacteria count, though production step 7 show a larger contamination with total bacteria count. *Enterobacter* and *Coliform* bacteria contamination is in step 5 more than ten times higher than in step 3. After step 5 the contamination with *Enterobacter* increases thirtyfold, the *Coliform* bacteria count actually increases sixtyfold. The count of *Enterococcus* and *Bacillus cereus* also increases during the production process. The *Bacillus cereus* count is more than two thousand times higher than after step 3. In case of yeast, there is no count in production step 3 and 5, but after step 7 the count increases up to almost 4.000 cfu (Tab.8). The thermophilic bacteria and spores show nearly the same count at step 3 and 5, which is very low compared with step 7. At production step 7 the contamination with thermophilic bacteria and spores increases up to tenfold. (Tab.8, Fig. 19)

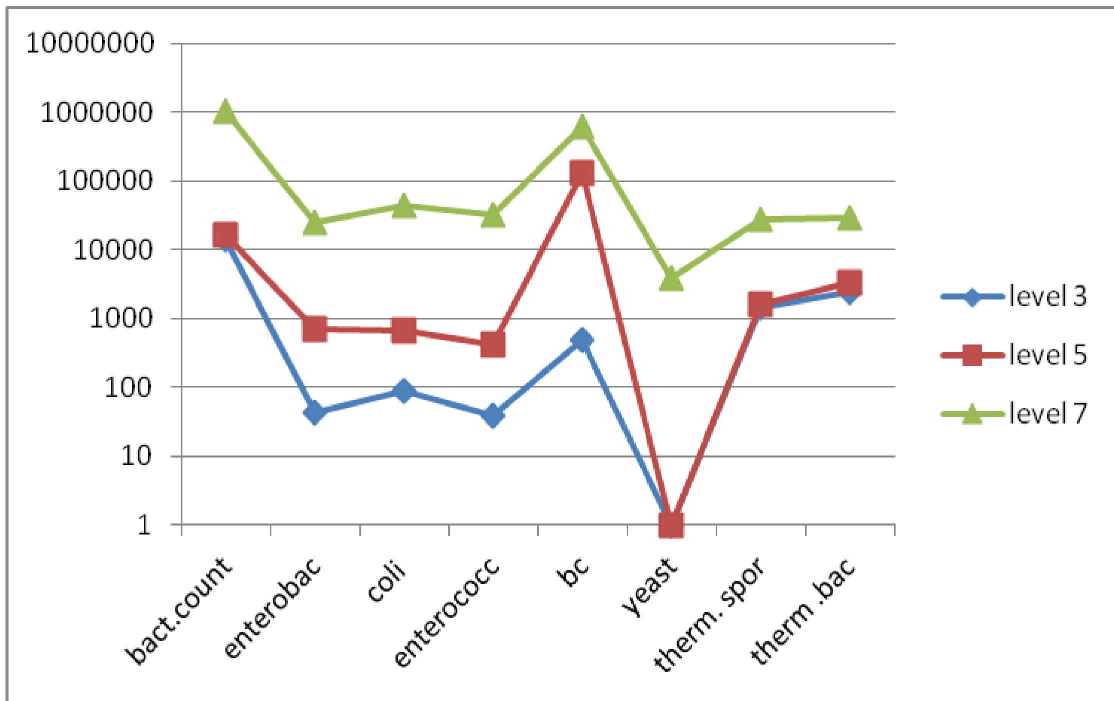


Fig.19: Bacterial contamination in operating level 3, 5 and 7

The samples for production step 5a and 7a are both taken after the heat holder plate; the only difference is that 5a is taken at batch beginning, 7a at batch end. The resulting counts are shown in Tab.9 and Fig.20.

Bacteria	Step 5a	Step 7a
Total Bac. Count	$4.5 \times 10^4 \pm 1.3 \times 10^5$	$6.3 \times 10^4 \pm 1.9 \times 10^5$
Enterobacter	20.0 ± 63.2	0.0 ± 0.0
Colioform Bacteria	0.0 ± 0.0	0.0 ± 0.0
Enterococcus	16.7 ± 31.6	0.0 ± 0.0
Bacillus Cereus	3.3 ± 6.8	33.0 ± 71.0
Yeast	0.0 ± 0.0	2.9 ± 6.3
Therm.Spores	$830.0 \pm 2.2 \times 10^3$	$3.0 \times 10^3 \pm 5.4 \times 10^3$
Therm. Bacteria	$1.4 \times 10^3 \pm 3.2 \times 10^3$	$4.3 \times 10^3 \pm 6.4 \times 10^3$

Tab. 9: Bacterial counts (average in cfu) and standard deviation (cfu) of production steps 5a and 7a (Unskimmed milk)

Total bacteria count is at the batch end, which means step 7a, approximately 40% higher than at batch beginning. The counts of *Enterobacter* and *Coliforms* are negligible in both production steps, the *Coliforms* are even not detected at all. In case of *Enterococcus,* the contamination is at step 5a is very low and at step 7a the bacterium is not detected. *Bacillus cereus* contamination increases during the production process tenfold, though the real value is still negligibly (Tab.9). The results for yeast investigation show the same picture, which means yeast is not detected at batch beginning and at batch end the counts are 3 cfu.

The results for thermophilic spores and bacteria are different from the other bacteria. The counts are already high at step 5a and rise even more during the productions step. As represented graphically in Fig.20, the counts of thermophilic bacteria and spores are approximately 1000 times higher than the counts of all the other bacteria species.

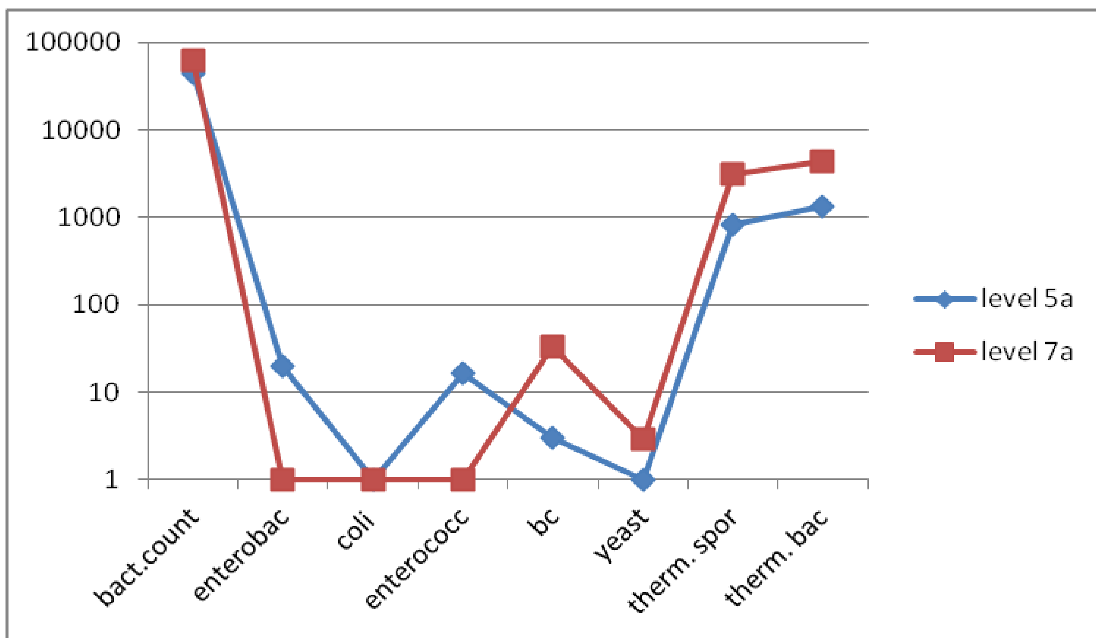


Fig.20: Bacterial contamination in operating level 5a and 7a

3.1.2 Raw Material and Final Products of Uskimmed Milk

The raw material for the unskimmed milk production came from different creameries but each creamery pasteurized the raw material before delivery. The total bacteria count contamination of the raw material differs from one to another case but was consistently very high. During the whole production process the bacterial count decreases on a large scale, the only exceptional cases are thermophilic spores and thermophilic bacteria.

Bacteria	Raw Material	Final Product	Reduction/Increase
Total Bac. Count	$1.7 \times 10^7 \pm 3.9 \times 10^7$	$2.2 \times 10^3 \pm 2.5 \times 10^3$	99.9 % Red.
Enterobacter	$3.3 \times 10^6 \pm 1.6 \times 10^7$	0.0 ± 0.0	100.0 % Red.
Colioform bacteria	$3.9 \times 10^6 \pm 2.2 \times 10^7$	0.0 ± 0.0	100.0 % Red.
Enterococcus	$1.4 \times 10^5 \pm 1.5 \times 10^4$	28.6 ± 75.6	99.9% Red.
Bacillus cereus	$3.8 \times 10^3 \pm 1.5 \times 10^4$	7.1 ± 12.5	99.8 % Red.
Yeast	$1.3 \times 10^7 \pm 7.5 \times 10^7$	0.0 ± 0.0	100.0 % Red.
Therm.Spores	155.3 ± 358.5	600.0 ± 847.6	409.1 % Inc.
Therm. Bacteria	$1.5 \times 10^3 \pm 5.7 \times 10^3$	$2.4 \times 10^3 \pm 3.9 \times 10^3$	157.6 % Inc.

Tab. 7: Bacterial counts (average in cfu) and standard deviation (cfu) in raw material/final products of unskimmed milk and their reduction/increase in percent

As indicated in Tab. 7, the count of spores and thermophilic bacteria increased by 409 % respectively 157 %. The contamination with all the other bacteria has been reduced by nearly 100 % (Tab.7). The large standard deviation reflects the major differences in raw product quality and also resulting in differences between the individual final product contaminations.

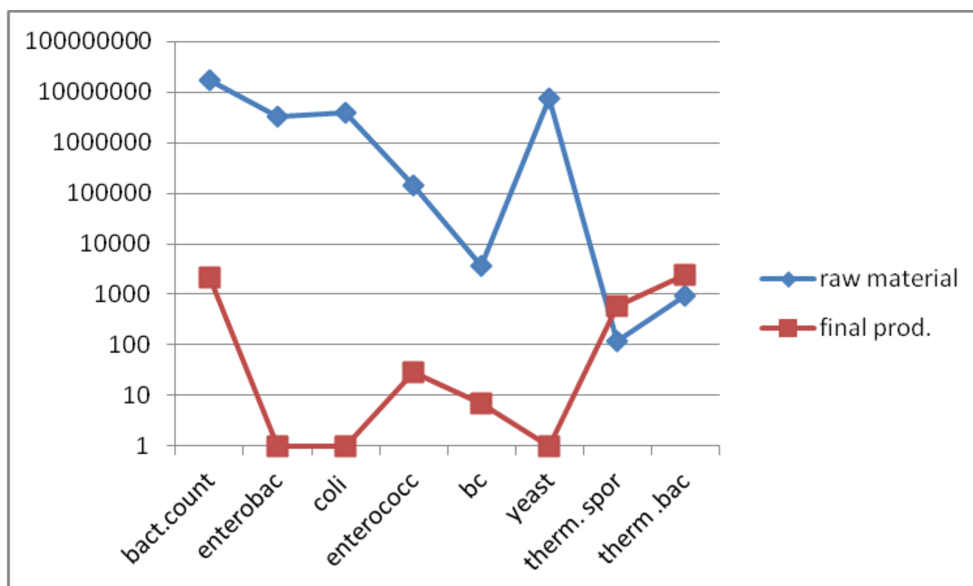


Fig.17: Comparison of raw material and final product contamination of unskimmed milk

The comparison of raw material and final product contamination (Fig.17) shows a large difference between raw material total bacteria count and final product bacteria count contamination. *Enterobacter, Coliform bacteria* and yeast contamination nearly completely disappears during the drying process. *Enterococcus* and *Bacillus cereuses* both declined in a large number but are still present in final products. Quite the contrary, the contamination with thermophilic spores and bacteria was very low in the raw material samples but increased in a large scale in the final product samples.

Bacteria	**Spring**	**Summer**	**Fall/Winter**
Total Bac. Count	500.0 ± 707.1	$2.8 \times 10^3 \pm 4.5 \times 10^3$	$6.0 \times 10^3 \pm 4.4 \times 10^3$
Enterobacter	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Colioform bacteria	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Enterococcus	0.0 ± 0.0	40.0 ± 89.4	0.0 ± 0.0
Bacillus cereus	10.0 ± 14.1	6.0 ± 13.4	0.0 ± 0.0
Yeast	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Therm.Spores	$1.3 \times 10^3 \pm 1.2 \times 10^3$	300.0 ± 565.7	$2.5 \times 10^3 \pm 4.2 \times 10^3$
Therm. Bacteria	$6.0 \times 10^3 \pm 6.2 \times 10^3$	$1.0 \times 10^3 \pm 2.0 \times 10^3$	$1.1 \times 10^4 \pm 2.1 \times 10^4$

Tab. 32: Comparison of bacterial contamination of unskimmed milk final product at different seasons (average and standard deviation in cfu)

The comparison of final product quality produced in fall/winter (Fig.18), spring or summer showed that there is no big difference in total bacteria count, but all the other investigated microorganisms show fluctuations during the seasons. The contaminations in fall and winter are nearly the same so the results are cumulated in fall/winter (Tab.32).
Enterobacter and *Coliform bacteria* contamination is not detected at all at final products; in contrast the *Enterococcus* count high in summer and not detected at spring. In this case there are no results for fall/winter available.
The *Bacillus cereus* contamination is not detectable at fall/winter and summer; the highest counts show the results of spring. The results for thermophilic bacteria and spores are high during all seasons compared with the contamination with the other bacteria. The highest counts are at fall/winter, the lowest contaminations are detected in summer.

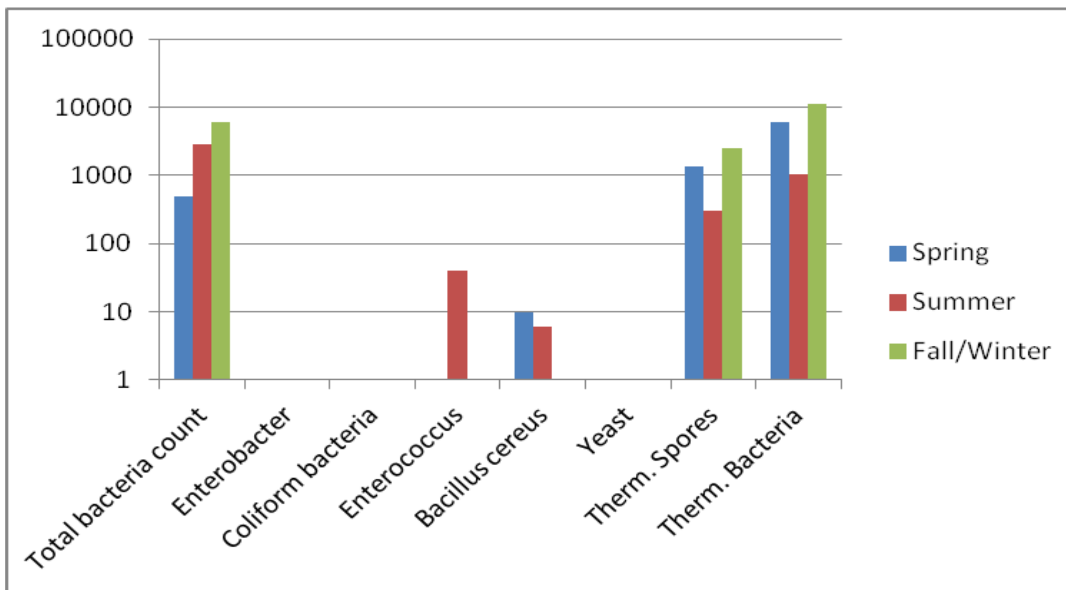


Fig. 18: Comparison of final product contaminations at different seasons

3.2 Low-Fat Milk Powder Results

In this study 81(n) samples of low fat-milk were taken. These samples include raw material, the production steps 3, 5, 5a, 7, 7a and the final low-fat milk powder product. The major parts of the samples were raw material samples (Tab.10), because different raw material was used to produce one final powder product. The distribution of the other samples is shown at Tab.10. In case of the production steps, just one sample per step has been taken and investigated, in case of the final products, each of the final powder has been investigated.

Distribution of samples		
Production step	Quantity (n)	Percentage
Raw material	36	44.4 %
3	6	7.4 %
5	7	8.6 %
5a	7	8.6 %
7	7	8.6 %
7a	7	8.6 %
Final product	11	13.6 %

Tab. 10: Distribution of low fat-milk samples during production

There was no positive *Cronobacter sakazakii* sample found during the whole production of low-fat milk powder.

3.2.1 Contamination during the Production Steps

After each of the production steps, 3, 5, 5a, 7 and 7a a sample has been taken and investigated. The counts of the different bacteria during the production steps 3, 5 and 7 are shown in Tab.11.

In general the highest contamination is detected at production step 7, except the contamination with yeast, which was at operation level 5 higher than after the other levels (Fig. 21, Tab.11).

Bacteria	Step 3	Step 5	Step 7
Total Bac. Count	$2.2 \times 10^8 \pm 5.3 \times 10^8$	$3.8 \times 10^7 \pm 9.8 \times 10^7$	$1.9 \times 10^8 \pm 4.9 \times 10^8$
Enterobacter	$6.8 \times 10^5 \pm 1.6 \times 10^6$	$1.5 \times 10^5 \pm 4.0 \times 10^5$	$5.7 \times 10^7 \pm 1.5 \times 10^8$
Colioform bacteria	$5.2 \times 10^5 \pm 1.2 \times 10^6$	$1.1 \times 10^5 \pm 2.8 \times 10^5$	$1.3 \times 10^8 \pm 3.4 \times 10^8$
Enterococcus	$6.3 \times 10^3 \pm 1.2 \times 10^5$	$8.8 \times 10^3 \pm 1.1 \times 10^4$	$3.4 \times 10^7 \pm 6.4 \times 10^7$
Bacillus cereus	13.3 ± 24.2	8.6 ± 22.7	12.9 ± 17.0
Yeast	$7.5 \times 10^3 \pm 1.6 \times 10^4$	$1.1 \times 10^5 \pm 3.0 \times 10^5$	50.0 ± 127.9
Therm.Spores	83.3 ± 116.9	200.0 ± 215.7	$3.9 \times 10^3 \pm 6.1 \times 10^3$
Therm. Bacteria	50.0 ± 83.7	$4.1 \times 10^6 \pm 1.1 \times 10^7$	$8.6 \times 10^3 \pm 1.9 \times 10^4$

Tab.11: Bacterial counts (average in cfu) and standard deviation (cfu) in production steps 3, 5 and 7of low-fat milk

As shown in Fig. 21 the total bacteria count is nearly equal at all three production steps. The counts of *Enterobacter*, *Coliform* bacteria and *Enterococcus* are much more at operation level 7 than at 3 and 5. Nevertheless, the contaminations with these three bacteria species are very high at all investigated production steps. In contrast *Bacillus cereus* counts are almost not detected during the whole production. As already mentioned, the contamination with yeast rises at the first two production steps but at level 7 the counts are insignificant low. Thermophilic spores contamination is more than thousand times higher at level 7 than at the other levels, in contrast the the thermophilic bacteria counts are much higher at level 5 (Fig.21, Tab.11)

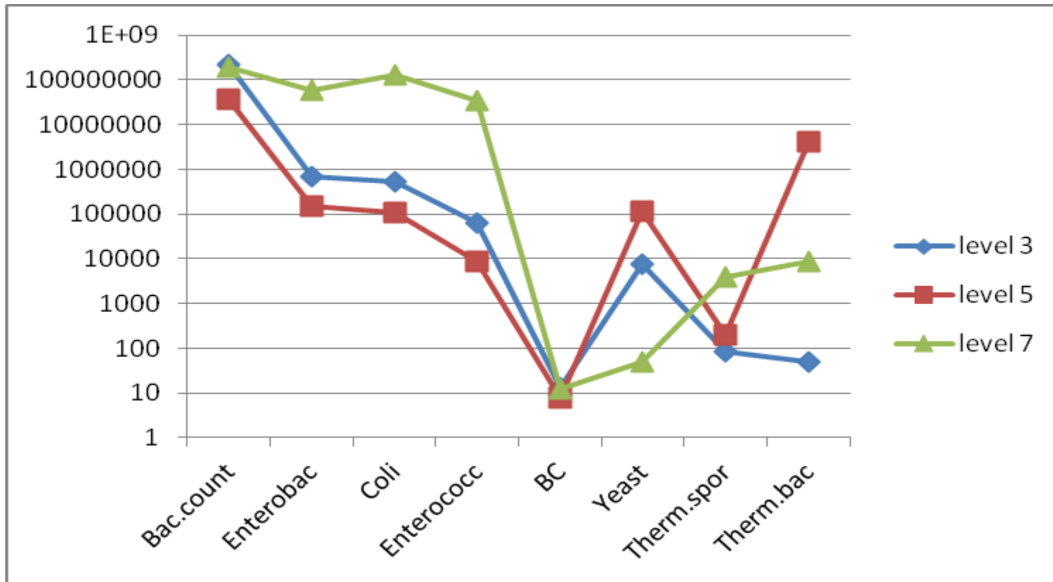


Fig. 21: Contamination profile at production steps 3, 5 and 7 at low fat-milk powder production

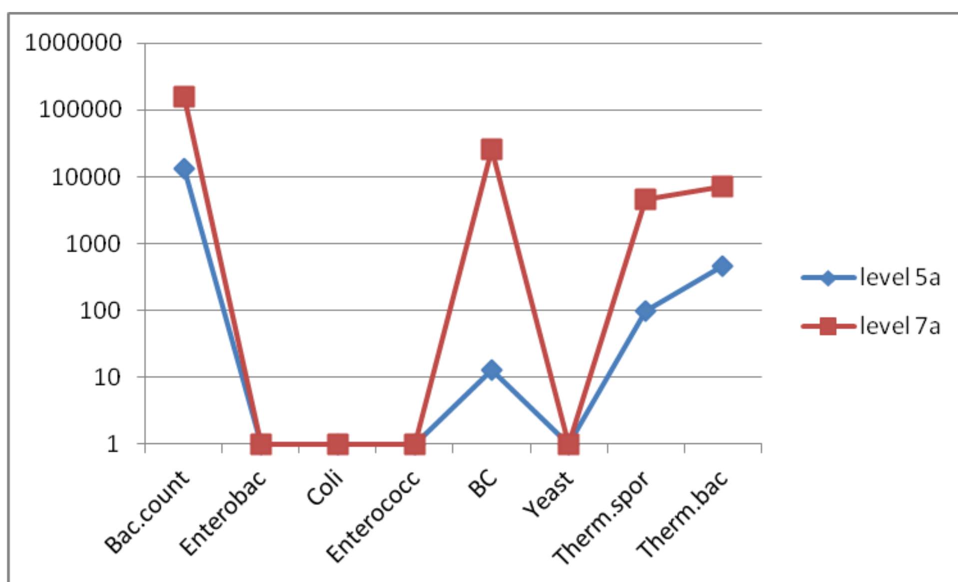
At this point the contamintation with all detected bacteria rises starting at the production beginning until the production ending is reached (Tab.12).

Bacteria	Step 5a	Step 7a
Total Bac. Count	$1.3 \times 10^4 \pm 2.4 \times 10^4$	$1.6 \times 10^5 \pm 4.2 \times 10^5$
Enterobacter	0.0 ± 0.0	0.0 ± 0.0
Colioform bacteria	0.0 ± 0.0	0.0 ± 0.0
Enterococcus	0.0 ± 0.0	0.0 ± 0.0
Bacillus cereus	12.9 ± 31.5	$2.6 \times 10^4 \pm 6.8 \times 10^4$
Yeast	0.0 ± 0.0	0.0 ± 0.0
Therm.Spores	100.0 ± 130.9	$4.6 \times 10^3 \pm 7.2 \times 10^3$
Therm. Bacteria	$471.42 \pm 1.0 \times 10^3$	$7.3 \times 10^3 \pm 1.8 \times 10^4$

Tab.12: Comparison of bacterial contamination production level 5a and 7a at low fat-milk production (average and standard deviation in cfu)

The total bacteria count is at production step 5a eleven times higher than at level 7a, although it is the production ending (Tab.12). As shown in Fig. 22 also *Bacillus cereus* count is about 25.000 CFU higher at the end of production than at the beginning. The contamination caused by thermophilic bacteria and spores is much larger at operating level

7a than at 5a. In contrast *Enterobacter, Coliform* bacteria*, Enterococcus* and yeast are not detected in both of the production steps.



Tab.22: Bacterial contamination at operation level 5a and 7a at low-fat milk production

3.2.2 Comparison of Raw Material and Final Low-Fat Milk Powder Product

The comparison of raw material and final product shows that in general the production process eliminates the majority of bacterial contamination .The only exceptions are *Bacillus cereus,* thermophilic bacteria and spores (Tab.13).

Bacteria	Raw Material	Final Product	Reduction/Increase
Total Bac. Count	$8.2 \times 10^7 \pm 1.6 \times 10^8$	$2.7 \times 10^4 \pm 6.9 \times 10^4$	99.9% Red.
Enterobacter	$8.0 \times 10^5 \pm 2.7 \times 10^6$	0.0 ± 0.0	100 % Red.
Colioform bacteria	$3.9 \times 10^6 \pm 1.7 \times 10^7$	0.0 ± 0.0	100 % Red.
Enterococcus	$3.1 \times 10^4 \pm 6.5 \times 10^4$	300.0 ± 315.1	99.0 % Red.
Bacillus Cereus	212.4 ± 1043.9	25.0 ± 45.4	88.2 % Red.
Yeast	$4.0 \times 10^3 \pm 1.8 \times 10^4$	0.00 ± 0.00	100 % Red.
Therm.Spores	$3.5 \times 10^3 \pm 1.1 \times 10^4$	$1.0 \times 10^4 \pm 1.9 \times 10^4$	197 % Inc.
Therm. Bacteria	$3.1 \times 10^4 \pm 8.9 \times 10^4$	$1.4 \times 10^3 \pm 1.9 \times 10^3$	95.6 % Red.

Tab.13: Bacterial counts (average cfu) and standard deviation (cfu) in raw material/final products of low-fat milk and their reduction/increase in percent.

The total bacteria count of the final product decreases by 99.9% of the raw material, the counts of *Enterobacter, Coliform* bacteria and *Enterococcus* become less than 1% (Tab.13). In case of yeast, the contamination was no longer detected in final product samples. Unlike the contamination with thermophilic spores which rises during the production process. The decrease of *Bacillus cereus* contamination is less distinct than the contamination with the other bacteria species. The same is true for the contamination with thermophilic bacteria, which is still reasonably high in the final product samples (Fig.23). The thermophilic spores count even rises at the final products; it is 197 times higher than the count in the investigated raw products.

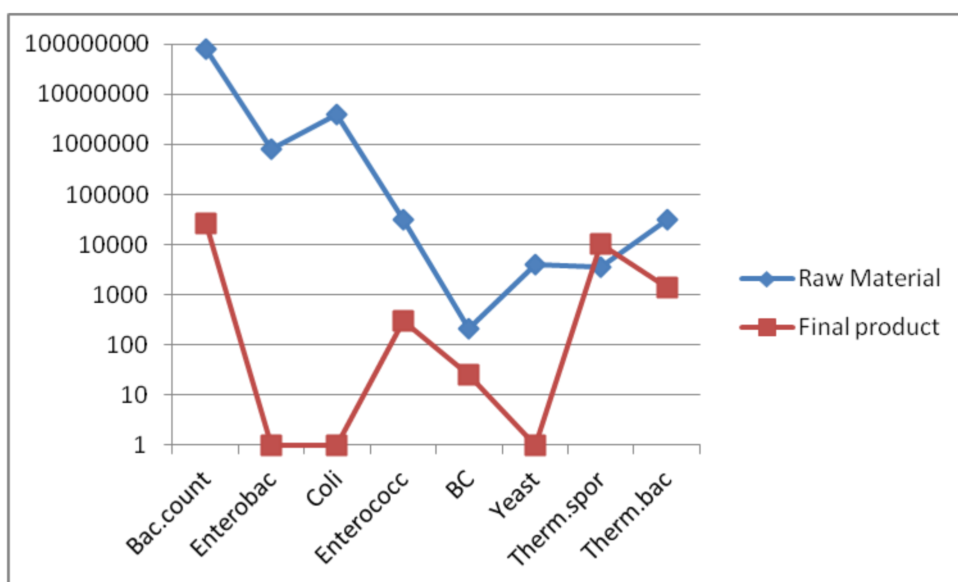


Fig.23: Comparison of contamination (average + standard deviation) of raw product and final product of low fat-milk powder

Bacteria	Summer	Winter
Total Bac. Count	$5.3 \times 10^4 \pm 8.4 \times 10^4$	$2.0 \times 10^3 \pm 2.4 \times 10^3$
Enterobacter	0.0 ± 0.0	0.00 ± 0.0
Colioform bacteria	0.0 ± 0.0	0.00 ± 0.0
Enterococcus	$3.5 \times 10^3 \pm 7.6 \times 10^3$	0.0 ± 0.0
Bacillus Cereus	38.3 ± 47.9	2.0 ± 4.5

Yeast	0.0 ± 0.0	0.0 ± 0.0
Therm.Spores	$1.3 \times 10^4 \pm 2.2 \times 10^4$	520.0 ± 311.5
Therm. Bacteria	$1.9 \times 10^3 \pm 1.9 \times 10^3$	320.0 ± 311.5

Tab.14: Comparison of final product results in summer and winter

In general, the bacterial counts are in summer higher than in winter (Fig.24). There are no counts of *Enterobacter*, *Coliform* bacteria and yeast in summer and winter (Tab.14). The count of *Enterococcus* is in summer relatively high; in winter *Enterococcus* is not detected. The counts of *Bacillus cereus* are insignificant in summer and winter season. In contrast, the spores and thermophilic bacteria counts are much higher in summer than in the colder season. The spore counts are about 25 times higher in summer than in winter. (Tab.14)

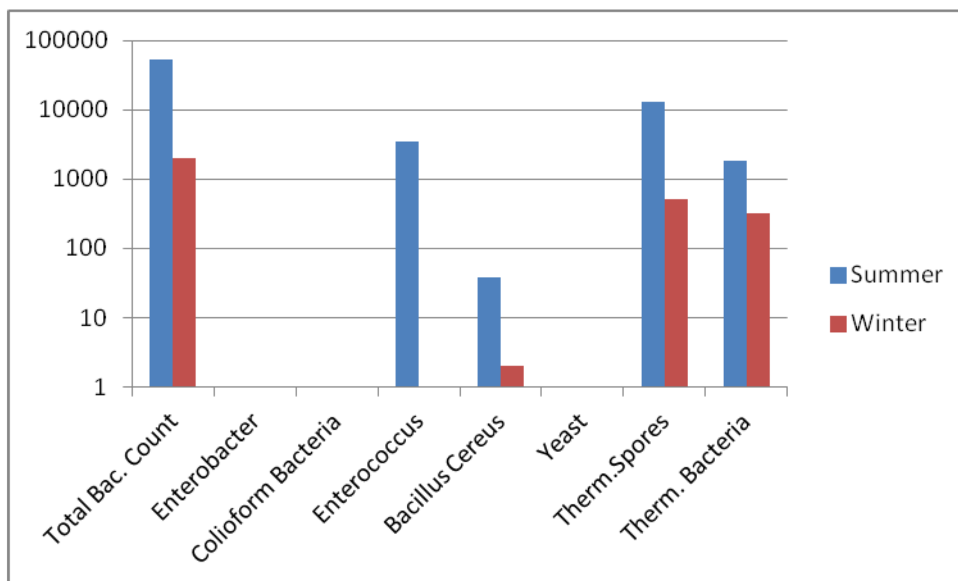


Fig. 24: Comparison of final product results in summer and winter

3.3 Results of WPC 35

In case of WPC 35 199 (n) samples were taken and investigated. These samples include all production steps, different raw material and also the final product WPC 35 powder. The major part of the investigation was raw material samples because different raw material was used to produce the final product. The production steps include steps 3, 5, 5a, 7, 7a, also step 1 at production start and step 1 at production end. The distribution of the WPC 35 samples is shown in Tab.15.

Distribution of samples		
Production step	Quantity (n)	Percentage
Raw material	49	24.6 %
1 start	18	9.1 %
1 end	18	9.1 %
3	18	9.1 %
5	19	9.5 %
5a	19	9.5 %
7	19	9.5 %
7a	19	9.5 %
Final product	20	10.1 %

Tab.15: Distribution of WPC 35 samples during production

Cronobacter sakazakii was detected in 23 of 49 (47 %) raw material samples. In all the other production steps and also final product *Cronobacter sakazakii* has not been found.

Contamination of Production Steps

After each production step at least one sample has been taken and investigated. The production steps include step 1 start and end, 3, 5, 5a, 7 and 7a. The contamination with different bacteria is shown in Fig. 25, 26 and. The counts of the different bacteria (average) and the standard deviation are shown in Tab.16, 17 and 18.

Bacteria	Step 3	Step 5	Step 7
Total Bac. Count	$5.0 \times 10^{6} \pm 1.9 \times 10^{7}$	$1.5 \times 10^{8} \pm 4.7 \times 10^{8}$	$8.2 \times 10^{7} \pm 2.5 \times 10^{8}$
Enterobacter	$6.1 \times 10^{5} \pm 2.6 \times 10^{6}$	$1.4 \times 10^{6} \pm 6.2 \times 10^{6}$	$6.2 \times 10^{7} \pm 2.0 \times 10^{8}$
Colioform bacteria	$8.4 \times 10^{5} \pm 3.5 \times 10^{6}$	$7.1 \times 10^{5} \pm 3.0 \times 10^{6}$	$7.6 \times 10^{7} \pm 2.5 \times 10^{8}$
Enterococcus	$3.7 \times 10^{6} \pm 1.3 \times 10^{7}$	$2.7 \times 10^{7} \pm 9.8 \times 10^{7}$	$2.7 \times 10^{5} \pm 7.7 \times 10^{5}$
Bacillus cereus	$7.1 \times 10^{4} \pm 2.3 \times 10^{5}$	$1.8 \times 10^{5} \pm 5.0 \times 10^{5}$	$1.8 \times 10^{5} \pm 4.6 \times 10^{5}$
Yeast	63.3 ± 225.6	$392.6 \pm 1.6 \times 10^{3}$	50.0 ± 102.0
Therm.Spores	344.4 ± 613.8	310.5 ± 598.1	294.7 ± 602.3
Therm. Bacteria	$6.4 \times 10^{3} \pm 2.6 \times 10^{4}$	194.7 ± 330.8	$1.7 \times 10^{3} \pm 6.6 \times 10^{3}$

Tab. 16: Bacterial counts (average cfu) and standard deviation (cfu) at production steps 3, 5 and 7of WPC 35

As shown in Fig.25 and Tab.16 the total bacteria count contamination rises during the production, the lowest value was counted at level 3 and the highest at level 5. *Enterobacter* and *Coliform* bacteria shown the same contamination profile, the counts are also higher at level 7 than at level 3. The *Enterococcus* counts are high at level 5 but they decrease during the last production steps and are lower at level 7. In contrast, the *Bacillus cereus* counts are low at production beginning, at level 3, but increase during the production levels. In general, the counts of yeast are very low; a temporary rise is noted only at level 5 but at level 7 the counts are lower than at production level 3 (Tab.16). The data for thermophilic spores are all about the same at all three production levels and the counts of thermophilic bacteria are the most at level 3, then the contamination increases at level 5, but decreases again at level 7 (Tab.16).

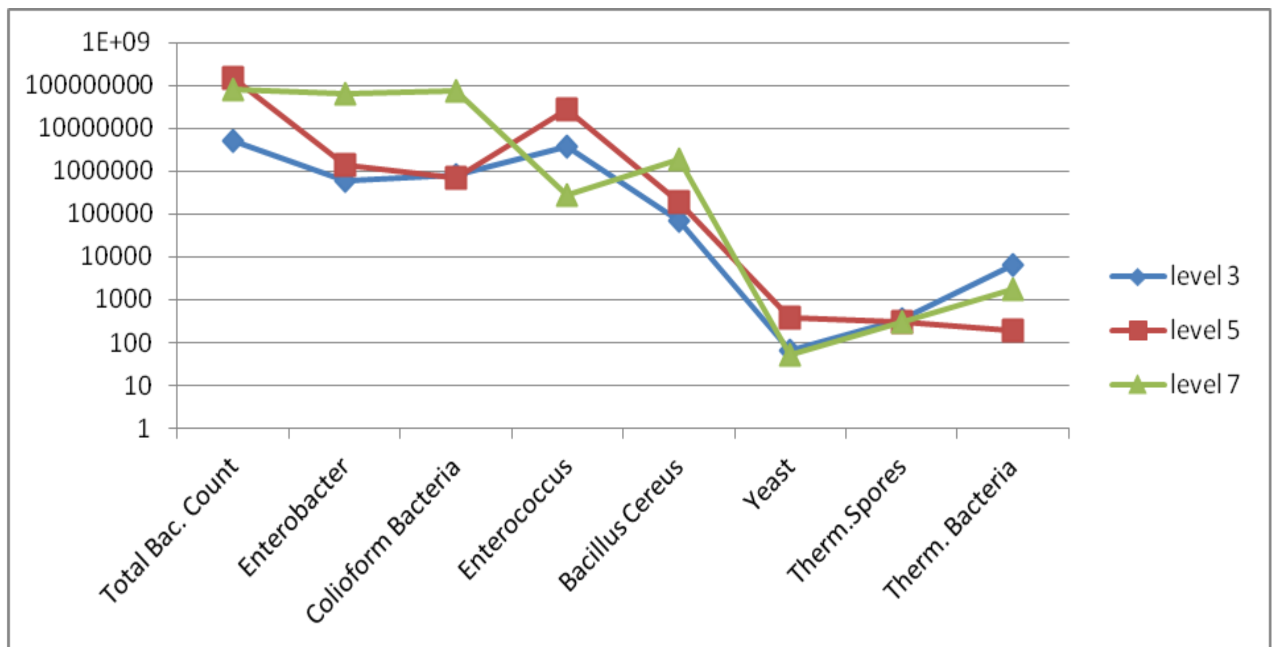


Fig.25: Comparison of contamination profiles of level 3,5 and 7 of WPC 35

Bacteria	1 Start	1 End
Total Bac. Count	$1.0 \times 10^8 \pm 2.3 \times 10^8$	$1.0 \times 10^8 \pm 2.0 \times 10^8$
Enterobacter	$2.8 \times 10^7 \pm 1.2 \times 10^8$	$7.4 \times 10^4 \pm 2.3 \times 10^5$
Colioform bacteria	$2.2 \times 10^7 \pm 9.2 \times 10^7$	$1.1 \times 10^5 \pm 2.8 \times 10^5$
Enterococcus	$2.1 \times 10^5 \pm 5.4 \times 10^5$	$5.2 \times 10^4 \pm 1.3 \times 10^5$
Bacillus cereus	16.1 ± 38.5	14.4 ± 32.9
Yeast	$8.2 \times 10^3 \pm 3.2 \times 10^4$	$8.2 \times 10^3 \pm 3.3 \times 10^4$
Therm.Spores	538.9 ± 847.2	$694.4 \pm 1.0 \times 10^3$
Therm. Bacteria	$2.0 \times 10^3 \pm 4.1 \times 10^3$	$638.9 \pm 1.0 \times 10^3$

Tab. 17: Bacterial counts (average cfu) and standard deviation (cfu) production steps 1 start and 1 end of WPC 35

At level 1 start and 1 end the counts of total bacteria are both very high (Tab.17), but it decreases until 1 end is reached. In case of *Enterobacteria* and *Coliform* bacteria the counts are high at level 1 start and during the production the counts decrease until a lower level of contamination at level 1 end is accomplished (Fig.26). *Enterococcus* contamination is 4 times higher at production start at level 1 start as at level 1 end. The contamination with *Bacillus cereus* is insigificant low at both operation levels. The counts of yeast are much higher than at levels 3,5 and 7 (Tab.16) and in general the counts do not

change during the production process. Thermophilic spores and bacteria counts are both relatively low at level 1 start as well as at level 1 end.

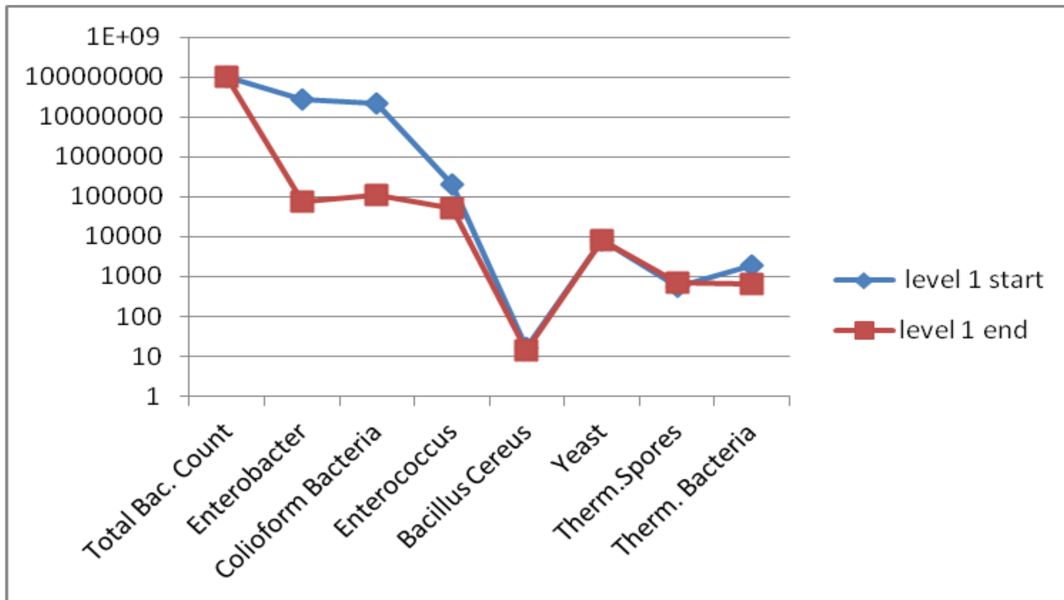


Fig.26: Comparison of contamination profiles of level 1 start and 1 end of WPC 35

Bacteria	Step 5a	Step 7a
Total Bac. Count	$3.6 \times 10^5 \pm 1.2 \times 10^6$	$4.0 \times 10^5 \pm 1.1 \times 10^6$
Enterobacter	178.9 ± 780.0	11.1 ± 47.1
Colioform bacteria	$1.4 \times 10^3 \pm 6.2 \times 10^3$	5.6 ± 23.6
Enterococcus	84.6 ± 167.6	50.0 ± 100.0
Bacillus cereus	$8.7 \times 10^4 \pm 2.4 \times 10^5$	$9.3 \times 10^4 \pm 3.0 \times 10^5$
Yeast	0.0 ± 0.0	0.6 ± 2.4
Therm.Spores	231.6 ± 463.1	200.0 ± 380.4
Therm. Bacteria	305.3 ± 485.9	$1.3 \times 10^3 \pm 2.6 \times 10^3$

Tab. 18: Bacterial counts (average cfu) and standard deviation (cfu) at production level 5a and 7a of WPC 35

Also at level 5a and 7a the total bacteria count is high (Tab.18, Fig.27), in contrast the contamination with *Enterobacter*, *Coliform* bacteria and *Enterococcus* is already moderate at level 5a but even decreases during the production until level 7a. *Bacillus cereus* shows a constant high count at both levels 5a and 7a (Fig.27). A contamination with yeast is not detected, there are no counts at level 5a and also insignificant low at level 7a (Tab.18). The

growth of thermophilic spores is unchanged at both operating levels but the counted colonies are quite low (Tab.18). Thermophilic bacteria count is much higher at the end of production step 7a as at production start at level 5a (Fig.27).

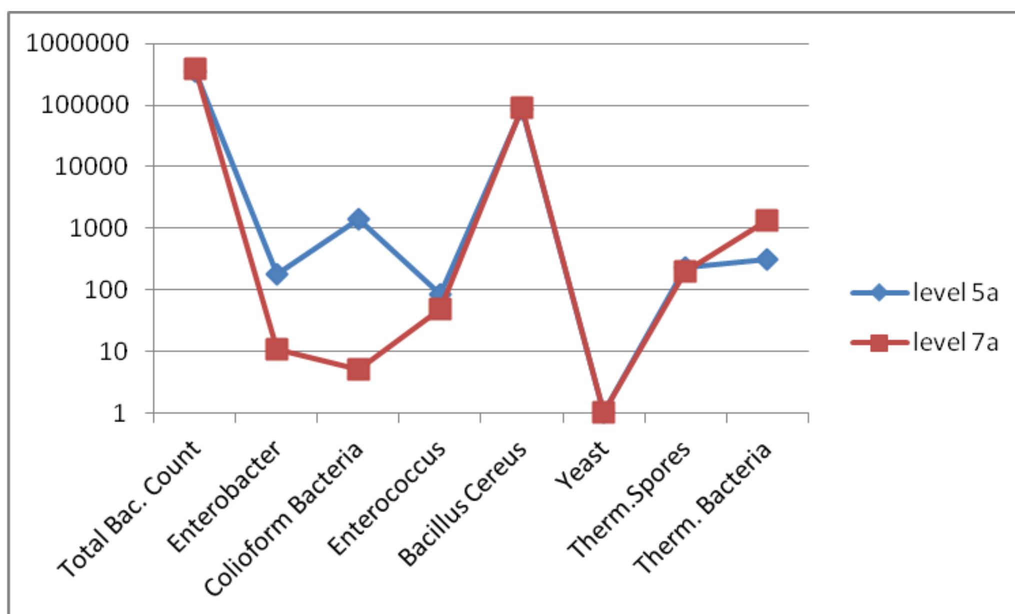


Fig.27: Contamination profile of operation level 5a and 7a at WPC 35 production

3.3.1 Comparison of Raw Material and WPC 35 Final Product

The comparison of raw products and final products show that the production process eliminates most of the bacterial contamination. In this case the only exceptions are thermophilic spores and *Bacillus cereus* (Tab.19), these are the bacteria which counts rise until the final product is reached.

Bacteria	**Raw material**	**Final product**	**Reduction/Increase**
Total Bac. Count	$1.0 \times 10^{8} \pm 1.9 \times 10^{8}$	$2.4 \times 10^{4} \pm 5.1 \times 10^{4}$	97.8 % Red.
Enterobacter	$2.1 \times 10^{6} \pm 6.4 \times 10^{6}$	0.00 ± 0.00	100 % Red.
Colioform bacteria	$3.4 \times 10^{6} \pm 1.1 \times 10^{7}$	0.00 ± 0.00	100 % Red.
Enterococcus	$7.9 \times 10^{5} \pm 3.7 \times 10^{6}$	69.2 ± 249.6	99.12 % Red.
Bacillus cereus	5.3 ± 16.1	47.5 ± 139.2	894.5 % Inc.
Yeast	$1.0 \times 10^{5} \pm 3.5 \times 10^{5}$	0.00 ± 0.00	100 % Red.

Therm.Spores	114.3 ± 244.9	300.0 ± 805.9	262.5 % Inc.
Therm. Bacteria	671.4 ± 2434.8	255.0 ± 708.9	62.0 % Red.

Tab.19: : Bacterial counts (average cfu) and standard deviation (cfu) of raw material and final product of WPC 35 and their reduction or increase

In general the bacterial counts for final WPC 35 products are much lower than the raw products (Tab.19, Fig.28). The contamination with *Enterobacter*, *Coliform* bacteria and yeast is 100 % reduced at the end of the production and the counts of *Enterococcus* and also the total bacterial count declined almost completely (99 % and 98 %, Tab.19). During the production process more than the half of the thermophilic bacteria could be eradicated; in contrast, the contamination with thermophilic spores increased up to more than 200 % of the raw product counts. *Bacillus cereus* increased ninefold during the production of WPC 35 final product (Fig.28, Tab.19).

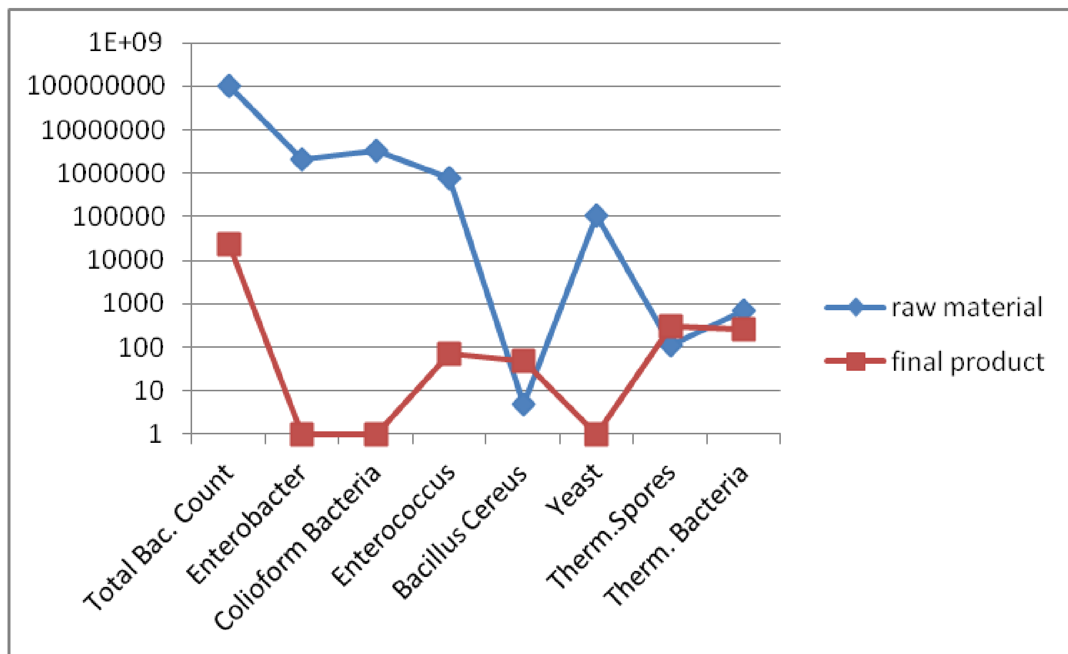


Fig. 28: Comparison of raw material and final product of WPC 35

Bacteria	Spring	Summer	Fall/Winter
Total Bac. Count	$2.7 \times 10^4 \pm 3.6 \times 10^4$	$4.9 \times 10^4 \pm 8.7 \times 10^4$	$5.8 \times 10^3 \pm 5.8 \times 10^3$
Enterobacter	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Colioform bacteria	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Enterococcus	0.0 ± 0.0	150.0 ± 367.4	0.0 ± 0.0

Bacillus cereus	2.0 ± 4.5	143.3 ± 239.2	8.9 ± 19.7
Yeast	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Therm.Spores	260.0 ± 527.3	666.7 ± 1.4 x 10^3	77.8 ± 164.2
Therm. Bacteria	180.0 ± 402.5	600.0 ± 1.2 x 10^3	66.7 ± 132.3

Tab.20: Bacterial profile of WPC 35 at different seasons (average + standard deviation of final products in cfu)

The comparison of final product bacterial profile during the seasons spring, summer and fall/winter showed that in general the contamination with bacteria is higher in summer than at the other seasons. The counts of fall and winter are nearly the same so it was summed up to fall/winter. In case of the total bacteria count the counted bacteria are in summer nearly twice as much at in spring, in winter the counts declined to a tenth (Tab.20). *Enterobacter*, *Coliform* bacteria and yeast were not detected at all at final product samples (Fig.29). *Enterococcus* and *Bacillus cereus* were not detected respectively negligible at spring and winter, but in summer much higher (Fig.29).The counts of thermophilic spores and bacteria are low in winter, but increase at the warmer seasons and reach their peak at summer.

Cronobacter sakazakii was not detected at final products, but in raw material 5 (10 %) samples were tested positive in winter, 10 (20 %) in spring and 8 (16 %) in summer.

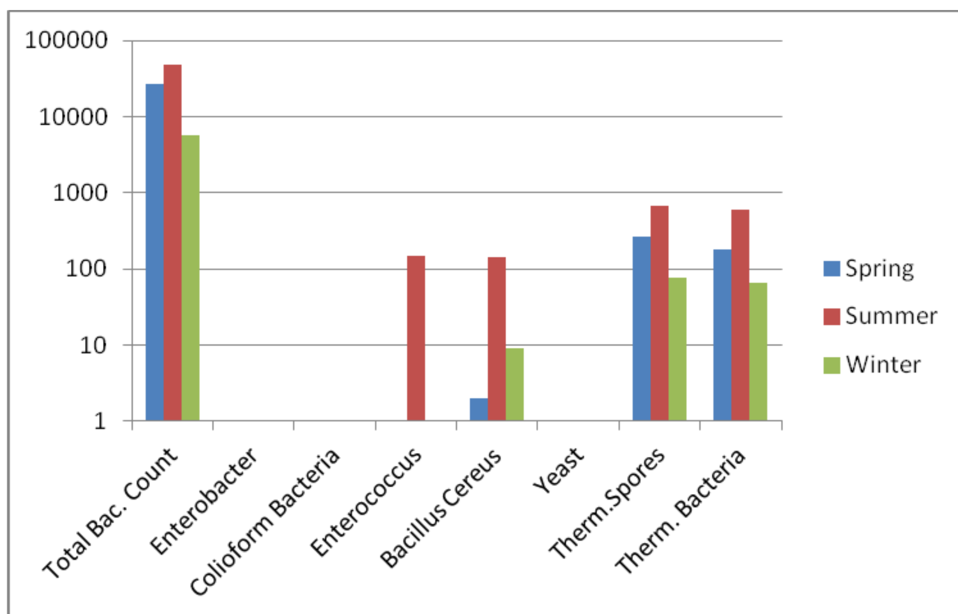


Fig. 29: Comparison of bacterial profile of WPC 35 final products at different seasons

3.4 Results of WPC 60

The investigation of WPC 60 comprises 95 (n) samples, including the production steps 1 start and 1 end, 3, 5, 5a, 7, 7a, and the raw material as well as final powder products. As shown in Tab.21 the major part of the samples are raw material samples, more precisely twice as much as final product samples. Final product samples were taken just once per each production run, the operating levels vary during the different production cycles.

Distribution of samples		
Production step	Quantity (n)	Percentage
Raw material	24	25.3 %
1 start	8	8.7 %
1 end	8	8.7 %
3	10	10.5 %
5	11	11.6 %
5a	5	5.3 %
7	11	11.6 %
7a	6	6.3 %
Final product	12	12.6 %

Tab. 21: Distribution of WPC 60 samples

During the production of WPC 60 in 12 (12.6 %) cases *Cronobacter sakazakii* was detected. In one case the raw material was tested positive and during the production also the levels 1 start and 1 end were still *Cronobacter sakazakii* positive.

3.4.1 Contamination of Production Levels of WPC 60

The samples of the production levels were taken after each step and include the levels 1 start, 1 end, 3, 5, 5a, 7, and 7a. The different bacteria counts (average and standard deviation) are shown in Tab. 22, 23 and 24 and are diagrammed in Fig. 30, 31 and 32.

The levels 3, 5 and 7 were taken at each production run, in contrast, to the other levels, which do not appear during every production run.

Bacteria	**Level 3**	**Level 5**	**Level 7**
Total Bac. Count	$2.1 \times 10^8 \pm 6.6 \times 10^8$	$1.3 \times 10^7 \pm 3.3 \times 10^7$	$5.6 \times 10^8 \pm 1.29 \times 10^8$
Enterobacter	$1.1 \times 10^4 \pm 3.4 \times 10^4$	$1.7 \times 10^5 \pm 5.4 \times 10^5$	$3.3 \times 10^7 \pm 7.4 \times 10^7$
Colioform bacteria	$1.7 \times 10^4 \pm 5.4 \times 10^4$	$7.1 \times 10^4 \pm 2.1 \times 10^5$	$2.8 \times 10^7 \pm 6.1 \times 10^7$
Enterococcus	$3.4 \times 10^6 \pm 9.5 \times 10^6$	$1.7 \times 10^5 \pm 3.5 \times 10^5$	$2.2 \times 10^7 \pm 59747715.20$
Bacillus cereus	215.0 ± 445.9	$364.6 \pm 1.2 \times 10^3$	$1.0 \times 10^3 \pm 3.3 \times 10^3$
Yeast	58.0 ± 111.8	$6.4 \times 10^3 \pm 2.1 \times 10^4$	$1.0 \times 10^3 \pm 3.0 \times 10^3$
Therm.Spores	20.0 ± 63.3	36.4 ± 67.4	236.4 ± 623.3
Therm. Bacteria	20.0 ± 42.2	0.0 ± 0.0	$600.0 \pm 1.9 \times 10^3$

Tab. 22: Bacterial count (average in cfu) and standard deviation (cfu) of production levels 3, 5 and 7 of WPC 60 production

The total bacteria count contamination of level 3 and 7 is about 43-times higher than at level 5 (Tab.22, Fig.30). In case of *Enterobacter*, *Coliform* bacteria and *Enterococcus* level 7 shows the most contamination, which means the bacteria counts rise during the production. The detected *Bacillus cereus* colonies have their peak at level 7, but the total counts of this bacteria is not as high as the previous described ones. The detected yeasts also rise until their maximum is reached at level and and then the counts decline (Fig.30). Thermophilic spores and bacteria reach nearly the same counts at level 3 and 5, which are insignificant low, but at level 7 the counts rise again (Tab.22, Fig.30).

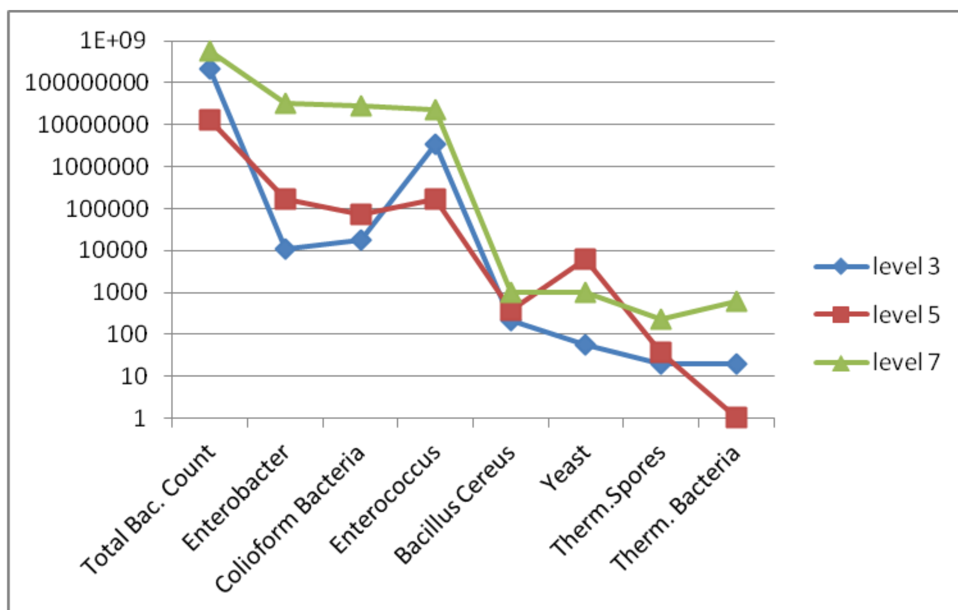


Fig.30: Contamination profile of production level 3, 5 and 7 of WPC 60

Bacteria	Level 5a	Level 7a
Total Bac. Count	$3.1 \times 10^4 \pm 2.9 \times 10^4$	$2.9 \times 10^4 \pm 3.2 \times 10^4$
Enterobacter	0.0 ± 0.0	$2.7 \times 10^3 \pm 6.5 \times 10^3$
Colioform Bacteria	0.0 ± 0.0	$6.9 \times 10^3 \pm 1.7 \times 10^4$
Enterococcus	$4.6 \times 10^3 \pm 1.0 \times 10^4$	$6.5 \times 10^3 \pm 1.2 \times 10^4$
Bacillus Cereus	354.0 ± 467.4	350.0 ± 763.5
Yeast	0.0 ± 0.0	13.3 ± 16.3
Therm.Spores	20.0 ± 44.7	16.7 ± 40.8
Therm. Bacteria	0.0 ± 0.0	66.7 ± 163.3

Tab. 23: Bacterial count (average + standard deviation in cfu) of production levels 5a and 7a of WPC 60

At level 5a and 7a the total bacteria counts are nearly the same with counts about 30.000 cfu. In case of *Enterobacter* and *Coliform* bacteria there are no counts detected at level 5a and during the production the counts rise two- respectively sixthousand-fold at level 7a (Tab.23). *Enterococcus* and *Bacillus cereus* show no serious variations during the productions levels 5a and 7a. The counts of yeast are not detected at level 5a, but also at level 7a the contamination is not significant. Thermophilic spores and bacteria contamination is not detected. There are no counts of thermophilic bacteria at level 5 and just 20 cfu of spores. At level 7a the counts of thermophilic spores decline to 16 cfu, the

counts of thermophilic bacteria indeed rise, but also the reached peak of about 67 colonies is insignificant.

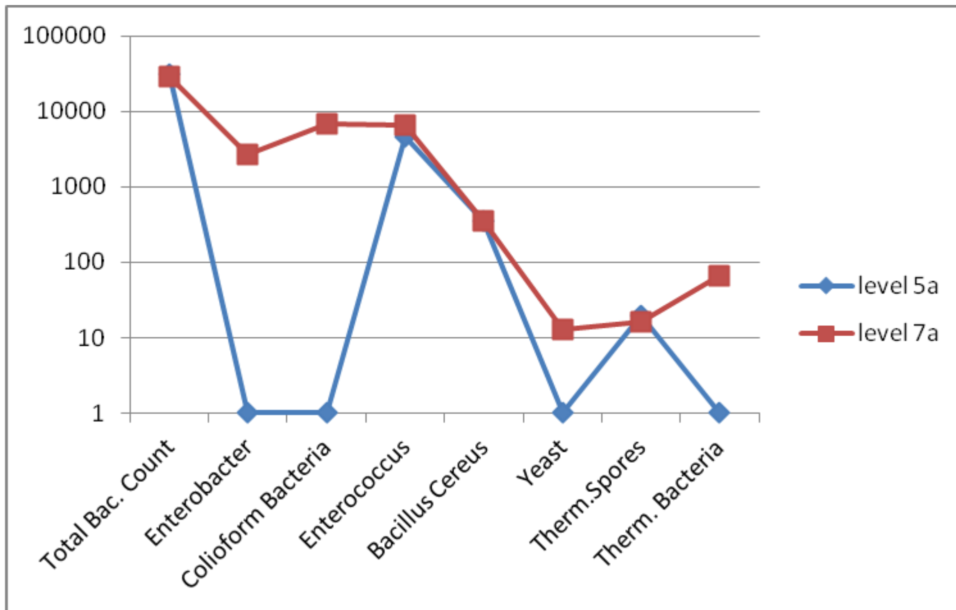


Fig. 31: Contamination profile of production level 5a and 7a during WPC 60 production

Bacteria	1 Start	1 End
Total Bac. Count	$2.9 \times 10^{7} \pm 8.1 \times 10^{7}$	$9.0 \times 10^{6} \pm 2.5 \times 10^{7}$
Enterobacter	$6.3 \times 10^{4} \pm 1.8 \times 10^{5}$	$2.0 \times 10^{3} \pm 5.6 \times 10^{3}$
Colioform bacteria	$1.3 \times 10^{4} \pm 3.5 \times 10^{4}$	$2.2 \times 10^{3} \pm 6.0 \times 10^{3}$
Enterococcus	$1.8 \times 10^{4} \pm 4.3 \times 10^{4}$	$3.1 \times 10^{4} \pm 7.5 \times 10^{4}$
Bacillus cereus	133.8 ± 350.9	7.5 ± 17.5
Yeast	187.5 ± 498.4	3.8 ± 7.4
Therm.Spores	87.5 ± 210.0	75.0 ± 116.5
Therm. Bacteria	12.5 ± 35.4	0.0 ± 0.0

Tab. 24: Bacterial count (average and standard deviation in cfu) of production level 1 start and 1 end of WPC 60 production

The total bacteria count is at both production levels high, but there is a more than 27 % decline during the process until level 1 end is reached (Tab.24).In case of *Enterobacter* and *Coliform* bacteria the counts at level 1 start are much higher than at level 1 end. The *Enterobacter* counts decline about 97 % during the production; the *Coliform* bacteria contamination decline about 84 % until level 1 end is achieved. *Enterococcus* counts

decrease sixfold during the process. The *Bacillus cereus* and yeast contamination in in general low, but declined until the counts are almost not detected (Tab.24, Fig.32). From the outset the thermophilic spore counts are low, but also do not change during the process (Fig.32). Thermophilic bacteria contamination is at these levels not detected; the counts of about 12 colonies at level 1 start are insignificant and at level 1 end there is no themophilic bacteria detected.

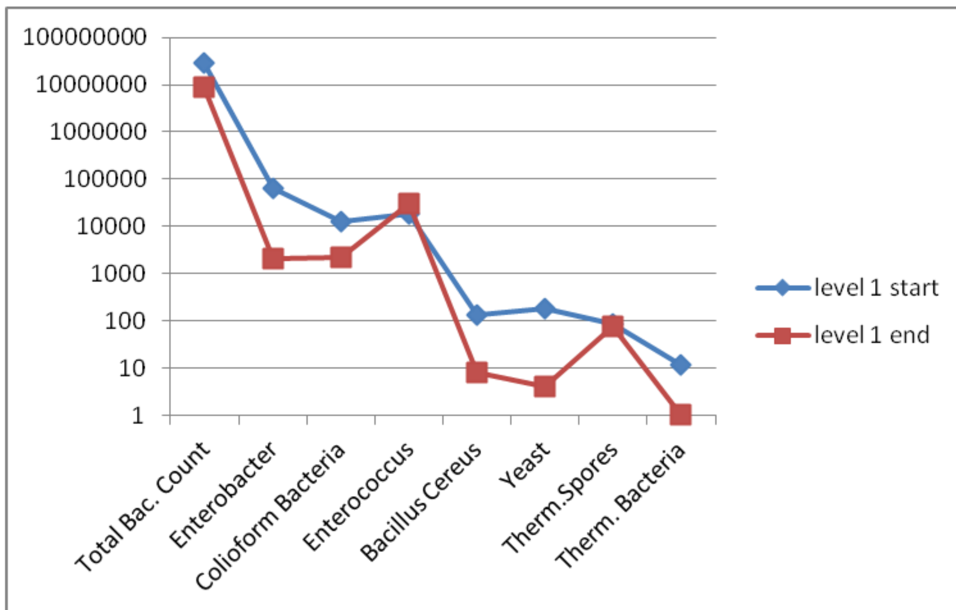


Fig.32: Contamination profile of production level 1 start and level 1 end at WPC 60 producion

3.4.2 Comparison Raw Material and Final WPC 60 Product

The comparison of bacterial profile is also at WPC 60 production important to monitor the the contamination profile and the hygienic standards. In the main, the results show a reduction of bacterial contamination of nearly 100 % until the final products are reached (Tab.25).

Bacteria	Raw material	Final product	Reduction/ Increase
Total Bac. Count	$2.9 \times 10^8 \pm 4.8 \times 10^8$	$2.5 \times 10^5 \pm 5.6 \times 10^5$	99.9 % Red.
Enterobacter	$4.3 \times 10^6 \pm 1.4 \times 10^7$	0.0 ± 0.0	100 % Red.
Colioform bacteria	$2.6 \times 10^6 \pm 7.5 \times 10^6$	8.3 ± 28.9	99.9 % Red.
Enterococcus	$1.1 \times 10^6 \pm 3.1 \times 10^6$	0.0 ± 0.0	100 % Red.
Bacillus cereus	39.6 ± 134.8	11.7 ± 13.3	70.5 % Red.
Yeast	$1.8 \times 10^4 \pm 5.6 \times 10^4$	0.8 ± 2.9	99.9 % Red.
Therm.Spores	200 ± 504.3	208.3 ± 460.1	4.2 % Inc.
Therm. Bacteria	400 ± 812.4	241.7 ± 475.67	39.6 % Red.

Tab.25: Bacterial counts (average in cfu) and standard deviation (cfu) in raw material/final products of WPC 60 and their reduction/increase in percent

The reduction of total bacteria count amounts to 99.9 %, but the effective value is still high in the final products (Tab.25). *Enterobacter, Coliform* bacteria, *Enterococcus* and yeast contamination could be almost eliminated during the drying process (Fig.33). *Bacillus cereus* contamination is already not significant in raw product samples and in final products the counts of 11.6 cfu is a minimal value. The counts of thermophilic spores rise during the production, but just for 4 % and the contamination with thermophilic spores declined to about 241 colonies, which equates a reduction of 39.6 %.

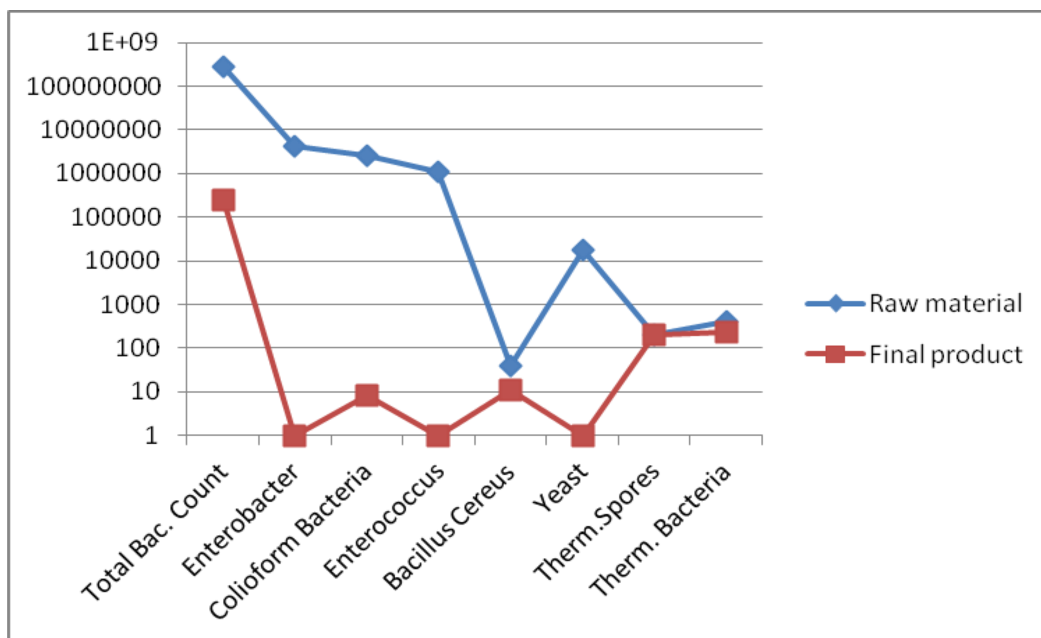


Fig.33: Comparison of the bacterial profile of WPC 60 raw material and final product

Bacteria	Spring	Summer	Fall/Winter
Total Bac. Count	$4.5 \times 10^5 \pm 8.3 \times 10^5$	$666.7 \pm 1.2 \times 10^3$	$2.3 \times 10^5 \pm 5.1 \times 10^5$
Enterobacter	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Colioform bacteria	0.0 ± 0.0	33.3 ± 57.7	0.0 ± 0.0
Enterococcus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Bacillus cereus	15.0 ± 19.1	6.7 ± 5.8	12.0 ± 13.0
Yeast	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 4.5
Therm.Spores	0.0 ± 0.0	166.7 ± 152.8	400.0 ± 692.8
Therm. Bacteria	75.0 ± 150.0	0.0 ± 0.0	520.0 ± 661.1

Tab.26: Comparison of bacterial profile of WPC 60 final product at different seasons (average + standard deviation in cfu)

The bacterial profile differs in a large way at the different seasons. Fall and winter results are nearly equal, so it is summed up in winter/fall results (Tab.26, Fig.34). Total bacteria count is at its minimum at summer, the highest contamination was detected in spring. *Enterobacter* and *Enterococcus* could not be detected at all in WPC 60 final products. In contrast, *Coliform* bacteria count is at its maximum at summer, but also in this case the count of 33 cfu in summer is very low. The profile of *Bacillus cereus* and yeast does not show large variation during the seasons, but is always at a low level. There are no

thermophilic spores detected at spring, but at summer the counts rise up to 166 colonies and in winter/fall, the contamination comes to its maximum with about 400 colonies. Also, the thermophilic bacteria have their maximal contamination at winter/fall, in spring the counts are at a low level and in summer no contamination could be detected (Fig.34, Tab.26).

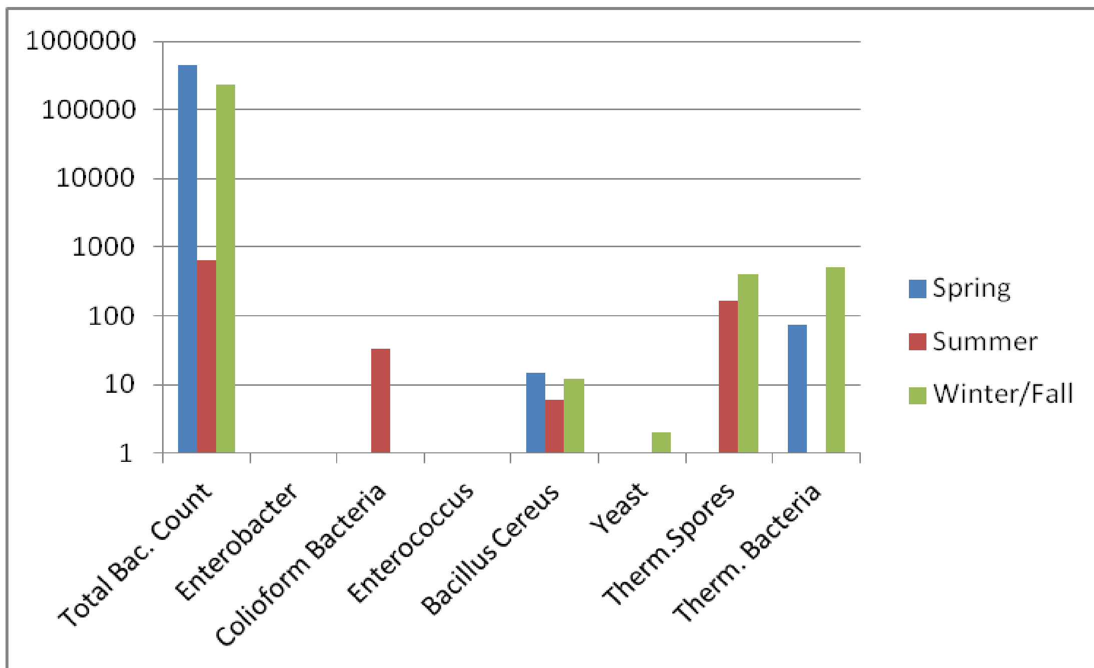


Fig.34: Bacterial profile of WPC 60 final product at different seasons

The contamination with *Cronobacter sakazakii* does not show a wide variation during the seasons. There are 4 counts (33.3 %) in winter/fall, 5 (41.7 %) in summer and 3 (25 %) in spring.

3.5 Results of WPC 80

In case of WPC 80 115 (n) samples were investigated. These samples include different raw material and the resulting final products as well as the different operating levels. At WPC 80 production the production levels 3, 5, 7, 1 start and 1 end were investigated. The distribution of the WPC 80 samples is shown in Tab. 27. The major part of the investigated samples was raw material; about 15 % of the samples were taken from final products. The other samples were taken after each production step; the major part was level 5.

Distribution of samples		
Production step	Quantity (n)	Percentage
raw material	23	20.0 %
3	15	13.0 %
5	21	18.3 %
7	18	15.7 %
1 start	11	9.6 %
1 end	10	8.7 %
final product	17	14.8 %

Tab.27: Distribution of samples of WPC 80

There are 15 samples (13 %) tested *Cronobacter sakazakii* positive. One of these positive tested samples was a final product; all the others were raw material.

3.5.1 **Contamination of the Production Levels during WPC 80 Production**

The samples of each production step were taken at the end of the production level. In case of WPC 80 operation levels 3, 5, 7, 1 start and 1 end were taken. The different bacteria counts including average and standard aviation are shown in Tab.28 and 29 and are graphically represented in Fig.35 and Fig.36.

Bacteria	Level 3	Level 5	Level 7
Total Bac. Count	$2.6 \times 10^8 \pm 4.2 \times 10^8$	$1.4 \times 10^8 \pm 2.1 \times 10^8$	$8.4 \times 10^7 \pm 1.1 \times 10^8$
Enterobacter	$2.5 \times 10^7 \pm 4.4 \times 10^7$	$1.0 \times 10^7 \pm 2.2 \times 10^7$	$1.1 \times 10^7 \pm 4.0 \times 10^7$
Colioform bacteria	$3.1 \times 10^7 \pm 5.2 \times 10^7$	$9.7 \times 10^6 \pm 2.0 \times 10^7$	$1.2 \times 10^7 \pm 4.2 \times 10^7$
Enterococcus	$6.5 \times 10^7 \pm 2.4 \times 10^8$	$4.5 \times 10^7 \pm 1.5 \times 10^8$	$3.6 \times 10^6 \pm 1.1 \times 10^7$
Bacillus cereus	$8.3 \times 10^4 \pm 3.1 \times 10^5$	$9.1 \times 10^4 \pm 2.7 \times 10^5$	$8.5 \times 10^3 \pm 2.4 \times 10^4$
Yeast	$2.5 \times 10^4 \pm 7.5 \times 10^4$	$4.3 \times 10^4 \pm 1.2 \times 10^5$	$3.9 \times 10^3 \pm 1.3 \times 10^4$
Therm.Spores	60.0 ± 124.2	323.8 ± 829.4	$3.2 \times 10^4 \pm 8.1 \times 10^4$
Therm. Bacteria	$2.0 \times 10^3 \pm 7.7 \times 10^3$	$3.6 \times 10^3 \pm 8.1 \times 10^3$	$5.0 \times 10^4 \pm 1.2 \times 10^5$

Tab.28: Bacterial counts (average and standard deviation in cfu) of operating levels 3, 5 and 7 of WPC 80

The operating level 3 is the first one during the production and therefor the total bacteria count, *Enterobacter* and *Coliform* bacteria are at this level at its maximum. Level 5 and 7 are a bit lower, but in general, the counts do not decrease in a significant way. The same could be decribed for *Enterococcus*, *Bacillus cereus* and yeast; there the counts are nearly the same at level 3 and 5, but decrease about tenfold at level 7 (Tab.28).
In contrast thermophilic spores and thermophilic bacteria rise about tenfold per each production level. The counts start at level 3 with 60 respectively 2006 cfu and rise during the production until counts of about 32 000 and 47 000 cfu are reached. This means an increase of more than 53 000 % (Tab.28, Fig.35).

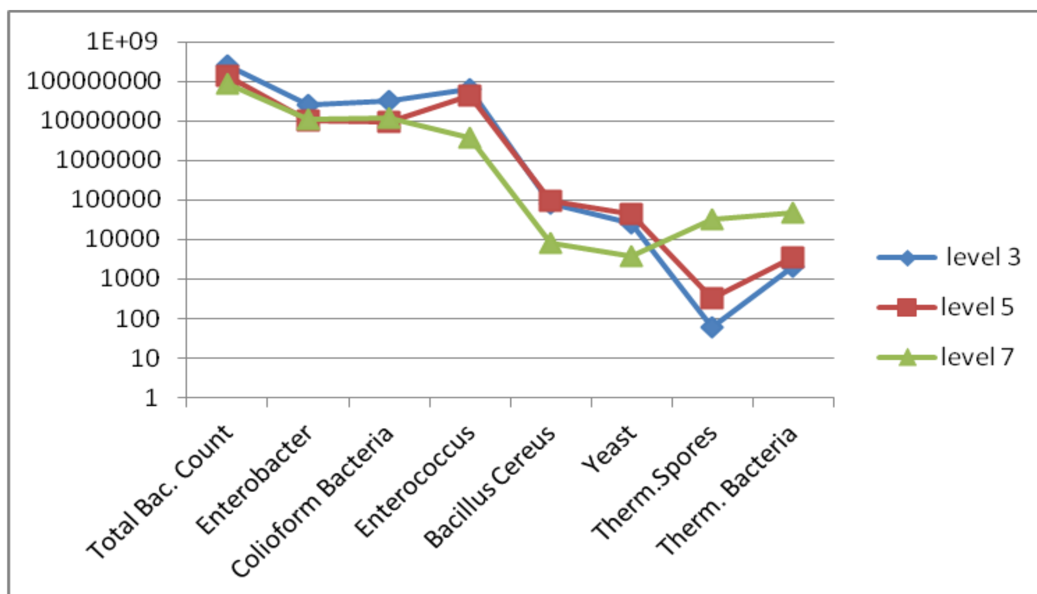


Fig.35: Bacterial profile of WPC 80 at operation levels 3, 5 and 7

Bacteria	1 Start	1 End
Total Bac. Count	$1.8 \times 10^7 \pm 5.7 \times 10^7$	$2.3 \times 10^8 \pm 4.5 \times 10^8$
Enterobacter	$3.6 \times 10^6 \pm 1.2 \times 10^7$	$8.2 \times 10^4 \pm 2.5 \times 10^5$
Colioform bacteria	$5.2 \times 10^6 \pm 1.7 \times 10^7$	$1.2 \times 10^5 \pm 2.8 \times 10^5$
Enterococcus	$7.4 \times 10^4 \pm 2.4 \times 10^5$	240.0 ± 447.7
Bacillus cereus	$4.7 \times 10^4 \pm 1.6 \times 10^5$	1.0 ± 3.2
Yeast	$1.1 \times 10^5 \pm 3.6 \times 10^5$	$6.7 \times 10^4 \pm 2.1 \times 10^5$
Therm.Spores	45.5 ± 103.6	20.0 ± 42.2
Therm. Bacteria	236.4 ± 783.9	10.0 ± 31.6

Tab.29: Bacterial count (average and standard aviation in cfu) of production levels 1 start and 1 end at WPC 80 production

The total bacteria count is at level 1 end more than 12-fold higher than at 1 start. In case of *Enterobacteria, Coliform* bacteria, *Enterococcus* and *Bacillus cereus* the counts of 1 end are much lower, but *Enterobacteria* and *Coliform* bacteria contamination is still at a high level at 1 end. The contamination with yeast is nearly bisected until operation level 1 end is reached. Thermophilic spores and bacteria at level 1 start low, but at level 1 end the counts are insignificant (Tab.29, Fig.36).

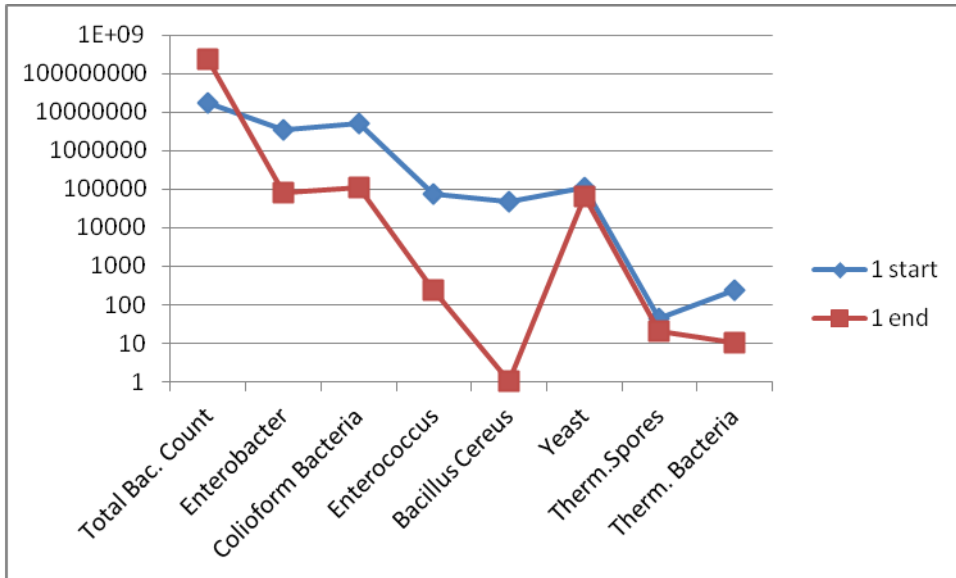


Fig.36: Bacteria contamination at levels 1 start and 1 end at WPC 80 production

3.5.2 Comparison of Raw Material and Final Product of WPC 80 Production

The heating steps during the production cause, that most of the contamination is eliminated until the final product is ready for packaging.

Bacteria	Raw material	Final product	Reduction/Increase
Total Bac. Count	$1.5 \times 10^{8} \pm 2.4 \times 10^{8}$	$2.9 \times 10^{6} \pm 1.0 \times 10^{7}$	98.0 % Red.
Enterobacter	$8.0 \times 10^{7} \pm 2.9 \times 10^{8}$	35.3 ± 105.7	99.9 % Red.
Colioform bacteria	$1.1 \times 10^{8} \pm 4.0 \times 10^{8}$	17.7 ± 72.8	99.9 % Red.
Enterococcus	$2.5 \times 10^{6} \pm 1.1 \times 10^{7}$	$7.3 \times 10^{5} \pm 2.9 \times 10^{6}$	70.6 % Red.
Bacillus cereus	$7.1 \times 10^{3} \pm 3.3 \times 10^{4}$	28.8 ± 46.8	99.6 % Red.
Yeast	$6.0 \times 10^{4} \pm 1.9 \times 10^{5}$	2.9 ± 7.7	99.9 % Red.
Therm.Spores	547.8 ± 2432.1	$1.0 \times 10^{5} \pm 4.1 \times 10^{5}$	18265.7 % Inc.
Therm. Bacteria	$7.4 \times 10^{3} \pm 3.1 \times 10^{4}$	$1.1 \times 10^{5} \pm 4.4 \times 10^{5}$	1425.6 % Inc.

Tab.30: Comparison of bacterial contamination (average and standard deviation in cfu) of raw material and final product at WPC 80 prodcution

Total bacteria count is still at a high level at the final product, but in fact 98.0 % of the contamination is eliminated (Tab.30). In case of *Enterobacter* and *Coliorm* bacteria the counts are very high at raw material, but the final products are nearly free of contamination; the production process killed about 99 % of the bacteria. *Enterococcus* counts could be reduced to less than 30 % of the level counted at raw material. *Bacillus cereus* and yeast contamination also could be nearly completely eliminated; the counts of 28 cfu and 3 cfu are insignificant low at the end of the production. Another picture shows the counts of thermophilic bacteria and spores; in these cases the counts rise during the production to a high level. The contamination increased during the production process by more than 1500 % (Tab.30, Fig.37).

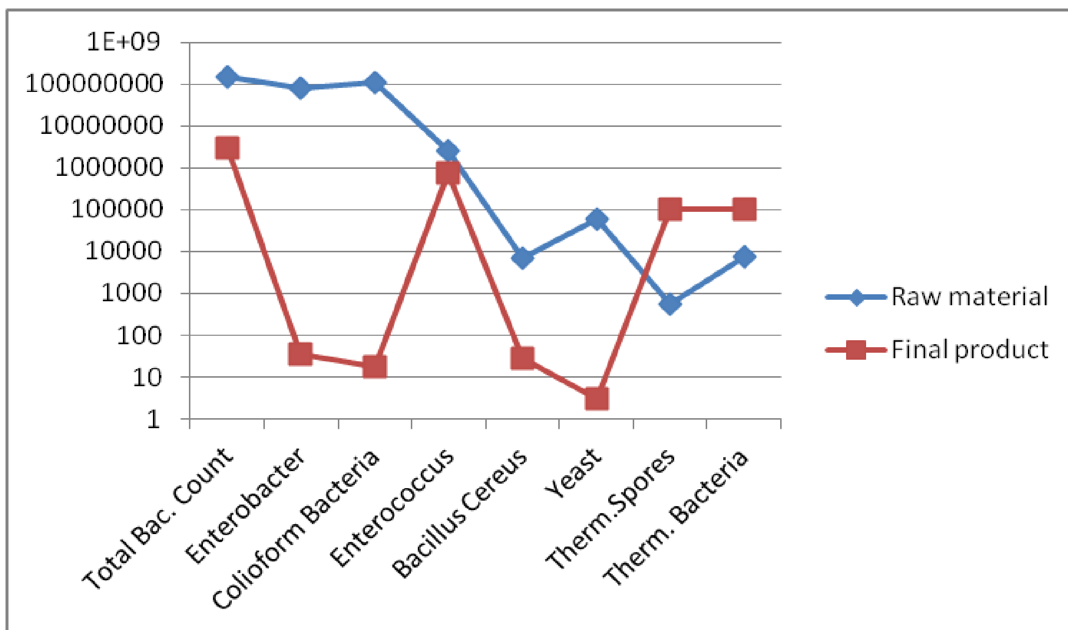


Fig.37: Bacterial profile of WPC 80 raw material and final product

The results of winter and fall do not differ in a large way from one antoher and so the results are summed up in fall/winter (Tab.31 and Fig. 38).

Bacteria	Spring	Summer	Fall/Winter
Total Bac. Count	$6.0 \times 10^5 \pm 1.4 \times 10^6$	$6.2 \times 10^6 \pm 1.6 \times 10^7$	$4.3 \times 10^5 \pm 9.7 \times 10^5$
Enterobacter	0.0 ± 0.0	28.6 ± 75.6	100.0 ± 200.0
Colioform Bacteria	0.0 ± 0.0	0.0 ± 0.0	75.0 ± 150.0
Enterococcus	$1.3 \times 10^4 \pm 3.2 \times 10^4$	$1.8 \times 10^6 \pm 4.5 \times 10^6$	0.0 ± 0.0
Bacillus Cereus	16.7 ± 19.7	32.9 ± 65.8	40.0 ± 43.0
Yeast	6.7 ± 12.1	1.4 ± 3.8	0.0 ± 0.0
Therm.Spores	$2.8 \times 10^5 \pm 6.9 \times 10^5$	42.9 ± 78.7	25.0 ± 50.0
Therm. Bacteria	$3.0 \times 10^5 \pm 7.3 \times 10^5$	100.0 ± 264.6	200.0 ± 182.6

Tab.31: Comparison of bacterial contamination of WPC 80 final product at different seasons (average and standard deviation in cfu)

As shown in Tab.31 and Fig.38 the bacterial contaminations fluctuate between the different seasons. In summer the contamination with *Enterococcus* and also the total bacteria count dominate the final products (Fig.38). Although, there seems to be very low contamination with *Coliform* bacteria and yeast in summer. At spring the total bacteria is still at a high level, but also *Enterococcus*, thermophilic spores and bacteria are the major problems. Otherwise there are no *Enterobacter*, *Coliform* bacteria and very low yeast and *Bacillus cereus* count (Tab.31). In fall/winter, except the total bacteria count, the contamination is in general very low (Tab.31). *Enterococcus* and yeast are not detected in fall/winter and the highest counts are thermophilic bacteria with a contamination of 200 cfu (Tab.31, Fig. 38).

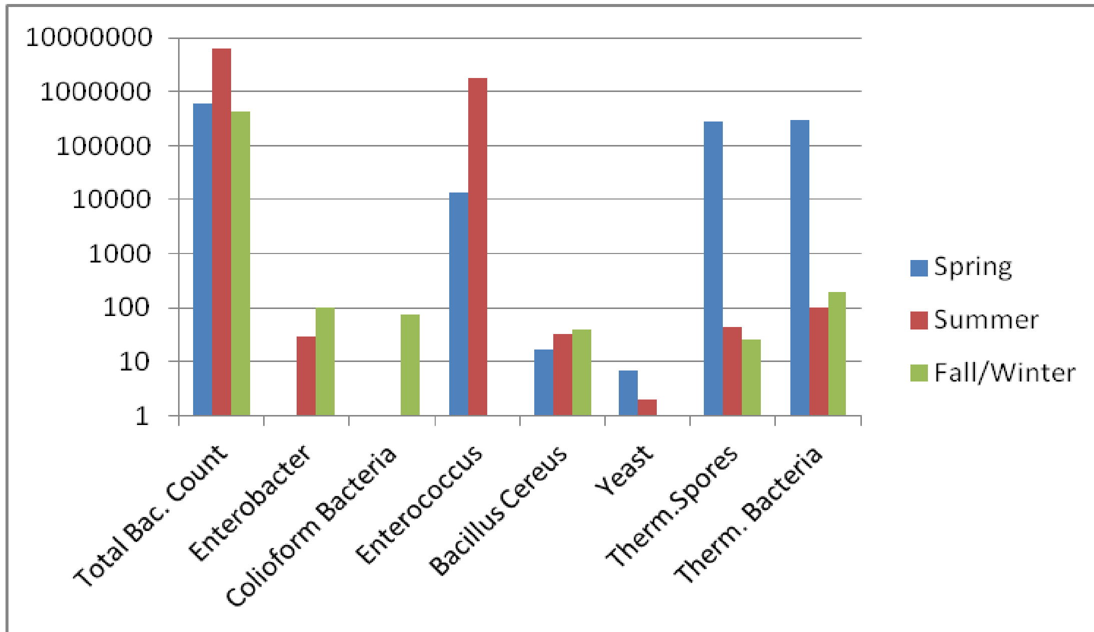


Fig.38: Bacterial contamination of WPC 80 final products at different seasons

53 % of the *Cronobacter sakazakii* cases are in summer (n=8), in spring and fall/winter there are each 2 cases of *Cronobacter sakazakii* (23 %).

4 Discussion

4.1 *Cronobacter sakazakii* in Milk Products

Cronobacter sakazakii is an opportunistic food borne pathogen and because of a heteropolysaccharide capsule it is able to survive throughthougt the long shelf-life of PIF. [4, 5, 14, 22, 23] The prevention of a contamination with *Cronobacter sakazakii* is also complicated by the fact that the bacterium is able to create or attach to biofilms, which support the resistance to disinfectants and cleaning agents. [14, 22, 23] The optimum growth temperature is 39 °C, but there are species of this genus, which can grow at temperatures between 6-47 °C. [14] These facts make it difficult for food producer to prevent contaminations with *Cronobacter*.

In this study the contamination with *Cronobacter sakazakii* has been investigated in raw milk and the resulting final products of low-fat milk, unskimmed milk, WPC 35, WPC 60 and WPC 80. The raw material for unsikimmed milk and low-fat milk seem to have a higher quality, because there could be detected no contamination with *Cronobacter sakazakii*. In case of WPC 35 47 % (n=23) of raw material was *Cronobacter* positive, WPC 60 raw material samples were positive in 12.6 % (n=12) of cases and WPC 80 show a 13 % (n=14) contamination of the investigated raw material. There was no contamination detected during the production levels, but in one case, it was found a contamination in WPC 80 final product.

The raw material quality seems to be very important to avoid contamination with such a dangerous bacteria like *Cronobacter sakzakii*. The facts, that it is relatively heat stable and the prevention is difficult because of the formation of biofilms, make it important for food producer to ensure a high quality raw material. The pasteurization step, which is perfomed at the dairies, seems to be insufficient for removing all *Cronobacter* contamination. [14, 22, 23]

Another problem could occure when the contaminated material comes in contact with the manufacturing equipment or with the production staff. The contamination could be distributed at the production hall and cause much more contaminated products or even

events of illnesses. The fact, that the biofilm of *Cronobacter sakazakii* support resistance to cleaning agents, it is difficult to get rid of this contamination. [22, 23]
In this study the results show, that the hygienic control makes an impact, but the raw material quality should be optimized to avoid *Cronobacter sakazakii* in general.

4.2 Raw Material Quality

The raw material for all products is pasteurized bovine milk, which is delivered from dairies. During the filling and delivery it is important for the milk quality to ensure a constant temperature of 4 °C. [269] Milk quality is affected by various contaminations and for this case, proper handling is very important, particularly because milk is a perishable material. [270]
In this study the different raw material samples for each final product have been investigated. In case of unskimmed milk and low-fat milk the results show a massive contamination with *Enterobacter, Coliform* bacteria, *Enterococcus*, yeast and as a result also the total bacteria count is very high. The raw material of the WPC products seems to be even worse than the raw material of the milk products, because the counts are, in average, tenfold higher than in unskimmed and low-fat milk. This seems to be the result of low hygienic standards at raw milk processing. The high results of *Coliform* bacteria and *Enterobacter* are an indication of post-pasteurization contamination, because they are not able to survive this heating process. [49, 55] *Coliform* bacteria are also used in the USA to observe the hygienic quality of dairy food. [48] Therefore a high number of these bacteria is a sign of unsanitary production or improper handling of milk or milk utensils. [51] The high number of yeast could lead to spoilage, which also reduces the final product quality and as a consequence the profit.
In many cases the standard deviation is much higher than the average of cfu, which is caused by the variation of raw material quality. There are huge differences in bacterial counts between the different samples. A possible explanation for this phenomenon could be the different suppliers, which pursue different hygienic standards and therefore sell different raw material quality.
The raw material quality depends on many different factors, such as the pasteurization step itself, the hygienic standards at the dairies the raw material comes from and the hygienic standards at delivery. The temperature at the whole transport, at the filling and

storage in cooling tanks always has to be at 4 °C to guarantee best raw material quality. [269, 270] Also important for better raw material quality is the sterile handling during the production process and filling. The sample taking should always take place under sterile conditions to ensure representative results.
If the lower price of low quality raw material is a real benefit or cause more costs during the production is one of the most difficult issues for food producer.

4.3 Production Steps

The production of the final products required different production steps, which are investigated in this study. After each operating level (Tab.5) samples have been taken and investigated. The levels 3, 5 and 7 are part of each spray drying process, no matter which final product is in progress. The levels 1 start and 1 end are part of the production of WPC; the production levels 5a and 7a are part of unskimmed and low-fat milk as well as WPC 35 and 60.
After a preheating step, the pasteurized raw material is stored in cooling tanks until the production starts. The fact, that the raw material is pasteurized and preheated before storage could be expected to lead to minimal total bacterial counts during the production.
Level 3 describes the process vessel after evaporation at the production process. The heating temperature at this point for unskimmed and low-fat milk is 72 °C, for WPC products it is 57 °C.
Level 5 samples are taken at the feed balance tank at the batch beginning, level 7 describes the same heating step, but at batch end. The temperature for this operating level is 66 °C for WPC 35 and 60, 72 °C for WPC 35 and for unskimmed and low-fat milk it is 74 °C (Tab.5).
The production level 5a and 7a are taken after the sampling valve after the heat holding tube, whereby 5a describes batch beginning and 7a batch end.
Operating level 1 start and 1 end describe the point after preheating at heat exchange plate at the beginning of a batch and at the end.

4.3.1 **Production Levels for Unskimmed and Low-Fat Milk**

The heating step 3, step after evaporation, shows the best results for all bacteria count for unskimmed milk, the counts rise during the production partly hundredfold until level 7 is reached. This means the counts are at a lower level at the production start and then rise until the batch end. At level 5, batch start, the counts for yeast are not detectable and at batch end the counts are at a ten thousandfold level. The same is true for *Bacillus cereus*, *Enterococcus, Coliform* bacteria and *Enterobacter.* A possible explanation could be the time of warm-keeping, which gives the bacteria time to grow. That could be the reason for lower counts at batch beginning and a rise until batch end. At level 5a and 7a, which means after the heat holding tube at batch beginning and end, the results show that the heat resistant bacteria have an advantage over the other bacterial species. The thermophilic bacteria and spores are much higher at batch end than at batch beginning. The storage at high temperatures kills the other bacteria and lead to a growth of thermophic bacteria. Also *Bacillus cereus* seems to survive the high temperatures during storage, the fact that some strains grow at 50 °C could be an advantage for the survival. (350) Maybe the heating temperature is not held constantly or the bulk is not heated homogeneously. Reasons which would indicate the hypothesis are that the results for low-fat milk show the same picture. The results for low-fat milk at level 3 are much higher than for unskimmed milk, at level 5 the counts dercease tenfold, but at level 7, the batch end, the counts are nearly the same as at level 3. Only the counts of *Bacillus cereus* are at a constant low level during the whole process, all the other bacteria seem to grow at a large number. In case of low-fat milk, it is the same as at unskimmed milk, the thermophilic spores and bacteria seem to grow during the heating and warm-keeping steps at a higher percentage as the other heat-unstable bacteria. The levels 5a and 7a, which are the levels after the heat holding tube, show low levels of contamination. The only exceptions are also in this case the thermophilic bacteria and spores, which seem to grow during the warm-keeping process. At batch beginning, step 5a, the counts are a low level but rise during the production until a tenfold contamination could be detected. This also could be a problem of insufficient heating at the beginning, which enables the thermophilic bacteria and bacterial spores to survive and the fact that the heat-unstable bacteria were killed at this process; they do not have any rivalry for the nutrients they need for growth. At good way to avoid these problems could be the

monitoring of the raw material, if less bacteria contaminate the raw material, less bacteria could grow during the production. Also a precise hygienic monitoring could help to avoid this extent of contamination. Milk and milk products are a high quality breeding ground for microbial contamination, but at the final product the bacterial growth is widely inhibited because of the drying process, except the growth of thermophilic bacteria. They are still able to grow and have significant consequences when the critical values are surpassed, because they downgrade the product. These bacteria could form extremely heat-stable spores which is one of the most important factors for the drying process and pasteurization. (284)

4.3.2 **Production Steps for WPC**

After the delivery with a truck samples were taken and afterwards the raw material is preheated at a plate heat exchange. This practice describes level 1 start, which means the point after delivery and 1 end, which describes the point after preheating. The temperature of 66 °C at the preheating is for WPC, which means all types of protein concentration, the same. The counts of total bacteria at level 1 start are for all types of WPC very high, which means after the long transport the bacteria had time to grow. This is traced back to the fact that the counts of *Enterobacter, Coliform* bacteria and *Enterococcus* are high after delivery. The contamination with thermophilic spores is for all three WPC types at a reasonably low level, which means the temperature at pasteurization and transport was not high enough for spore-formation. At level 1 end, after the preheating step, the counts for *Enterobacter, Enterococcus* and *Coliform* bacteria decline about hundredfold.
However, there are differences between the WPC raw materials at level 1 start. In case of WPC 35 the counts for yeast and thermophilic bacteria are high and in contrast the contamination with *Bacillus cereus* is insignificant. After the preheating step the counts for yeast seem to be stable, there is no significant improvement. Maybe the contamination with yeast is caused by the environment at the point of sample taking. The contamination with thermophilic bacteria is tenfold higher after the preheating step, which make sense because of their ability to grow at high temperatures.
WPC 60 shows another profile of contamination in relation to yeast. The contamination is at a constant low level and at level 1 end a further decline is detected. Also the counts for

thermophilic bacteria and spores are insignificant, which means the heating step and the sterile sample taking performed its task.
In case of WPC 80, the contamination with *Bacillus cereus* is at the delivery at a high level but after the preheating step the contamination could no longer be detected. The counts for thermophilic bacteria and spores are constant low, respectively they could be reduced by the heating. Noticable is the high contamination with yeast, which could be caused by the environment at the filling at the dairy or it was a dusty environment, caused by agriculuture at the sample taking point.
After the preheating step and the storage the pduction starts. Level 3 is the point after evaporation; level 5 and 7 are located after the feed balance tank, at batch beginning level 5 and 7 at batch end. After the evaporation the contamination in general, but also in special with *Enterobacter, Coliform* bacteria and *Enterococcus* is high. *Bacillus cereus* contamination plays a role at WPC 80 and WPC 35 production at level 3. Yeast is a problem after the evaporation only at WPC 80 production. The counts of thermophilic bacteria are at level 3 are at a relatively high level for WPC 35 and 80, for WPC 60 the counts are nearly not detectable. Spores are low at all three WPC types. The evaporation step causes the concentration of WPC, which means water is removed. This seems to cause a rise of bacteria contamination, a concentration of the bacteria causing the contamination. The only way to protect the material from such a high bacterial count is to try to remove them before the evaporation step. The temperature of 57 °C seems to be not enough to remove contamination at this heating step. Although *Enterobacteria, Coliform* bacteria and *Enterococcus* are not heat stable, they survive the thickening temperature at a high number. Also level 5 and 7 show a high contamination with nearly all types of bacteria, except thermophilic spores. The thermophilic spores rise more than tenfold until the batch end is reached. This is the same effect as decribed for unskimmed and low-fat milk. In general the contamination decline a bit until level 7 is reached, maybe the long heating step during the production inhibit the bacterial growth a little. Also could be that residues, which contaminate the production pipelines are mostly removed until batch end.

In case of WPC 80 there are no samples taken at 5a and 7a.
At level 5a, the beginning of the batch after heat holding tube, the samples show a high contamination with total bacteria count, which do not change in counts until batch end. Noticeable is the fact, that in WPC 35 the counts of *Enterobacter, Coliform* bacteria and

Enterococcus decline at level 7a, but at WPC 60 *Enterobacter* and *Coliform* bacteria are not detected at level 5a, but rise thousandfold at level 7a. Just *Enterococcus* counts at WPC 60 do not change during the production, they are stable but at a high level. The contamination with thermophilic bacteria and spores is at level 5a low or rather not detected and rises at level 7a. Especially for WPC 35 the counts of thermophilic bacteria rise tenfold during batch end is reached. It seems that WPC 60 is a good nutrient medium for *Enterobacter* and *Coliform* bacteria, so they could grow at the heat holding tube and rise until the samples of batch end were taken. At this point of production yeast do not play a role, the environment of the heat holding tube seems to inhibit their growth. As described before the critical factor is a good hygienic monitoring to prevent contaminations. As seen at the results the standard deviation is equal or even higher as the average of the counts, which means the individual results of each production line differ at a great extent from one another. A proper incoming control of raw material could help to creat a stable high quality product and could inhibit the contamination of production equipment or pipelines.

4.4 Comparison of Different Seasons

In this study final product samples were taken during the seasons to find out if the different temperatures influence the bacterial contamination.The counts of fall and winter were nearly equal and so the results are summed up to fall/winter. *Enterobacteria* and *Coliform* bacteria contamination was not detected at all at unskimmed and low-fat milk as well as at WPC 35. WPC 60 shows counts of *Coliform* bacteria at summer and WPC 80 at fall/winter. In case of WPC 80 there are detected *Enterobacteria* as well as in summer and fall/winter. *Enterococcus* shows the highest counts at summer. This was verified at all final products, which means that this bacterium is at the warmer season a huge problem for food production. The counts rise and contaminate the final products. In the last years the infection with these bacteria became more and more serious because of the multiple resistances of some *Enterococci* strains and the resulting difficulties threatening these infections. [136] Researchers now try to find out whether *Enterococcus* can be transmitted by food and cause infections. [160]

The same results seem to hold for *Bacillus cereus,* the higher temperatures at spring and summer seem to support the growth. An exception is the result of WPC 80 final product,

which rises at fall/winter a bit more than at summer or spring. *Bacillus cereus* is one of the most common food pathogens. It is found in about 25 % of food products tested and frequently led to food poisoning outbreaks. [186] A possible way of contamination of milk products could be cows, which get contaminated feed and excrete spores, which contaminate the raw milk. [179,186]

WPC 80 final products are also the only ones, which show a contamination with yeast. Most of the counts are detected at spring, but a few also at summer. An explanation could be a dusty environment at the filling or sample taking. On the other hand, this kind of contamination is just detected at WPC 80, which maybe means the high protein concentration support the growth of yeast at some way.

Thermophilic spores and bacteria seem to have always their season. It could be proved that the counts are higher at warm seasons like spring and summer, but they also could be found at fall/winter. This means that the surrounding temperatures namely influence the counts of the thermphilic bacteria and spores, but cold ambient temperatures do not inhibit the growth at all. The conclusion of the investigation should be that the seasons influence the growth of some bacteria, but a proper hygienic monitoring should always be obvious.

4.5 Final Products

In general the results of the final products are satisfactory. The not heat-stable bacterial growth decline, compared with the raw material, to nearly 1% of the contamination at production beginning. This means that the drying process is an effective way to create a high quality final product. On the other side, the counts of total bacteria are also too high at final products. Despite the heating steps the bacterial growth is not inhibited, there are still pathogen bacteria detected. The major problems are caused by thermophilic bacteria and spores. One of the spore forming bacteria is *Bacillus cereus,* which was also detectable at final products. If these bacteria surivive the heating steps they can sporulate and lead to serious infections. [186] To improve the final product quality food producers could focus on these spore-forming bacteria. They could try to ensure, that the raw material is not contaminated with this kind of bacteria, because it seems to be impossible to get rid of them. The other bacterial contamination seems to vanish during the production process, so the hygienic monitoring performed.

The high standard deviation at all results show a wide range of raw material quality, which also influences the resulting final product. If the raw material quality could be improved, the food producer would have less trouble to produce contamination free final product. The drying process eliminates about 99 % of bacterial contamination, but the point is how high the counts are at the beginning.
All in all, a proper hygienic monitoring, cleaning and sterile handling prevent unnecessary contaminations at the factory and support the production of high quality dryed milk products.

List of references

1. www.gesund.at/f/lebensmittelvergiftung

2. Volker Krömker, Rupert M. Bruckmaier, 2007: Kurzes Lehrbuch Milchkunde und Milchhygiene, Parey.

3. H.Weber (Hrsg.), 2006: Milch und Milchprodukte, Behr`s Verlag, 2.Auflage.

4. Nazarowec-White M, Farber JM: Thermal resistance of *Enterobacter sakazakii* in reconstituted dried infant formula, LettApplMicrobiol1997a, 24:9–13

5. Iversen C, Mullane N, McCardell BD, Tall BD, Lehner A, Fanning S, Stephan R and Joosten H, 2008: *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii* and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. 6 nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1 and of 8 three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov., International Journal of Systematic and Evolutionary Microbiology 58, 1442–1447.

6. Aldova E, Malamou-Ladas E, Kostalos C, Staikou A and Koutsia C, 1984: A fatal case of sepsis in a premature newborn associated with *Enterobacter sakazakii* bacteremia, *Acta Microbiologica Hellaa* **29,** 402–407.

7.Willis J, Robinson JE: *Enterobacter sakazakii* meningitis in neonates, *Pediatr Infect Dis J*1988, *7*: 196–199.

8. Van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A, Lauwers S: Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *J Clin Microbiol* 2001, 39: 293–297.

9. Muytjens HL, Zanen CH, Sonderkamp JH, Kollee LA, Wachsmuth K, Farmer JJ: Analysis of Eight Cases of Neonatal Meningitis and Sepsis, *J ClinMicrobiol* 1983, 18 (1): 115-20.

10. Himelright I, Harris E, Lorch V and Anderson M: *Enterobacter sakazakii* infections associated with the use of powdered infant formula, Tennessee, *J Am Med Ass* 2002; 287: 2204–05.

11. Pagotto FJ, Farber JM: *Cronobacter* spp. (*Enterobacter sakazakii*): Advice,policy and research in Canada, *Intl J Food Microbiol,* 2009; 136 (2): 238-245.

12. Kim H, Ryu J-H, Beuchat LR: Effectiveness of disinfectants in killing *Enterobacter sakazakii* in suspension, dried on the surface of stainless steel and in a biofilm, *Appl Environ Microbiol* 2007; 73: 1256–65.

13. Kahn AA, Jones RA, Cerniglia CE: Rapid method for the detection of genetically engineered microorganisms by polymerase chain reaction from soil and sediments, *J Ind Microbiol Biotech* 1998; 20: 90-94.

14. Iversen C, Forsythe S: Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends Food Sci Technol* 2003; 14 (11): 443-454.

15. Kim H, Beuchat LR: Survival and growth of *Enterobacter sakazakii* storage temperature on fresh cut fruits and vegetables and in unpasteurized juices as affected by storage temperature, *J Food Protec*2005; 68 (12): 2541-52.

16. Beuchat LR, Kim H, Gurtler JB, Lin LC, Ryu JH, Richards GM: *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation, *Intl J Food Microbiol* 2009*;* 136 (2): 204-13.

17. Md. Fakruddin, Md. Mizanur R, Monzur MA, Md. Mahfuzul H: *Cronobacter sakazakii* (*Enterobacter sakazakii*): An Emerging Food borne Pathogen, *International Journal of Biomedical And Advance Research* ISSN: 2229-3809.

18. Iversen C, Lehner A, Mullane N, Bidlas E, Cleenwerck I, Marugg J *et al*.: The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb.nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomo species, *BMC Evol Biol* 2007; 64 (7): 1471-2148.

19. Iversen C, Druggan P, Forsythe S: A selective differential medium for *Enterobacter sakazakii*, a preliminary study, *Intl J Food Microbiol* 2004a; 96 (2): 133-139.

20. Farmer JJ, Asbury MA, Hickman FW, Brenner DJ: The *Enterobacteriaceae* Study Group. *Enterobacter sakazakii*, new species of *Enterobacteriaceae* isolated from clinical specimens, *Intl J Sys Bacteriol* 1980; 30: 569-84.

21. Beuchat LR, Kim H, Gurtler JB, Lin LC, Ryu JH, Richards GM: *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation, *Intl J Food Microbiol* 2009; 136 (2): 204-13.

22. Kim H, Ryu J-H, Beuchat LR: Attachment and biofilm formation by *Enterobacter sakazakiion* stainless steel and enteral feeding tubes. *Appl Environ Microbiol* 2006; 73: 5846–56.

23. Lehner A, Tasara T and Stephan R: 16S rRNA gene based analysis of *Enterobacter sakazakii* strains from different sources and development of a PCR assay for identification. *BMC Microbiology* 2004; 4:43.

24. Lehner A, Nitzsche S, Breeuwer P, Diep B, Thelen K, Stephan R: Comparison of two chromogenic media and evaluation of two molecular-based identification systems for *Enterobacter sakazakii* detection. *BMCMicrobiol*2006; *6*: 15.

25. Iversen C, Waddington M, Farmer JJ, Forsythe SJ: The biochemical differentiation of *Enterobacter sakazakii* genotypes. *BMC Microbiol* 2006; 6: 94.

26. Iversen C, Druggan P, Schumacher S, Lehner A, Feer C, Gschwend K *et al*.: Development of a novel screening method for the isolation of '*Cronobacter*' spp. [*Enterobacter sakazakii*]. *Appl Environ Microbiol* 2008b; 74 (8), 2550-2553.

27. Pagotto FJ, Farber JM: *Cronobacter* spp. (*Enterobacter sakazakii*): Advice,policy and research in Canada. *Intl J Food Microbiol* 2009; 136 (2): 238-245.

28. MacLean LL, Pagotto F, Farber JM, Perry MB: The structure of the Oantigen in the endotoxin of the emerging food pathogen *Cronobacter* (*Enterobacter*) *muytjensii* strain 3270. *Carbohydrate Res* 2009; 344 (5): 667-671.

29. Zogaj X, Bokranz W, Nimtz M, Romling U: Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract. *Infect Immun* 2003; 71:4151–8.

30. Mullane N, O'Gaora P, Nally JE, Iversen C, Whyte P, Wall PG, Fanning S: Molecular analysis of the *Enterobacter sakazakii* antigen gene locus. *App Environ Microbiol* 2008a; 74(12): 3783-94.

31. Farmer JJ, Asbury MA, Hickman FW, Brenner DJ: The *Enterobacteriaceae* Study Group. *Enterobacter sakazakii*, new species of *Enterobacteriaceae* isolated from clinical specimens. *Intl J Sys Bacteriol* 1980; 30: 569-84.

32. Lockwood DE, Kreger AS, Richardson SH: Detection of toxins produced by *Vibrio fluvialis. Infect Immun* 1982; *35*: 702-708.

33. Kandhai MC, Reij MW, Gorris LG, Guillaume-Gentil O, van Schothorst M: Occurrence of *Enterobacter sakazakii* in food production environments and households. Lancet 2004; 363: 39–40.

34. Kothary MH, McCardell BA, Frazar CD, Deer D, Tall BD: Characterization of the zinc-containing metalloprotease encoded by zpx and development of a species-specific detection method for *Enterobacter sakazakii*. *Appl Environ Microbiol* 2007; 73 (13): 4142-4151.
35. Raghav M, Aggarwal PK: Purification and characterisation of *Enterobacter sakazakii* enterotoxin. *Can J Microbiol* 2007; 53:750-755

36. Chenu JW, Cox JM. *Cronobacter* ("*Enterobacter sakazakii*"): current status and future prospects. *Let Appl Microbiol* 2009*;* 49 (2): 153-9.

37. Centers for Disease Control and Prevention (CDC): *Enterobacter sakazakii* infections associated with the use of powdered infant formula--Tennessee, 2001. *MMWR Morb Mortal Wkly Rep* 2002; 51 (14): 297–300.

38. Gurtler JB, Kornacki JL, Beuchat LR: *Enterobacter sakazakii:* a coliform of increased concern to infant health. *Intl J Food Microbiol* 2005; 104:1–34.5

39. Iversen C, Forsythe SJ. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula and related products. *Food Microbiol* 2004b; 21: 771–776.

40. Mullane N, Healy B, Meade J, Whyte P, Wall PG, Fanning S: Dissemination of *Cronobacter* spp. [*Enterobacter sakazakii*] in a powdered milk protein manufacturing facility. *App EnvironMicrobiol*2008b; 74(19): 5913-17.

41. European Food Safety Authority: Opinion of the Scientific Panel on Biological Hazards on a request from the Commission related to the microbiological risks in infant formulae and follow-on formulae. *EFSAJ* 2004; 13:1–34.

42. Dennison SK, Morris J: Multi resistant *Enterobacter sakazakii* wound infection in an adult, *Infect Med* 2002; 533–535.

43. Block C, Peleg O, Minster N, Bar-Oz B, Simhon A, Arad I, Shapiro M: Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. *European J Clin Microbiol Infect Dis* 2002; 21:613–16.

44. Muytjens HL, van der Ros, van der Repe J: Comparative studies of 13-Lactam Antibiotics. *Antimicrobial Agents Chemo* 1982; 21(6): 6925-934.

45. Muytjens HL, Kollee LAA: *Enterobacter sakazakii* meningitis in neonates: causative role in formula. *JPediat Infect Dis* 1990; 9 : 372–373.

46. Baron S, 1996: Medical Microbiolgy- 4th edition – Galveston (TX). University of Texas Medical Branch at Galveston.

47. Feng, P., S. D. Weagant, M. A. Grant, and W. Burkhardt, 2002: Enumeration of *Escherichia coli* and the coliform bacteria. Chapter in Bacteriological Analytical Manual. 8th ed. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD. Accessed May 20, 2015. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm.

48. C. J. Hervert, A. S. Alles, N. H. Martin, K. J. Boor, and M. Wiedmann: Evaluation of different methods to detect microbial hygiene indicators relevant in the dairy industry. 2016, THE AUTHORS. Published by FASS and Elsevier Inc. on behalf of the American Dairy Science Association®. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

49. Imhoff, J. 2005: Enterobacteriales. Pages 587–850 in Bergey's Manual of Systematic Bacteriology, ed. Springer, New York, NY.

50. Boor KJ, Brown DP, Murphy SC, Bandler DK, 1998: Microbial and chemical quality of raw milk in New York State, J. Dairy Sci., 81: 1743-1748.

51. EL-zubeir IEM, Ahmed MI, 2007: The hygienic quality of raw milk produced by some dairy farms in Khartoum-Sudan. J. Microbiol., 2: 988-991.

52. Murphy SC, Boor KJ, 2003: Basic dairy Bacteriology. Microbiological quality defects in fluid milk products: The evaluation of shelf life. Cornell University, Ithaca, NY.

53. Wessels D, PJ Jooste and J F Mostert, 1989: Psychrotrophic, proteolytic and lipolytic properties of Enterobacteriaceae isolated from milk and dairy products. Int. J. Food Microbiol. 9:79–83.
54. Martin NH, NR Carey, SC Murphy, M Wiedmann and KJ Boor, 2012: A decade of improvement: New York State fluid milk quality. J. Dairy Sci. 95:7384–7390.

55. Perez NF, Zappa V: Mastite em vacas leiteira: revisão de literatura. Rev Cient Eletron Med Vet. 2011; 16: 1–28.

56. Jiang Q, Yang Y, Xin K: Microbial diversity analysis of subclinical mastitis in dairy cattle in Northeast China. Afr J Microbiol Res. 2015:687–694.

57. Hogan JS, Gonzalez RN, Harmon RJ: National Mastitis Council, Inc.; Madison, WI, USA: 1999. Laboratory Handbook on Bovine Mastitis.

58. Hogan JS, Weiss WP, Smith KL, Todhunter DA, Schoenberger PS, Sordillo LM: Effects of an *Escherichia coli* J5 vaccine on mild clinical coliform mastitis. J Dairy Sci. 1995;78:285–290.

59. Bosco KJ, Kaddu-Mulindwa DH, Asiimwe BB, 2012: Antimicrobial drug resistance and plasmid profiles of Salmonella isolates from humans and foods of animal origin in Uganda. Adv. Infect. Dis. 2, 151–155. 10.4236/aid.2012.24025

60. Fleming LR, Bolzan DN, Nascimento JS, 2010: Antimicrobial substances produced by coliform strains active against foodborne pathogens. Foodborne Pathog. Dis. 7, 243–247. 10.1089/fpd.2009.0333

61. Murphy CP, Fajt VR, Scott HM, Foster MJ, Wickwire P, McEwen SA, 2016: Scoping review to identify potential non-antimicrobial interventions to mitigate antimicrobial

resistance in commensal enteric bacteria in North American cattle production systems. Epidemiol. Infect. 144, 1–18. 10.1017/S0950268815000722

62. Rolain J., 2013: Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. Front. Microbiol. 4:173. 10.3389/fmicb.2013.00173

63. Grotiuz G, Sirok A, Gadea P, Varela G, Schelotto F, 2006: Shiga toxin 2-producing Acinetobacter haemolyticus associated with a case of bloody diarrhea. J. Clin. Microbiol. 44, 3838–3841. 10.1128/JCM.00407-06

64. Verraes C, Van Boxstael S, Van Meervenne E, Van Coillie E, Butaye P, Catry B et al., 2013: Antimicrobial resistance in the food chain: a review. Int. J. Environ. Res. Public Health 10, 2643–2669. 10.3390/ijerph10072643

65. Chauhan S., Farooq U., Singh V., Kumar A., 2013: Determination of prevalence and antibacterial activity of ESBL (Extended Spectrum Beta-lactamases) producing *Klebsiella* species isolated from raw milk of Doon Valley in India. Int. J. Pharm. Biol. Sci. 4, 417–423.

66. Fakruddin Md., Rahaman M., Ahmed M. M., Hoque M., 2014: Antimicrobial resistance and virulence factors of Enterobacteriaceae isolated from food samples of Bangladesh. Int. J. Microbiol. Immunol. Res. 3, 12–18.

67. Thenmozhi S., Moorthy K., Sureshkumar B. T., Suresh M., 2014: Antibiotic resistance mechanism of ESBL producing Enterobacteriaceae in clinical field: a review. Int. J. Pure App. Biosci. 2, 207–226.

68. Tekiner I. H., Özpinar H., 2016: Occurrence and characteristics of extended spectrum beta-lactamases-producing Enterobacteriaceae from foods of animal origin. *Braz*. J. Microbiol. 47, 444–451. 10.1016/j.bjm.2015.11.034

69. Angelo M. B. Amorim and Janaína dos Santos Nascimento, 2017: A Highlight for Non-Escherichia coli and Non-Salmonella sp. Enterobacteriaceae in Dairy Foods Contamination. Front Microbiol. 2017; 8: 930.

70. Munoz-Price L. S., Weinstein R. A., 2008: Acinetobacter infection. N.Engl. J. Med. 358, 1271–1281. 10.1056/NEJMra070741

71. Saito R., Koyano S., Nagai R., Okamura N., Koike K., 2010: Evaluation of a Chromogenic medium for extended-spectrum beta-lactamase-producing Enterobacteriaceae. Lett. Appl. Microbiol. 51, 704–706. 10.1111/j.1472-765X.2010.02945.

72. Dahmen S., Métayer V., Gay E., Madec J.-Y., Haenni M., 2013: Characterization of extended-spectrum beta-lactamase (ESBL)-carrying plasmids and clones of Enterobacteriaceae causing cattle mastitis in France. Vet. Microbiol. 162, 793–799. 10.1016/j.vetmic.2012.10.015

73. Sudarwanto M., Akinedem O., Odenthal S., Gross M., Usleber E., 2015: Extended-spectrum β-lactamase (ESBL)–producing *Klebsiella pneumoniae* in bulk tank milk from dairy farms in Indonesia. Foodborne Pathog. Dis. 12, 585–590. 10.1089/fpd.2014.1895

74. Mogha K. V., Shah N. P., Prajapati J. B., Chaudhari A. R., 2014: Biofilm - A threat to dairy industry. Indian J. Dairy Sci. 67:6.

75. Teh K. H., Flint S., Palmer J., Andrewes P., Bremer P., Lindsay D., 2014: Biofilm − An unrecognised source of spoilage enzymes in dairy products? Int. Dairy J. 34, 32–40. 10.1016/j.idairyj.2013.07.002

76. Flint S., Jamaludin N. M., Somerton B., Palmer J., Brooks J., 2015: The effect of milk composition on the development of biofilms, in Biofilms in the Dairy Industry, eds Teh K. H., Flint S., Brooks J., Knight G., editors. (Chichester: John Wiley & Sons;), 36–48. 10.1002/9781118876282.ch3

77. Cherif-Antar A., Moussa-Boudjemâa B., Didouh S., Medjahdi K., Mayo B., Flórez A. B., 2016: Diversity and biofilm-forming capability of bacteria recovered from stainless steel pipes of a milk-processing dairy plant. Dairy Sci. Technol. 96, 27–38. 10.1007/s13594-015-0235-4

78. Malek F., Moussa-Boudjemâa B., Khaouani-Yousfi F., Kalai A., Kihel M., 2012: Microflora of biofilm on Algerian dairy processing lines: an approach to improve microbial quality of pasteurized milk. Afr. J. Microbiol. Res. 6, 3836–3844. 10.5897/AJMR11.1120

79. Zajác P., Čapla J., Vietoris V., Zubrická S., Čurlej J., 2015: Effects of storage on the major constituents of raw milk. Potravinarstvo 9, 375–381. 10.5219/518

80. Masiello S. N., Martin N. H., Trmčić A., Wiedmann M., Boor K. J., 2016: Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk. J. Dairy Sci. 99, 130–140. 10.3168/jds.2015-9728

81. Caldera L., Arioli S., Stuknyte M., Scarpellini M., Franzetti L., 2015: Setup of a rapid method to distinguish among dead, alive, and viable but not cultivable cells of *Pseudomonas* spp. in mozzarella cheese. J. Dairy Sci. 98, 8368–8374. 10.3168/jds.2015-9677

82. Böhme K., Fernández-No I. C., Barros-Velázquez J., Gallardo J. M., Calo-Mata P., Cañas B., 2013: Species identification of food spoilage and pathogenic bacteria by MALDI-TOF mass fingerprinting. J. Prot. Res. 9, 3169–3183. 10.1021/pr100047q

83. Carpiné D., Dagostin J. L. A., Santa H. S. D., Alvarez D. C., Terra N. N., Santa O. R. D., 2010: Atividade proteolítica e lipolítica de bactérias lácticas isoladas de salames artesanais. Ambiência 6, 125–132.

84. Krewinkel M., Baur C., Kranz B., Von Neubeck M., Wenning M., Scherer S., et al., 2016: A sensitive and robust method for direct determination of lipolytic activity in natural milk environment. Food Anal. Method 9, 646–655. 10.1007/s12161-015-0233-4

85. Bhardwaj A., Kapila S., Mani J., Malik R. K., 2009: Comparison of susceptibility to opsonic killing by in vitro human immune response of *Enterococcus* strains isolated from dairy products, clinical samples and probiotic preparation. *Int. J. Food Microbiol.* 128 513–515. 10.1016/j.ijfoodmicro.2008.10.010

86. Moreno M. F., Sarantinopoulos P., Tsakalidou E., De Vuyst L., 2006: The role and application of enterococci in food and health. *Int. J. Food Microbiol.* 106 1–24. 10.1016/j.ijfoodmicro.2005.06.026

87. Arias C. A., Murray B. E., 2012: The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10 266–278. 10.1038/nrmicro2761

88. Björkroth J., Ristiniemi M., Vandamme P., Korkeala H., 2005: *Enterococcus* species dominating in fresh modified-atmosphere-packaged, marinated broiler legs are overgrown by *Carnobacterium* and *Lactobacillus* species during storage at 6°C. *Int. J. Food Microbiol.* 97 267–276. 10.1016/j.ijfoodmicro.2004.04.011

89. Giraffa G., 2003: Functionality of enterococci in dairy products. *Int. J. Food Microbiol.* 88 215–222. 10.1016/S0168-1605(03)00183-1

90. M'hir S., Minervini F., Di Cagno R., Chammem N., Hamdi M., 2011: Technological, functional and safety aspects of enterococci in fermented vegetable products: a mini-review. *Ann. Microbiol.* 62 469–481. 10.1007/s13213-011-0363-x

91. Thiercelin, M.E., 1899: Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathoge`ne. C.R. Se´ances Socie´te´ Biologie 5, 269–271. food. Int. J. Food Microbiol. 35, 57–66.

92. Thiercelin, M.E., Jouhaud, L., 1903: Reproduction de l'en- te´rocoque; taches centrales; granulations pe´ripheriques et mi- croblastes. C.R. Se´ances Socie´te´ Biologie 55, 686–688.

93. Devriese, L.A., Pot, B., Collins, M.D., 1993: Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J. Appl. Bacteriol. 75, 399–408.

94. Hardie, J.M., Whiley, R.A., 1997: Classification and overview of the genera *Streptococcus* and *Enterococcus*. J. Appl. Microbiol. Symposium Supplement 83, 1S–11S.

95. Morrison, D., Woodford, N., Cookson, B., 1997: Enterococci as emerging pathogens of humans. J. Appl. Microbiol. Symposium Supplement 83, 89S–99S.

96. Stiles, M.E., Holzapfel, W.H., 1997: Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1–29.

97. Dack, G.M., 1956: Food Poisoning, 3rd ed., The University of Chicago Press, Chicago, IL.

98. Stiles, M.E., 1989: Less recognized or presumptive foodborne pathogenic bacteria. In: Doyle, M.P. (Ed.), Foodborne Bacterial Pathogens, Marcel Dekker Inc, New York, pp. 674–735.

99. Ruoff, K.L., 1990: Recent taxonomic changes in the genus *Enterococcus*. Eur. J. Clin. Microbiol. Infect. Dis. 9, 75–79.

100. Devriese, L.A., Pot, B., Van Damme, L., Kersters, K., Haesebrouck, F., 1995: Identification of *Enterococcus* species isolated from foods of animal origin. Int. J. Food Microbiol. 26, 187–197.

101. Murray, B.E., 1990: The life and times of the *Enterococcus*. Clin. Microbiol. Rev. 3, 46–65.

102. Noble, C.J., 1978: Carriage of group D streptococci in the human bowel. J. Clin. Pathol. 4, 1182–1186.

103. Devriese, L.A., Laurier, L., De Herdt, P., Haesebrouck, F., 1992: Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows. J. Appl. Bacteriol. 72, 29–31.

104. Devriese, L.A., Hommez, J., Pot, B., Haesebrouck, F., 1994: Identification and composition of the streptococcal and en- terococcal flora of tonsils, intestines and faeces of pigs. J. Appl. Bacteriol. 77, 31–36.

105. Mundt, J.O., 1961: Occurrence of enterococci: Bud, blossom, and soil studies. Appl. Microbiol. 9, 541–544.

106. Mundt, J.O., 1963: Occurrence of enterococci on plants in a wild environment. Appl. Microbiol. 11, 141–144.

107. Niemi, R.M., Niemela¨, S.I., Bamfort, D.H., Hantula, J., Hyva¨rinen, T., Forsten, T., Raateland, A., 1993: Presumptive fecal streptococci in environmental samples characterized by one dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. 59, 2190–2196.

108. Jay, J.M. (Ed.), 1996: Modern Food Microbiology, 5th ed.,
Chapman & Hall, New York, pp. 395–400.

109. Klaenhammer, T.R., 1993: Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39–86.

110. Jack, R.W., Tagg, J.R., Ray, B., 1995: Bacteriocins of Gram positive bacteria. Microbiol. Rev. 59, 171–200.

111. Delves-Broughton, J., 1990: Nisin and its uses as a food preservative. Food Technol. 44 (11), 100–117.

112. Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink,V., Holo, H., 1996: Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 70, 113–128.

113. Aymerich, T., Holo, H., Havarstein, L.S., Hugas, M., Garriga, M., Nes, I.F., 1996: Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. 62, 1676–1682.

114. Casaus, P., Nilsen, T., Cintas, L.M., Nes, I.F., Herna´ndez, P.E., Holo, H., 1997: Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology 143, 2287–2294.

115. Cintas, L.M., Casaus, P., Havarstein, L.V., Herna´ndez, P.E., Nes, I.F., 1997: Biochemical and genetic characterization of en- terocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63, 4321–4330.

116. Cintas, L.M., Casaus, P., Holo, H., Hernandez, P.E., Nes, I.F., Havarstein, L.S., 1998: Enterocins L50A and L50B, two novel phylobacteriocins from *Enterococcus faecium* L50 are related to staphylococcal hemolysins. J. Bacteriol. 180, 1988–1994.

117. Giraffa, G., 1995: Enterococcal bacteriocins: Their potential as anti-Listeria factors in dairy technology. Food Microbiol. 12, 291–299.

118.http://www.bio.nite.go.jp/pks/cluster/view/63d589ac0a8b21875a10d239e71cbc978f92d06c

119. http://www.sci-news.com/biology/enterococci-ordovician-period-04852.html

120. http://blog.microbiologics.com/environmental-isolate-case-file-cronobacter-sakazakii/

121. Leclercq, R., 1997: Enterococci acquire new kinds of resistance. Clin. Infect. Dis. 24 (Suppl. 1), S80–S84.

122. Moellering, R.C., 1990: The enterococci: An enigma and acontinuing therapeutic challenge. Eur. J. Clin. Microbiol. Infect. Dis. 9, 73–74.

123. Simjee, S., Gill, M.J., 1997: Gene transfer, gentamycin resistance and enterococci. J. Hosp. Infect. 36, 249–259.

124. Arthur, M., Courvalin, P., 1993: Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob. Agents Chemother. 37, 1563–1571.

125. Vincent, S., Knight, R.G., Green, M., Sahm, D.F., Shlaes, D.M., 1991: Vancomycin susceptibility and identification of motile enterococci. J. Clin. Microbiol. 29, 2335–2337.

126. Dutka-Malen, S., Evers, S., Courvain, P., 1995: Detection of glycopeptide resitance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33, 24–27.

127. Leclercq, R., 1997: Enterococci acquire new kinds of resistance. Clin. Infect. Dis. 24 (Suppl. 1), S80–S84.

128. Landman, D., Quale, J.M., 1997: Management of infections due to resistant enterococci: A review of therapeutic options. J. Antimicrob. Chemother. 40, 161–170.

129. Fraise, A.P., 1996: The treatment and control of vancomycin resistant enterococci. J. Antimicrob. Chemother. 38, 753–756.

130. Clewell, D.B., 1990: Movable genetic elements and antibiotic resistance in enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 9, 90–102.

131. Simjee, S., Gill, M.J., 1997: Gene transfer, gentamycin resistance and enterococci. J. Hosp. Infect. 36, 249–259.

132. Dunny, G.M., 1990: Genetic functions and cell–cell interactions in the pheromone inducible plasmid transfer system of *Enterococcus faecalis*. Mol. Microbiol. 4, 689–696.

133. Clewell, D.B., 1993: Bacterial sex pheromone-induced plasmid transfer. Cell 73, 9–12.

134. Wirth, R., 1994: The sex pheromone system of *Enterococcus faecalis*. More than just a plasmid-collection mechanism? Eur. J. Biochem. 222, 235–246.

135. Booth, M.C., Bogie, C.P., Sahl, H.-G., Siezen, R.J., Hatter, K.L., Gilmore, M.S., 1996: Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. Mol. Microbiol. 21, 1175–1184.

136. Charles M.A.P. Franza, Wilhelm H. Holzapfel , Michael E. Stilesa 1999: Enterococci at the crossroads of food safety?. International Journal of Food Microbiology 47 (1999) 1–24

137. Jordens, J.Z., Bates, J., Griffiths, D.T., 1994: Faecal carriage and nosocomial spread of vancomycin-resistant *Enterococcus faecium*. J. Antimicrob. Chemother. 34, 515–528.

138. Chenoweth, C., Schaberg, D., 1990: The epidemiology of enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 9, 80–89.

139. Jett, B.D., Huyke, M.M., Gilmore, M.S., 1994: Virulence of enterococci. Clin. Microbiol. Rev. 7, 462–478.

140. Johnson, A.P., 1994: The pathogenicity of enterococci. J. Anti-microb. Chemother. 33, 1083–1089.

141. Zareba, T.W., Pascu, C., Hryniewicz, W., Wadstro, T., 1997: Binding of extracellular matrix proteins by enterococci. Curr. Microbiol. 34, 6–11.

142. Guzma`n, C.A., Pruzzo, C., Lipira, G., Calegari, L., 1989: Role of adherence in pathogenesis of *Enterococcus faecalis* urinary tract infection and endocarditis. Infect. Immun. 57, 1834–1838.

143. Guzma`n, C.A., Pruzzo, C., Plate, M., Guardati, M.C., Calegari, L., 1991: Serum dependent expression of *Enterococcus faecalis* ahesins involved in the colonization of heart cells. Microbial Pathogenesis 11, 399–409.

144. Kreft, B., Marre, R., Schramm, U., Wirth, R., 1992: Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. Infect. Immun. 60, 25–30.

145. Olmsted, S.B., Dunny, G.M., Erlandsen, S.L., Wells, C.L., 1994: A plasmid-encoded surface protein on *Enterococcus faecalis* augments its internalization by cultured intestinal epithelial cells. J. Infect. Dis. 170, 1549–1556.

146. Wells, C.L., Jechorek, R.P., Erlandsen, S.L., 1990: Evidence for the translocation of *Enterococcus faecalis* across the mouse intestinal tract. J. Infect. Dis. 162, 82–90.

147. Ember, J.A., Hugli, T.E., 1989: Characterization of the human neutrophil response to sex pheromones from *Streptococcus faecalis*. Am. J. Pathol. 134, 797–805.

148. Sannomiya, P.A., Craig, R.A., Clewell, D.B., Suzuki, A., Fujino, M., Till, G.O., Marasco,W.A., 1990: Characterization of a class of nonformylated *Enterococcus faecalis*-derived neutrophil chemotactic peptides: The sex pheromones. Proc. Natl. Acad. Sci. USA 87, 66–70.

149. Usui, Y., Ichiman, Y., Suganuma, M., Yoshida, K., 1991: Platelet aggregation by strains of enterococci. Microbiol. Immun. 35, 933–942.

150. Jett, B.D., Jensen, H.G., Nordquist, R.E., Gilmore, M.S., 1992: Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. 60, 2445–2452

151. Ike, Y., Hashimoto, H., Clewell, D.B., 1984: Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. 45, 528–530.

152. Stevens, S.X., Jensen, H.G., Jett, B.D., Gilmore, M.S., 1992: A hemolysin-encoding plasmid contributes to bacterial virulence in experimental *Enterococcus faecalis* endophthalmitis. Invest. Ophthal. Vis. Sci. 33, 1650–1656.

153. Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., Zervos, M.J., 1993: Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. 37, 2474–2477.

154. Batish, V.K., Grover, S., Ranganathan, B., 1990: Toxigenic characteristics of enterococci isolated from milk and milk products. Microbiologie-Aliments-Nutrition 8, 135–142.

155. Ike, Y., Hashimoto, H., Clewell, D.B., 1987: High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. J. Clin. Microbiol. 25, 1524–1528.

156. Coque, T.M., Patterson, J.E., Steckelberg, J.M., Murray, B.E., 1995: Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from the feces of hospitalized and community-based persons. J. Infect. Dis. 171, 1223–1229.

157. Kühnen, E., Richter, F., Richter, K., Andries, L., 1988: Establishment of a typing system for group D streptococci. Zentralbl. Bakteriol. Hygiene A 267, 322–330.

158. Moellering, R.C., 1991: The enterococcus: A classic example of the impact of antimicrobial resistance on therapeutic options. J. Antimicrob. Chemother. 28, 1–12.

159. Gordts, B., van Landuyt, H., Ieven, M., Vandamme, P., Goossens, H., 1995: Vancomycin-resistant enterococci colonizing the intestinal tracts of hospitalized patients. J. Clin. Microbiol. 33, 2842–2846.

160. Bates, J., Jordens, Z., Selkon, J.B., 1993: Evidence for an animal origin of vancomycin-resistant enterococci. Lancet 342, 490– 491.

161. Bates, J., Jordens, J.Z., Griffiths, D.T., 1994: Farm animals as a putative reservoir for vancomycin-resistant enterococcal in infection in man. J. Antimicrob. Chemother. 34, 507–516.

162. Klare, I., Heier, H., Claus, H., Reissbrodt, R., Witte, W., 1995: VanA-mediated high-level glycopeptide resistance in *En*- microbiologi*terococcus faecium* from animal husbandry. FEMS Microbiol. Lett. 125, 165–172.

163. Das, I., Fraise, A., Wise, R., 1997: Are glycopeptide-resistant enterococci in animals a threat to human beings? Lancet 349, 997–998.

164. Devriese, L.A., Ieven, M., Goossens, H., VanDamme, P., Pot, B., Hommez, J., Haesebrouck, F., 1996: Presence of vancomycin- resistant enterococci in farm and pet animals. Antimicrob. Agents Chemother. 40, 2285–2287.

165. Chadwick, P.R., Woodford, N., Kaczmarski, E.B., Gray, S., Barrell, R.A., Oppenheim, B.A., 1996: Glycopeptide-resistant enterococci isolated from uncooked meat. J. Antimicrob. Chemother. 38, 908–909.

166. Morrison, D., Woodford, N., Cookson, B., 1997: Enterococci as emerging pathogens of humans. J. Appl. Microbiol. Symposium Supplement 83, 89S–99S.

167. McDonald, L.C., Kuehnert, M.J., Tenover, F.C., Jarvis, W.R., 1997: Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. Emerg. Infect. Dis. 3, 311–317.

168. Coque, T.M., Tomayko, J.F., Ricke, S.C., Okhyusen, P.C., Murray, B.E., 1996: Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob. Agents Chemother. 40, 2605–2609.

169. Rice, L.B., 1996: The theoretical origin of vancomycin-resistant enterococci. Clin. Microbiol. Newslett. 17, 189–192.

170. Teuber, M., Perreten, V., Wirsching, F., 1996: Antibiotikum-resistente Bakterien: Eine neue Dimension in der Lebensmittelmikrobiologie. Lebensmitteltechnologie 29, 182–199.

171. Arihara, K., Cassens, R.G., Luchansky, J.B., 1993: Characterization of bacteriocins from *Enterococcus faecium* with activity against *Listeria monocytogenes*. Int. J. Food Microbiol. 19, 123–134.

172. Batish, V.K., Ranganathan, B., 1986: Antibiotic susceptibility of deoxyribonuclease-positive enterococci isolated from milk and milk products and their epidemiological significance. Int. J. Food Microbiol. 3, 331–337.

173. Knudtson, L.M., Hartman, P.A., 1993: Antibiotic resistance Bacamong enterococcal isolates from environmental and clinical sources. J. Food Prot. 56, 486–492.

174. Perreten, V., Teuber, M., 1995: Antibiotic resistant bacteria in fermented dairy products – a new challenge for raw milk cheeses? in: Residues of Antimicrobial Drugs and Other Inhibitors in Milk (International Dairy Federation Brussels). Special Issue [9505. pp. 144–148.

174. Perreten, V., Kolloffel, B., Teuber, M., 1997: Conjugal transfer of the Tn916-like transposon TnF01 from *Enterococcus faecalis* isolated from cheese to other Gram-positive bacteria. Syst. Appl. Microbiol. 20, 27–38.

175. Wegener, H.C., Madsen, M., Nielsen, N., Aarestrup, F.M., 1997: Isolation of vancomycin resistant *Enterococcus faecium* from food. Int. J. Food Microbiol. 35, 57–66.

176. Suksuwan, M. 1983: The incidence of Bacillus cereus in foods in Central Thailand. Southeast Asian Journal of Tropical Medicine and Public Health.14:324-9.

177. TeGiffel, M.C., Beumer, R.R., Granum,P.E. and Rombouts, F.M.1997: Isolation and characterization of Bacillus cereus from pasteurized milk in household refrigerators in the Netherlands. International Journal of Food Microbiology 34:307-318.

178. Dierick,K.,Van Collie,E.,Swiecicka,I.,Meyfroidt,G.,Devieger,H.and Meulemans,a.2005: Fatal family outbreak of Bacillus cereus associated food poisoning.Journal of Clinical Microbiology 43:4277-4279.

179. Kramer ,J. M. and Gilbert ,R. J. 1989: Bacillus cereus and other Bacillus species. Foodborne bacterial pathogens. Marcel Dekker Inc, New York, USA.

180. Jay, M., Loessner, J .and Golden, A.2005: Modern food Microbiology.7th Edition.Springer Science and Buisness Media,Inc.New York,USA.

181. Mahendra Pal, Meseret Asefa, Asefa Deressa,Redwan Muzein 2014: Processed Foods and Bacillus Cereus Poisoning. BEVERAGE & FOOD WORLD - Vol. 41 - No. 12

182. Mehrdad ,T. 2007: *Bacillus cereus*. Journal of Clinical Microbiology 23: 332-360.

183. Schneider, K. R., Mickey, E. P., Renée, M. G. and Taylor, C., 2004: Preventing Foodborne Illness: *Bacillus cereus and Bacillus anthracis.* University of Florida. IFAS Extention. Pp: 1-5.

184. Christiansson, A. 1993: Enterotoxin production in milk by *Bacillus cereus*: a comparison of methods for toxin detection. Netherlands Milk Dairy Journal 47:79–87.

185. Wijnands, L., Dufrenne, J., Zwietering ,M. H., and Leusden F., 2006: Spores from mesophilic *Bacillus cereus* strains germinate better and grow faster in simulated gastrointestinal conditions than spores from psychrotrophic strains. International Journal of Food Microbiology 112: 120-128.

186. Kotiranta A., Lounatmaa K., and Haapasalo M. 2000: Epidemiology and pathogenesis of Bacillus cereus infections. Microbes and Infection 2: 189-198.

187. Barrie ,D., Wilson, J. A., Hoffman ,P. N., Kramer ,J. M. 1992: *Bacillus cereus* meningitis in two neurosurgical patients: an investigation into the source of the organism. Journal of Infection 25:291-297.

188. https://en.wikipedia.org/wiki/Bacillus_cereus#/media/File:Bacillus_cereus_SEM-cr.jpg Mogana Das Murtey and Patchamuthu Ramasamy

189. Notermans ,S., Dufrenne, J., Teunis, P., Beumer ,R., Giffel ,M. T. and Peeters W. P.1997: A risk assessment study of *Bacillus cereus* present in pasteurized milk. Food Microbiology 14:143- 151.

190. Jensen ,G., Hansen, B., Eilenberg ,J. and Mahillon ,J. 2003: The hidden lifestyles of Bacillus cereus and relatives. Environmental Microbiology 5: 631–640.

191. Pal, M., Seid, H., Karanfil,O. and Woldemariam,T. 2013: Importance of microbial films in food processing plants. Beverage and Food World 40:49-50.

192. Carlin ,F., Fricker, M. and Pielaat ,A. 2006: Emetic toxin-producing strains of Bacillus cereus show distinct characteristics within the Bacillus cereus group. International Journal of Food Microbiology 109:132–138.

193. Stenfors Arnesen L. P., Fagerlund , A. and Granum, P. E. 2008: From soil to gut: Bacillus cereus and its food poisoning toxins. FEMS Microbiology Reviews.32: 579–606.

194. Pal M, Bekele T and Feleke A, 2012: Public health significance of pasteurized milk. Beverage and Food World 39:55-56.

195. Bassett, J. and McClure, P., 2008: A risk assessment approach for fresh fruits. Journal of Applied Microbiology 104:925–943.

196. Schneider, K. R., Mickey, E. P., Renée, M. G. and Taylor, C., 2004: Preventing Foodborne Illness: Bacillus cereus and Bacillus anthracis. University of Florida. IFAS Extention. Pp: 1-5.

197. Magan, N., Olsen, M., 2004: Mycotoxins in Food: Detection and Control. Woodhead Publishing, United Kingdom.

198. Engel, G., & Teuber, M., 1991: Heat resistance of ascospores of Byssochlamys nivea in milk and cream. International Journal of Food Microbiology, 12, 225-233.

199. Sant'Ana, A. S., Rosenthal, A., & Massaguer, P. R., 2009: Heat resistance and the effects of continuous pasteurization on the inactivation of Byssochlamys fulva ascospores in clarified apple juice. Journal of Applied Microbiology, 107, 197-209.

200. Tournas, V., 1994: Heat-resistant fungi of importance to the food and beverage industry. Critical Reviews in Microbiology, 20, 243-263.

201. Noreddine Benkerroum, 2016: Mycotoxins in dairy products: A review
Institut Agronomique et Veterinaire Hassan II, D_epartement des Sciences Alimentaires et Nutritionnelles, BP 6202, Instituts, 10101, Rabat, Morocco.

202. Scott, P. M., & Kennedy, P. C., 1976: Analysis of blue cheese for roquefortine and other alkaloids from Penicillium roqueforti. Journal of Agricultural and Food

Chemistry, 24, 865-868.

203. Hymery, N., Masson, F., Barbier, G., & Coton, E., 2014: Cytotoxicity and immunotoxicity of cyclopiazonic acid on human cells. Toxicology in Vitro, 28, 940-947.

204. Nielsen, K. F., Dalsgaard, P.W., Smedsgaard, J., & Larsen, T. O., 2005: Andrastins A-D, Penicillium roqueforti metabolites consistently produced in blue-mould-ripened cheese. Journal of Agricultural and Food Chemistry, 53, 2908-2913.

205. Ali, M. A., El Zubeir, I. E., & Fadel Elseed, A. M., 2014: Aflatoxin M1 in raw and imported powdered milk sold in Khartoum state, Sudan. Food Additives and Contaminants Part B Surveillance, 7, 208-212.

206. Burdock, G. A., & Flamm, W. G., 2000: Review article: Safety assessment of the mycotoxin cyclopiazonic acid. International Journal of Toxicology, 19, 195-218.

207. Fontaine, K., Passero, E., Vallone, L., Hymery, N., Coton, M., Jany, J.-L., et al., 2015: Occurrence of roquefortine C, mycophenolic acid and aflatoxin M1 mycotoxins in blue-veined cheeses. Food Control, 47, 634-640.

208. Ismail, A., Akhtar, S., Levin, R. E., Ismail, T., Riaz, M., & Amir, M., 2016: Aflatoxin M1: Prevalence and decontamination strategies in milk and milk products. Critical Reviews in Microbiology, 42, 418-427.

209. Sengun, I. Y., Yaman, D. B., & Gonul, S. A., 2008: Mycotoxins and mould contamination in cheese: A review. World Mycotoxin Journal, 1, 291-298.

210. Kokkonen, M., Jestoi, M., & Rizzo, A., 2005: Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry. Food Additives and Contaminants, 22, 449-456.

211. Murphy, P. A., Hendrich, S., Landgren, C., & Bryant, C. M., 2006: Food mycotoxins: An update. Journal of Food Science, 71, R51-R65.

212. Prandini, A., Tansini, G., Sigolo, S., Filippi, L., Laporta, M., & Piva, G., 2009: On the occurrence of aflatoxin M1 in milk and dairy products. Food and Chemical Toxicology, 47, 984-991

213. Pattono, D., Grosso, A., Stocco, P. P., Pazzi, M., & Zeppa, G., 2013: Survey of the presence of patulin and ochratoxin A in traditional semi-hard cheeses. Food Control, 33, 54-57.

214. Cakmakci, S., Gurses, M., Hayaloglu, A. A., Cetin, B., Sekerci, P., & Dagdemir, E., 2015: Mycotoxin production capability of Penicillium roqueforti in strains isolated from mould-ripened traditional Turkish civil cheese. Food Additives and Contaminants. Part A, Chemistry, Analysis, Control, Exposure and Risk Assessment, 32, 245-249.

215. O'Brien, M., Nielsen, K. F., O'Kiely, P., Forristal, P. D., Fuller, H. T., & Frisvad, J. C., 2006: Mycotoxins and other secondary metabolites produced in vitro by Penicillium paneum Frisvad and Penicillium roqueforti Thom isolated from baled grass silage in Ireland. Journal of Agricultural and Food Chemistry, 54, 9268-9276

216. Gowda, N. K. S., Swamy, H. V. L. N., & Mahajan, P., 2013: Recent advances for control, counteraction and amelioration of potential aflatoxins in animal feeds. In M. Razzaghi-Abyaneh (Ed.), Aflatoxins e Recent advances and future prospects (pp. 129-140). New Delhi, India: InTech.

217. Battacone, G., Nudda, A., Cannas, A., Cappio Borlino, A., Bomboi, G., & Pulina, G., 2003: Excretion of aflatoxin M1 in milk of dairy ewes treated with different doses of aflatoxin B1. Journal of Dairy Science, 86, 2667-2675.

218. Fallah, A. A., Rahnama, M., Jafari, T., & Saei-Dehkordi, S. S., 2011: Seasonal variation of aflatoxin M1 contamination in industrial and traditional Iranian dairy products. Food Control, 22, 1653-1656.

219. Flores-Flores, M. E., Lizarraga, E., L_opez de Cerain, A., & Gonzalez-Penas, E., 2015: Presence of mycotoxins in animal milk: A review. Food Control, 53, 163-176.

220. Murphy, P. A., Hendrich, S., Landgren, C., & Bryant, C. M., 2006: Food mycotoxins: An update. Journal of Food Science, 71, R51-R65.

221. Govaris, A., Roussi, V., Koidis, P. A., & Botsoglou, N. A., 2001: Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese. Food Additives and Contaminants, 18, 437-443.

222. Lopez-Díaz, T. M., Rom_an-Blanco, C., García-Arias, M. T., García-Fern_andez, M. C., & García-L_opez, M. L., 1996: Mycotoxins in two Spanish cheese varieties. International Journal of Food Microbiology, 30, 391-395.

223. Scaglioni, P. T., Becker-Algeri, T., Drunkler, D., & Badiale-Furlong, E., 2014: Aflatoxin B1 and M1 in milk. Analytica Chimica Acta, 829, 68-74.

224. Sengun, I. Y., Yaman, D. B., & Gonul, S. A., 2008: Mycotoxins and mould contamination in cheese: A review. World Mycotoxin Journal, 1, 291-298

225. Iqbal, S. Z., Jinap, S., Pirouz, A. A., & Ahmad Faizal, A. R., 2015: Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review. Trends in Food Science and Technology, 46, 110-119.

226. Prandini, A., Tansini, G., Sigolo, S., Filippi, L., Laporta, M., & Piva, G., 2009: On the occurrence of aflatoxin M1 in milk and dairy products. Food and Chemical Toxicology, 47, 984-991.

227. Fink-Gremmels, J., 2008: Mycotoxins in cattle feeds and carry-over to dairy milk: A review. Food Additives and Contaminants. Part A, Chemistry, Analysis, Control, Exposure and Risk Assessment, 25, 172-180.

228. Maul, R., Warth, B., Kant, J. S., Schebb, N. H., Krska, R., Koch, M., et al., 2012:

Investigation of the hepatic glucuronidation pattern of the Fusarium mycotoxin deoxynivalenol in various species. Chemical Research in Toxicology, 25, 2715-2717.

229. Shephard, G. S., Burger, H. M., Gambacorta, L., Gong, Y. Y., Krska, R., Rheeder, J. P., et al., 2013: Multiple mycotoxin exposure determined by urinary biomarkers in rural subsistence farmers in the former Transkei, South Africa. Food and Chemical Toxicology, 62, 217-225.

230. Winkler, J., Kersten, S., Valenta, H., Meyer, U., Engelhardt, U. H., & Danicke, S., 2015: Development of a multi-toxin method for investigating the carryover of zearalenone, deoxynivalenol and their metabolites into milk of dairy cows. Food Additives and Contaminants: Part A, 32, 371-380.

231. Becker-Algeri, T. A., Castagnaro, D., de Bortoli, K., de Souza, C., Drunkler, D. A., & Badiale-Furlong, E., 2016: Mycotoxins in bovine milk and dairy products: A review. Journal of Food Science, 81, R544-R552.

232. Ashiq, S., 2015: Natural occurrence of mycotoxins in food and feed: Pakistan perspective. Comprehensive Reviews in Food Science and Food Safety, 14, 159e175.

233. Dorner, J. W., & Cole, R. J., 2002: Effect of application of nontoxigenic strain of Aspergillus flavus and Aspergillus parasiticus on subsequent aflatoxin contamination of peanuts in storage. Journal of Stored Products Research, 38, 329-339.

234. Mark Busman, John R. Bobell, Chris M. Maragos, 2015: Determination of the aflatoxin M_1 (AFM_1) from milk by direct analysis in real time – mass spectrometry (DART-MS). Food Control Volume 47, January 2015, 592-598

235. Galvano, F., Galofaro, V., & Galvano, G., 1996: Occurrence and stability of aflatoxin M1 in milk and milk products: A worldwide review. Journal of Food Protection, 59, 1079-1090.

236. Jard, G., Liboz, T., Mathieu, F., Guyonvarc'h, A., & Lebrihi, A., 2011: Review of

mycotoxin reduction in food and feed: From prevention in the field to detoxification by adsorption or transformation. Food Additives and Contaminants Part A, 28, 1590-1609.

237. El-Nezami, H., Mykkanen, H., Kankaanpaa, P., Salminen, S., & Ahokas, J., 2000: Ability of Lactobacillus and Propionibacterium strains to remove aflatoxin B, from the chicken duodenum. Journal of Food Protection, 63, 549-552.

238. Haskard, C. A., El-Nezami, H. S., Kankaanpaa, P. E., Salminen, S., & Ahokas, J. T., 2001: Surface binding of aflatoxin B(1) by lactic acid bacteria. Applied and Environmental Microbiology, 67, 3086-3091

239. Shetty, P. H., Hald, B., & Jespersen, L., 2007: Surface binding of aflatoxin B1 by Saccharomyces cerevisiae strains with potential decontaminating abilities in indigenous fermented foods. International Journal of Food Microbiology, 113, 41-46.

240. Sweeney, M. J., & Dobson, A. D., 1998: Mycotoxin production by Aspergillus, Fusarium and Penicillium species. International Journal of Food Microbiology, 43, 141-158.

241. Bata, A., & Lasztity, R., 1999: Detoxification of mycotoxin contaminated food and feed by microorganisms. Trends in Food Science and Technology, 10, 223-228.

242. Karlovsky, P., 2014: Enzymatic detoxification of mycotoxins for healthy food. New Food, 17, 66-68.

243. Rasic, J. L., Skrinjar, M., & Markov, S., 1991: Decrease of aflatoxins B1 in yoghurt and acidified milks. Mycopathologia, 113, 117-119.

244. ROSE,A .H. & HARRISONJ.,S, 1970: *The Yeasts,* Vol. 3, *Yeast Technology.* London: Academic Press.

245. TODD, E.C., 1983: Foodborne disease in Canada-a 5 year summary. *Journal UJ Food Protection 46,* 65C-657.

246. TAYLORS, L., 1980: Food allergy-the enigma and some potential solutions. *Journal of Food Protection* 43, 300-306.

247. ROBINSONR, .K., 1981: *Dairy Microbiology* Vol. 2. London & New Jersey: Applied Science Publishers.

248. COUSINM, .A., 1982: Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. *Journal* of *Food Protection* 45, 172-207.

249. Rasic, J. L., 1983: The role of dairy foods containing bifido and acidophilus bacteria in nutrition and health? North European Dairy Journal, 4, 1–5.

250. Fleet, G. H., & Mian, M. A., 1987: The occurrence and growth of yeasts in dairy products. International Journal of Food Microbiology, 4, 145–155.

251. Jakobsen, M., & Narvhus, J., 1996: Yeasts and their beneficial and
negative effects on the quality of dairy products. International Dairy Journal, 6, 755–768.

252. McFarland, L., & Bernasconi, P., 1993: Saccharomyces boulardii: a Review of an innovative biotherapeutic agent. Microbial Ecology in Health and Disease, 6, 157–171.

253. Gedek, B. R., 1991: Regulierung der Darmflora über die Nahrung. Zentralblatt für Hygiene und Umweltmedizin, 191, 277–301.

254. Fleet, G. H., 1990: Yeasts in dairy products — a review. Journal of Applied Bacteriology, 68, 199–211.

255. Giudici, P., Masini, G., & Caggia, C., 1996: The role of galactose fermenting yeast in plain yogurt spoilage. Annali di Microbiologia ed Enzimologia, 46, 11–19.

256. Lang, F., & Lang, A., 1975: Acidophilus milk products: little known cultured milks of great potential. The Milk Industry, 77, 4–6.

257. Subramanian, P., & Shankar, P. A., 1983: A note on lactose fermenting yeasts in milk products. Journal of Food Science and Technology, 20, 181–183.

258. A. Lourens-Hattingh, B.C. Viljoen., 2001: Growth and survival of a probiotic yeast in dairy products. Food Research International 34, 791–796.

259. Pal, M., 2007: Veterinary and Medical Mycology. India Council of Agricultural Research, New Deli, India.

260. Ainsworth, G.C. and Austwick, P.K.C., 1973: Fungal Diseases of Animals. C.A.B. Farnham Royal Slough, England.

261. Chahota, R., Katoch, R., Mahajan, A. and Verma, S., 2001: Vet.Arh. 71:197.

262. Tarfarosh, M.A. and Purohit,S.K., 2008: Vet.Scandinavia. 3:14.

263. Radostits, O., Gay, C.C., Hincheliff, K.W. and Constable, P.D., 2007: Veterinary Medicine: A textbook of diseases of cattle, horses, sheep, pigs and goats. 10th edn., W B Saunders, Philadelphia, USA.

264. Schallibaum, M., Nicolet, J. and Koning, H., 1980: Sabouraudia. 18:33.

265. Pal, M., 1997: Buffalo J. 13:91.

266. Gaudie, C.M., Wrag, P.N., Barber, A. M., 2009: Vet. Record. 165:535.

267. Seker, E., 2010: Mycopathologia. 169:303.

268. Konte M, 1999: Le lait et les produits laitiers. Développement de systèmes de productions intensives en Afrique de l’ouest. Université de Nouakchott (R.I.M) Faculté des Sciences et Technologies des aliments, B. P. 5026. ISRA/ URV – LNERV/FEVRIER : 2-25.

269. Kohler G, 2013: Note d’information relative à la vente directe de lait cru. Projet des Pyrénées. Atlantiques: pp.1 – 4.

270. Guetouache, Mourad, Guessas, Bettache and Medjekal, Samir, 2014: Composition and nutritional value of raw milk. Issues in Biological Sciences and Pharmaceutical Research Vol. 2(10),pp .115-122.

271. Adib A, Bertrand S, 2009: Risk Analysis transfer pesticides to milk. Institute of Livestock. National Interprofessional Centre

272. Leseur R, Melik N, 1990: Lait et produits laitiers. Edition : Cedex. Paris : pp.2 – 7.

273. Gautheron M, Lepouze A, 2012: Le lait, un aliment indispensable.

274. Fillion MM, 2006: Amélioration de la stabilité thermique du lait par modulation du potentiel d’oxydoréduction. Thèse : pp.23 – 447.

275. De Wit JN, 1981: Structure and junction al behavior of whey proteins. Netherlands Milk and dairy journal. 35 : 47 – 54.

276. Grand Pierre C, Ghisolfi J, Thomsent TH, 1988: Etude biochimique du lait de chèvre. Edition CCAN. Nutr. DIET. 23 : 364 – 374.

277. Florence CL, 2010: Qualité nutritionnelle du lait de vache et de ses acides gras, voies d’amélioration par l’alimentation. Ecole nationale vétérinaire d’ALFOR. thèse. Doctorat vétérinaire. P 51.

278. Mireille L, 2006: Les produits laitiers allégés : murage ou panages : aspects technologiques et nutritionnels. Thèses de Doctorat vétérinaire. Diplôme d’état, Université Paul-Sabatier de Toulouse, Ecole nationale vétérinaire Toulouse, Tou. (31-34) : 3-4007.

279. Schrodes MJA, 1982: Effect of oxygen on the keeping quality of milk, I. Oxidized flavor development and oxygen uptake in relation to oxygen availability, J. Dairy Res. (49): 407– 424.

280. https://www.quora.com/Is-lactose-an-invention-of-evolution-to-prevent-adults-from-drinking-the-milk-thats-made-for-the-youngest-people

281. Robert, M.K., Gerald, F.F., Colin, H., Catherine, S. and Paul, R.R., 2015: Novel approaches to improve the intrinsic microbiological safety of powdered infant milk formula. Nutrients 7: 1217-1244.

282. Falegan, C.R. and Oluwaniyi, T.T., 2015: Microbial composition, antibiotic sensitivityand proximate composition of popular imported powdered infant milk formulas sold in Ado Ekiti, Nigeria. International Journal of Microbiology, Genetics and Monocular Biology Research 1:10-24.

283. Carlos, A.F.O., Lucinéia, M., Marcos, V.S., José, F.M., Aleksandrs, S. and Pedro, M.L.G., 2000: Effect of microbiological characteristics of raw milk on the quality of whole milk powder. Brazilian Journal of Microbiology 31: 95-98.

284. Anup, S., Atanu, H. J .and Rupesh, S.C., 2012: Functionality of milk powders and milk based powders for end use applications. Comprehensive Reviews in Food Science and Food Safety 11: 1-11.

285. László, V., 2007: Microbiological quality of commercial dairy products. Communicating Current Research and Educational Topics and Trends in Applied Microbiology 1: 1-8.

286. Ron, S.R., Andreas, R.K. and Hugh, W.M., 2006: Survival of thermophilic spore-forming bacteria in a 90 + year old milk powder from Ernest Shackelton's Cape Royds Hut in Antarctica. Journal of Dairy Research 73: 235–243.

287. Pal,M. and Jadhav,V.J., 2013: Microbial contamination of various Indian milk products. Beverage and Food World 40: 43-44.

288. Ahmed, M.M.M., Hafez, E.E., Mona, M.H, Hagar, A. A. and Mabrouk, Y.M., 2014: Detection of baby milk powder contamination by microorganisms. World Applied Sciences Journal 1: 93-98.

289. Hafsa ,A., Fouzia, S., Fakruddin, M.D., Kamrunnahar, Zahed ,U.M.K. and Suvamoy, D., 2013: Isolation of Escherichia coli and Staphylococcus aureus from full cream powder milk sold under market conditions at Dhaka, Bangladesh and their antibiotic susceptibility. Journal of Advanced Scientific Research 4: 27-31

290. Mahendra Pal, Jemberu Alemu*, Selamawit Mulu, Olga Karanfil, B.C.Parmar, J. B. Nayak, 2016: Microbial and Hygienic aspects of Dry Milk Powder. BEVERAGE & FOOD WORLD - Vol. 43 - No. 7: 28-31.

291. U.S. Dairy Export Council Product specifications. Arlington, VA, USA, 1999.

292. Harper W.J., 2000: Biological Properties of Whey Components: A Review. American Dairy Product Institute, Chicago IL.pp. 1–67.

292. Boye J.I., Alli I., Ramaswamy H., Reghvan V.G.S., 1997: Interactive effects of factor affecting gelation of whey proteins. J. Food Sci. 62: 57–65.

293. Corradini C., 1998: Functional properties of whey proteins in foods. Sci. Tecn. Latt.-Cas. 49: 204–213.

294. Huffman L.M., 1996: Processing whey protein for use as a food ingredient. Food Technol. 50: 49–52.

295. Nakai S., Li-Chane E., 1993: Recent advances in structure and function of food proteins. QSAR approach, Crit. Rev. Food Sci. 33. 477–499.

296. Galazka V.B., Dickinson E., Ledward D.A., 1996: Effect of high-pressure on the emulsifying behaviour of β-lactoglobulin. Food Hydrocoll. 10 : 213–219.

297. Knorr D., Advantages, 1998: possibilities and challenges of high pressure applications in food processing, in. Reid D.S. (Ed.). The Properties of Water in Foods. Blackie, New York, USA. pp. 419–437.

298. Tewari G., Jayas D.S., Holley R.A., 1999: High pressure processing of foods: an overview. Sci. Aliment 19. 619–661.

299. Marshall K., 2004: Therapeutic applications of whey protein. Altern Med Rev., 9(2): 136-156.

300. Wong C.W., Seow H.F., Husband A.J., Regester G.O., Watson D.L., 1997: Effects of purified bovine whey factors on cellular immune functions in ruminants. Vet. Immunol. Immunopathol., 56 (1-2): 85-96.

301. Matsumoto H., Shimokawa Y., Ushida Y., Toida T., Hayasawa H., 2001: New biological function of bovine alpha-lactalbumin: protective effect against ethanol- and stress-induced gastric mucosal injury in rats. Bioscience, biotechnology, and biochemistry., 65 (5): 1104-11.

302. Parodi P.W.; 2007: A role for milk proteins and their peptides in cancer prevention. Curr Pharm Des., 13(8): 813-28.

303. Krissansen G.W., 2007: Emerging Health Properties of Whey Proteins and Their Clinical Implications, Journal of the American College of Nutrition., 26(6): 713S-723S.

304. Korhonen H., Marnila P., Gill H.S., 2000: Bovine milk antibodies for health. Br J Nutr., 84(Suppl 1): S135-146.

305. Beucher S., Levenez F., Yvon M., Corring T., 1994: Effects of gastric digestive products from casein on CCK release by intestinal cells in rat. J Nutr Biochem., 5 (12):578-584.

306. Manso M.A., Lopez-Fandino R., 2004: Kappa-Casein macropeptides from cheese whey: Physicochemical, biological, nutritional, and technological features for possible uses. Food Reviews International., 20: 329-355.

307. Besse, N. G., A. Leclercq, V. Maladen, C. Tyburski, and L. Bertrand, 2006: Evaluation of the International Organization for Standardization-International Dairy Federation (ISO-IDF) draft standard method for detection of Enterobacter sakazakii in powdered infant food formulas. J. AOAC Int. 89:1309-1316.

308. International Organization for Standardization, 2006: Milk and milk products -- Detection of Enterobacter sakazakii. ISO/TS 22964|IDF/RM 210:2006.

309. International Organization for Standardization, 2015: Microbiology in the food chain - Horizontal method for the detection of *Cronobacter* spp. ISO/DIS 22964.

310. Iversen, C., P. Druggan, S. Schumacher, A. Lehner, C. Feer, K. Gschwend, H. Joosten, and R. Stephan, 2008: Development of a novel screening method for the isolation of "*Cronobacter*" spp. (*Enterobacter sakazakii*). *Appl. Environ. Microbiol.* 74:2550-2553.

311.http://www.mibius.de/out/oxbaseshop/html/0/images/wysiwigpro/Chromocult_E_Sakazakii_100873_De.pdf

312. Technical Data Sheet Buffered Peptone Water. Diluent Enrichment Broth. Biokar diagnostics. http://www.solabia.com/Produto_166,9/BIOKAR-Diagnostics/BUFFERED-PEPTONE-WATER.html

313. Guillaume-Gentil et al., 2005: *J. Food. Prot*. **68**. 64-69

314.http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM1133&org=66&c=UK&lang=EN. Technical Data Sheet Dehydrated Culture Media Vancomycin Medium. Oxoid. Thermo Fisher Scientific Inc.

315. http://www.merckmillipore.com/AT/de/product/Enterobacter-Sakazakii-Agar,MDA_CHEM-100873#anchor_APPL. Technical Data Sheet Chromocult® Enterobacter Sakazakii Agar. Merck Microbiology Manual 12th Edition.

316. Jackson EE, 2016: Techniques for the identification and differentiation ofcronobacter species. A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy. 2: 20-22

317. https://www.biomerieux.de/klinische-diagnostik/apir-id-teststreife

318. https://www.biomerieux-industry.com/food/api-id32-microbial-identification

319. Burkwall M. K. and Hartman P. A., 1964: *Appl. Microbiol*. 12. 18-23.

320. Nordic Committee on Food Analysis, 1968: *Leaflet 68*.

321. Taylor E. W. and Burman N. P., 1964: *J. Appl. Bact*. 27. 294-303.

322. Mead G. C., 1966: *Proc. Soc. Wat. Treat. Exam*. 15. 207-221.

323. http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0377&c=uk&org=68&print=Y

324. http://www.solabia.com/Produto_188,9/BIOKAR-Diagnostics/TRYPTO-CASEIN-SOY-AGAR-TSA-.html

325.
https://dongnamlab.com/publics/product/918/files/Violet_red_bile_agar_(VRBL)_TDS_BK152_BM034_035_v6.pdf

326. http://solabia.com/solabia/produitsDiagnostic.nsf/0/2534514325C567C8C125 74950048C168/$file/TDS_BK011_BM075_v12.pdf

327. Mossel, D.A.A., Koopman, M.J., and Jongerius, E., 1967: Enumeration of Bacillus cereus in Foods. App. Microb., 15, (3): 650-653.

328. http://solabia.com/solabia/produitsDiagnostic.nsf/0/EAF67E323B23554 DC12574C600343ED7/$file/TDS_BK116_BM038_v7.pdf

329. http://www.microbes-edu.org/glossaire/images/ACFE3A.jpg

330. https://www.mediray.co.nz/media/15796/om_biomerieux_test-kits_ot-50300_package_insert-50300.pdf . Manual insert Biomerieux

331. http://www.merckmillipore.com/AT/de/product/YGC-agar,MDA_CHEM-116000#anchor_DS. Application sheet Merck Millipore.

332. http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=CM0075&org =141&c= uk&lang=en. Data sheet Oxoid.

333. http://www.dongnamlab.com/publics/product/918/files/Violet_red_bile_agar_ (VRBL)_TDS_BK152_BM034_035_v6.pdf. VRB Agar Biokar Diagnostics, Technical Data Sheet.

334. https://www.bioser.com/p/46/vrbg-agar-violet-red-bile-glucose-agar/. Biokar Diagnostic agar.

335. Foto von Biomerx Laboratorium®

336. http://solabia.com/solabia/produitsDiagnostic.nsf/0/5F49F476B0956812C125 74C700313A31/$file/TDS_BK144_BM015_033_v8.pdf. Technical data sheet Biokar Diagnostics.

337. Jayaraman, K.S. and Das Gupta, D.K., 1992: Dehydration of fruits and vegetables-recent developments in principles and techniques.Drying Technology, 10(1): 1-50.

338. Mothibe, K. J., Zhang, M., Nsor-atindana, J. and Wang, Y.C., 2011: Use of ultrasound pretreatment in drying of fruits: drying rates, quality attributes, and shelf life extension, Drying Technology, 29(14): 1611-1621.

339. Parihari, A., 2009: Performance study of spray dryer using various salt solutions. Master Thesis, National Institute of Technology, Rourkela.

340. Masters, K., 1997: Spray Dryers. In C. G. J. Baker (Ed.), Industrial drying of foods. London, UK: Blackie Academic and Professional.

341. Bowen, M., Turok, R. and Maa, Y.F., 2013: Spray drying of monoclonal antibodies: investigating powder-based biologic drug substance bulk storage. Drying Technology, 31(13-14): 1441-1450.

342. Donz, E., Boiron, P. and Courthaudon, J.L., 2014: Characterization of industrial dried whey emulsions at different stages of spray-drying.Journal of Food Engineering, 126:190-197.

343.https://www.researchgate.net/profile/Antonio_Segura_Carretero/publication/305081927/figure/fig1/AS:614479834411008@1523514817747/Schematic-view-of-spray-drying-process.png

344. Broadhead, J, Edmond Rouan, S. K. and Rhodes, C.T. (1992). The spray drying of pharmaceuticals. Drug Development and Industrial Pharmacy, 18(11-12): 1169-1206.

345. Hede, P.D., Bach, P. and Jensen, A. D., 2008: Two-fluid spray atomisation and pneumatic nozzles for fluid bed coating/agglomeration purposes: A review. Chemical Engineering Science, 63(14): 3821-3842.

346. Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A. and Saurel, R., 2007: Applications of spray-drying in microencapsulation of food ingredients: An overview. Food Research International, 40(9): 1107-1121.

347. Cheuyglintase, K., 2009: Spray drying of fruit juice with vegetable fibre as a carrier. University of Canterbury, Christchurch, New Zealand,URI: http://hdl.handle.net/10092/3101.

348. Corrigan, O. I., 1995: Thermal analysis of spray dried products. Thermochimicaacta, 248: 245- 258.

349. M. Amdadul Haque,Yakindra Prasad Timilsena and Benu Adhikari, 2015: Spray Drying. Drying Technologies for Foods: Fundamentals & Applications, 79-106.

350. https://www.bfr.bund.de/de/bacillus_cereus-54344.html