MASTERARBEIT

Titel der Masterarbeit

Antibakterielle Eigenschaften der Klasse der nichtionischen Surfactanten Lutensol und die Konsequenzen für die Probenvorbereitung

Antibacterial properties of the non-ionic surfactant class Lutensol and the consequences for sample-preparation methods

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2015

Studienkennzahl lt. Studienblatt:	A 066 830
Studienrichtung lt. Studienblatt:	Masterstudium Molekulare Mikrobiologie und Immunbiologie
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Acknowledgements

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Abbreviations

BHQ 1	Black Hole Quencher™ 1
DNA	deoxyribonucleic acid
dNTP	nucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FAM	6-carboxyfluorescein
HCl	hydrochloric acid
L. monocytogenes	*Listeria monocytogenes*
M	molar (mol/l)
mg	milligram
min.	minute
ml	milliliter
mM	millimolar (mmol/l)
µl	microliter
µM	micromolar (µmol/l)
n.a.	non-accountable
OD	optical density
PCR	polymerase chain reaction
pH	pondus Hydrogenii also known as potentia Hydrogenii
PBS	phosphate buffered saline
pM	picomolar (pmol/l)
rpm	revolution per minute
RT	room temperature
SDS	sodium dodecyl sulphate
***S.* Typhimurium**	*Salmonella* Typhimurium
TAMRA	5-carboxytetramethylrhodamin
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TSA + Y	Trypton soy agar with yeast extract
TSB + Y	Trypton soy broth with yeast extract

Abstract

Lutensol is the umbrella term for a class of ethoxylated non-ionic surfactants, which are used for detergents, cleaners and industrial formulators. The different grades of Lutensols include oxo-alcohol ethoxylates that are based on predominately linear alcohols as well as alkylphenol ethoxylates and different types of branched or non-linear alcohols. The different grades of Lutensols can be used as wetting agents, emulsifiers, detergents and cleaners with a yearly production of more than 1.000.000 tons. Although Lutensols are used for many applications, there is only very limited detailed information publicly available.

The aim of this master thesis was to study the influence of different types of Lutensol on model organisms, such as *Escherichia coli* and *Listeria monocytogenes,* in order to contribute more detailed knowledge concerning the bactericidal activity of Lutensols and the underlying mechanisms. A combinational effect that was first reported by Kwisda (2012) was of special interest for this study. It was surprisingly found that Lutensol AO 7 (of the AO grade) in combination with certain salts shows a dramatically increased toxicity towards bacteria. So far no explanation has been available for such an effect.

In cooperation with BASF, first a screening for antimicrobial effects of Lutensol grades representatives was done. By applying the chemical concept of T-SAR (Thinking in structure-activity relationship) in the second phase and based on the screening results, the underlying principles of bactericidal effects of different classes of Lutensols on the cellular level were investigated.

Furthermore, it was discovered that the combinational effect is neither an unique feature of Lutensol AO 7, nor it is an unique feature of Lutensol grades in general. This effect was also detected in combination of inorganic salts with diverse surfactants. No universal pattern of this effect was observed, thus it can be said that the combinational effect depends on all three variables: inorganic salt, surfactant and model organism. It was shown that the plasma membrane damage is the main cause of bacterial death.

A particular research question was to determine the Lutensol grade with the least combinational effect which would make it a good candidate for the Matrix-Lysis procedure. In this respect, Lutensol TO 12 was found to have the least bactericidal effect.

Zusammenfassung

Lutensol ist der Überbegriff für die Klasse der ethoxylierten nicht-ionischen Surfactanten, die als Detergenzien und Waschmittel Anwendung finden. Unterschiedliche Typen von Lutensol beinhalten ethoxylierte Oxo-Alkohole deren Struktur überwiegend auf linearen Alkoholen, ethoxylierten Alkylphenolen und unterschiedlichen Typen von verzweigten bzw. linearen Alkoholen beruht.

Lutensole kommen als Befeuchtungsmittel, emulgierende Substanzen, Detergenzien und Waschmittel zur Anwendung. Die jährliche Produktion liegt bei über 1.000 000 Tonnen. Trotz der breiten Anwendung von Lutensolen ist die öffentlich zugängliche Information über deren Zusammensetzung bzw. Wirkung sehr gering.

Ziel der vorliegenden Arbeit war die Untersuchung der Wirkung von unterschiedlichen Lutensol-Typen anhand von zwei Modellorganismen – *Escherichia coli* und *Listeria monocytogenes* – um zum besseren Verständnis der bakteriziden Wirkung der Lutensole als auch der zugrunde liegenden Mechanismen beizutragen.

Der Kombinationseffekt, über den Kwisda (2012) zum ersten Mal berichtet hat, war von besonders großem Interesse für die vorliegende Studie. Überraschenderweise wurde festgestellt, dass Lutensol des AO Typs – Lutensol AO 7 - in Kombination mit bestimmten Salzen einen dramatischen Anstieg der Toxizität gegenüber Bakterien aufweist. Soweit gibt es keine Erklärung für diesen Effekt.

In Zusammenarbeit mit BASF wurde ein Screening von antimikrobiellen Wirkungen von Lutensol-Typen durchgeführt. Mittels Anwendung des chemischen Prinzips von T-SAR (Thinking in structure-activity relationship) in der zweiten Phase der Studie und basierend auf den Screening-Ergebnissen, wurden die zugrunde liegenden Effekte der unterschiedlichen Klassen von bakterizid wirkenden Lutensolen auf zellulärer Ebene untersucht.

Es wurde festgestellt, dass der Kombinationseffekt weder für Lutensol AO 7 noch für Lutensole im Allgemeinen ein spezifisches Merkmal ist. Dieser Effekt wurde auch bei der Kombination anderer Salze mit verschiedenen Surfactanten beobachtet. Es wurde kein universell anwendbares Muster von diesem Effekt beobachtet, was darauf schließen lässt, dass der Kombinationseffekt von allen drei Variablen abhängt: vom anorganischen Salz, dem Surfactant sowie vom Modellorganismus. Es konnte gezeigt werden, dass die Ursache für den Zelltod die Beschädigung der Plasmamembran ist.

Ein weiteres Ziel war die Identifizierung der Lutensol-Typen mit dem kleinsten Kombinationseffekt, was sie zu guten Kandidaten für die Matrix-Lysis machen würde. Beim Lutensol TO 12 wurde in diesem Zusammenhang die niedrigste bakterizide Wirkung beobachtet.

1 Introduction

1.1 Microbes in the food environment

Food- and waterborne pathogens influence human health worldwide to a large extent and have a great impact on economy. Due to continuous human population growth, expanding poverty and insufficient standards of hygiene, these pathogens have a great impact on economy.

Moreover, people became more sensitive against infections in the last decades due to malnutrition, insufficient medical care and immunosuppression (Egli et al. 2002). Last but not least, people have changed their lifestyle and eating habits (e.g. increased demanding of ready-to-eat food). These changes come along with the reappearance of emerging pathogens (Egli et al. 2002). Variable host species can act as reservoir of an emerging pathogen, where the pathogen is well established and able to survive over a long time period. Human diseases caused by a pathogen persisting in an animal´s body are known as zoonoses (Meslin et al. 2000). Laura H. Kahn defines zoonoses as diseases transmitted from wild or domestic animals, posing threat for worldwide public health (Kahn 2006). The animals, acting as reservoirs, do not show any clinical symptoms (Woolhouse et al. 2005). A good example for the existence of such reservoirs is the reptile´s intestine acting as an ecological niche for *Salmonella enterica* serovars, (Geue and Löschner 2002). Switches from the host acting as a reservoir to a new host organism have led to pandemics in the past and pose also a major threat nowadays, e.g. *myxomatosis* causing a reduction of the rabbit population in Europe in the mid-twentieth century or the reduction of African ruminants by rinderpest during the late nineteenth century (Woolhouse et al. 2005). In humans, variant Creutzfeldt-Jakob disease (vCJD), one of four types of CJD, emerged after transmission of bovine spongiform encephalopathy (BSE) from cattle to human (Sikorska et al. 2012; Woolhouse et al. 2005).

High flexibility of pathogens is based on a horizontal transfer of genetic material to other microorganisms. This phenomenon was observed not only in the environment, but also in the intestine of warm-blooded organisms, where enterococci, commensal bacteria belonging to a normal intestinal flora, became multi-resistant (Egli et al. 2002). Altered strains may spread from animals to humans via the food chain. Investigation for the presence of the antibiotic-resistant bacteria, done by Boehme et al., has shown that common vegetables, such as tomatoes, salads or mushrooms, contain coliform bacteria ($10^4 - 10^7$ CFU / g) whereas a small percentage of them shows resistance against antibiotics ($0 - 10^5$ CFU / g) (Boehme et al. 2004).

In the food-processing industry, members of bacterial strains, such as *Salmonella enteritidis*, *Escherichia coli* serovar O157:H7 or *Campylobacter* species, raise concern due to outbreaks, occuring in the past. A well-known example is an international outbreak of listeriosis (between 2009/2010), when Quargel (acid curd cheese) has been distributed and sold in Austria, Germany, Czech Republic, Poland and Slovakia. Consumption of contaminated cheese has led to 34 clinical cases (8 of them were lethal), 25 of them in Austria, eight in Germany and one in Czech Republic. The examination of recalled cheese has revealed 16 samples of red smear type positive for *L. monocytogenes*. According to these results the conclusion has been done that contamination with *L. monocytogenes* has occurred during red smear process, whereas the growth of pathogen could have been easily supported by quargel cheese (Schoder et al. 2012; Fretz et al. 2010).

The bacterial quality and food safety are not only based on the final control, but it is a result of the chain of subsequent events. The guarantee of quality can be ensured only if the entire process, including the production of raw materials, production, distribution and handling, is strictly controlled. The sum of all control points is known under the abbreviation HACCP (hazard analysis and critical control points) (Hoorfar 2011). Additionally, control is not the exclusive factor which plays a crucial role. Quite in contrast, the entire chain of processing needs to be designed precisely (Hofstra et al. 1994).

Looking closer at the problem of raw material production, we realize that microbial communities have a great influence on many factors (e.g. health, productivity). On the one hand, microbes cause infections leading to the loss of a great amount of crop. On the other hand, nitrifying bacteria living in symbiosis with roots of distinct plants play a crucial role in the nitrogen fixation and thus have an influence on growth. Further, bacteria can provide high resistance against different types of stress and diseases (Morrissey et al. 2004).

Food production is a further step in the entire chain of a process. In case of fermentation, a bacterial culture (e.g. lactic acid bacteria) has to be used to start the process of acidification. Beside the main product (lactic acid), bacteria produce a large variety of aroma compounds, saccharides, enzymes, etc. These products are not only necessary for a longer storage of a product, but also for its unique design (Leroy and De Vuyst 2004).

1.1.1 *Escherichia coli*

Escherichia coli, previously known as *Bacterium coli*, is a gram-negative, facultative anaerobic, rod-shaped bacterium, commonly present in colon. It was named after its discoverer Theodor Escherich, a German-Austrian pediatrician who described its properties as first. Nowadays, five virotypes are known to cause diarrheal diseases: enterotox-

igenic *E.coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E.coli* (EAEC) (Madigan and Martinko 2006).

ETEC is the main causative agent of infant´s diarrhea, the symptoms vary from a minor discomfort to severe cholera-like syndrome (Gaastra and Svennerholm 1996).

Pathogenic mechanisms and kinds of clinical illness of EIEC resemble Shigella´s infections. EIEC is an intracellular pathogen, replicating within the host´s cytoplasm while (mostly colon epithelial cell) causing a destruction of the host cell (Madigan and Martinko 2006).

EPEC, as causative agent of a watery or even bloody diarrhea, leads to an infantile diarrhea in countries with low standards of hygiene (Kenny et al.1997). EPEC infection is characterized by small microcolonies on the surface of infected intestinal cell. Microvilli of the infected cell will be degraded successively (Kenny et al. 1997)

A characteristic feature of EAEC is the host cell invasion to colonize the intestinal mucosa, whereas EAEC can survive intracellularly up to 72 hours. This strategy helps EAEC to escape the clearance mechanisms of the host organism (Pereira et al. 2008).

EHEC primarily causes hemorrhagic colitis (HC), which can further develop into more severe hemolytic uremic syndrome (HUS). The largest reported outbreak of HUS epidemic in Germany was in 2011, when more than 90% of infected persons were adults (800 adults, according to Kemper 2012; over 830 adults, according to Mellmann et al. 2011), with a median age of 42, compared to 90 children. Further, in adult population, women were overrepresented. The causative agent was Shiga-toxin producing *E. coli* (STEC) O104:H4, with an incubation period of two or three days leading to watery diarrhea. After further incubation (approximately one week), HUS has developed (10% to 15% of children younger than ten were affected) (Mellmann et al. 2011). According to Markus J. Kemper, HUS is regarded to infect children mostly, causing an acute kidney injury. Additionally, the situation has become more serious due to neurological complications (e.g. convulsions or altered mental status of children) (Kemper 2012). As reported by Mellmann et al., the HUS-associated strains contained genes typical for enteroaggregative *E. coli* and enterohemorrhagic *E. coli*. A high grade of similarity with the chromosome of EAEC (serotype O104:H4) was detected in its chromosomal backbone. But EAEC-infection does not lead to severe diseases such as a HUS. Further, the genome of the HUS-associated strains did not contain *eae*-gene, coding for intimin, a protein typical for EHEC which is necessary for the adhesion to epithelial cells (Mellmann et al. 2011).

EHEC, as a moderately invasive bacterium producing verotoxins, can be spread via water and food (e.g. raw milk, sandwiches and unpasteurized juice). Fukushima et al.

have described the importance of cattles as reservoir for the pathogenic strain of EHEC, producing Shiga-toxin (Fukushima et al. 1999).

1.1.2 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, facultative anaerobic microorganism, named after its discoverer Joseph Lister, an English surgeon. The name *monocytogenes* indicates the ability of this bacterium to induce an increase of a number of monocytes in the blood stream upon infection. Groups that are especially at risk include newborns, pregnant women and elderly and immunocompromised persons (Slutsker and Schuchat 1999). *L. monocytogenes* can be detected throughout the environment in soil, vegetables and animals (Allerberger and Wagner 2010) and can be transmitted by the consumption of contaminated food. This bacterium is able to adapt to a variety of stress conditions such as very low temperatures, vacuum, high salt concentrations and others. It enables growth not only during the processing of food but also during the storage. Thus, it can overcome food preservation and persist in processed food products (Lou and Yousef 1999; Gandhi and Chikindas 2007). To be able to survive and grow, specific mechanisms, such as biofilm formation and quorum sensing are of great importance (Gandhi and Chikindas 2007). Listeriosis can become lethal in the worst case, as reported by Fretz et al. (Fretz et al. 2010). Clinical manifestations of listeriosis included a febrile gastroenteritis and severe invasive forms, such as meningitis, rhombencephalitis, sepsis, perinatal infections and abortion (Allerberger and Wagner 2010). Due to the long incubation time of listeriosis, it is difficult to retrospectively identify the original contaminated food product (Gandhi and Chikindas 2007). The incidence of listeriosis in the last decade has increased in some countries due to changes in dietary practices, when minimally processed and ready-to-eat food is requested (Rocourt and Bille 1997).

The adaptation to a wide range of temperature (2°C – 45°C) and the ability to survive and grow at 2°C - 4°C make it difficult to control *L. monocytogenes* in food.

Another challenge is its ability to withstand a low pH value (e.g. in the stomach or within the phagolysosome of macrophage). Experiments have shown that *L. monocytogenes* induces a specific program called acid tolerance response (ATR) upon exposure to a mild acidic environment (pH = 5.5) (O´Driscoll et al. 1996). Despite of the acidic conditions outside of the cell, the cytoplasmic pH has to be maintained. For this purpose, transport of H^+ across the plasma membrane has to occur either by coupling a proton transport with the transport of an electron in a respiratory chain (aerobic bacteria) or via proton ATPases, utilizing energy from ATP hydrolysis. *L. monocytogenes*, as a facultative anaerobic microorganism, can use both means (Shabala et al. 2002). Another way how to survive in an acidic environment is the activation of glutamate decarboxylase (GAD). This mechanism is encoded by three genes (*gadA,B,C*), whereas A and B en-

code the enzyme decarboxylase and C encodes an antiporter transporting glutamate into cytoplasm and γ-aminobutyrate in the opposite direction (Cotter et al. 2001). Gamma-aminobutyrate is slightly alkaline causing a pH increase within the environment. Further, utilization of one intracellular proton leads to an increase of cytoplasmic pH (Small and Waterman 1998).

Any kind of stress is sensed by a system with two components, consisting of a membrane-bound histidine kinase and a cytoplasmic response regulator inducing the alteration of gene expression (Cotter et al. 1999).

An important mechanism of protection is the formation of a biofilm. These complex structures can be formed on a wide range of surfaces (e.g. medical devices or equipment of food processing facilities). Bacteria contained within the biofilm can be transferred easily to the processed food. Observation done by Hefford et al. in 2005 has shown that the physiology of biofilm-grown bacteria differs from that of free living (planktonic) cells (Hefford et al. 2005). Further, *L. monocytogenes* is able to create monoculture biofilms and mixed culture biofilms (e.g. together with *Flavobacterium*) (Bremer et al. 2001). A biofilm formation is a material- and energy consuming process, thus it is started first when cell density threshold is reached. The sensing of the cell density is provided by cell-to-cell communication, also known as a quorum sensing, when an autoinducer produced and released by one cell is recognized by a neighbour cell (Xavier and Bassler 2003; Bassler 2002). Gram-positive bacteria produce oligopeptides acting as an autoinducer, whereas autoinducers of gram-negative bacteria are acylated homoserin lactones (Winans and Bassler 2002).

1.1.3 *Salmonella enterica, ssp. enterica, serovar* Typhimurium

Salmonella enterica, ssp. enterica, serovar Typhimurium is a gram-negative rod-shaped, flagellated, aerobically growing bacterium. Non-typhoidal *Salmonella* is a causative agent of mild and severe infections and a leading cause of human gastroenteritis (Helms et al. 2005; McClelland et al. 2001). Further, it is used as a mouse model of human typhoid fever (Neidhardt et al. 1996). *Salmonella enterica* serovars have a wide range of hosts within mammals and birds (Rabsch et al. 2002). A natural ecological niche is the intestine of warm-blooded vertebrates, but *Salmonella* was detected also in cold-blooded vertebrates (e.g. reptiles) from which it can be dispersed into the environment. Thus, reptiles and mammals are natural reservoirs showing no clinical symptoms. A reptile´s intestine is populated by various serovars of many *Salmonella* subspecies, whereas different serovars can be present simultaneously (Geue and Löschner 2002). According to the report written by S. M. Long, between 1992 and 2000 more than 1500 outbreaks of infectious intestinal diseases caused by foodborne pathogens have been recorded, whereas Salmonella´s serovars were reported to be the most frequent causa-

tive agent (41.0%), followed by Norwalk-like virus (15.7%). In 83 cases (5.5%) only, consumption of salad vegetables or fruits was the reason for an infection (Long et al. 2002). Ethelberg et al. report about a restaurant-associated outbreak of *S.* Typhimurium infection caused by a multidrug-resistant strain. Laboratory tests confirmed 67 positive cases, but the number of patients was estimated to be greater up to six times. The investigation has revealed the assistant chef to be the infected person whereas the infection has been spread via the buffet prepared by him (Ethelberg et al. 2004). Naqid et al. have shown in their study that probiotic *Lactobacillus plantarum B2984*, as part of a diet (10^{10} cfu per day and animal) induces an increased production of IgM and IgG detectable in serum, and thus an enhanced response to *S.* Typhimurium infection. On the other hand, simultaneous administration of pro- and prebiotic in a symbiotic diet did not lead to a synergic effect. This suggests that a symbiotic diet does not have to lead to the immune response of much higher efficiency compared to a diet when either pro- or prebiotics are administered separately. Based on these results, using lactic acid bacteria could be one of the strategies how to enhance the immune response of a pig against zoonotic pathogenic bacteria (Naqid et al. 2015). In the study done by Graziani et al., the resistance of *S.* Typhimurium strains, isolated in Italy within the period of 2002 – 2004 has been examined. According to the results, two-thirds of all isolated strains have shown a multiple resistance against four and more antibiotics. Further, *S.* Typhimurium is a main cause of human infections in Italy. On the contrary, in other European countries, *S.* Enteritidis serovar is the agent, mostly isolated from infected persons. In case of *S.* Typhimurium, resistance against ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline were phenotypes observed most frequently (Graziani et al. 2008).

1.2   Detection of foodborne pathogens

Pathogenic bacteria mentioned in the previous chapter raise concern for human health. Thus, it is necessary to develop methods detecting pathogens efficiently during food processing. To detect foodborne pathogens, microbiological methods and methods of molecular biology can be applied. Culture methods are based on plating out of the sample with a putative foodborne pathogen on agar and an overnight incubation (or even longer, in case of *L. monocytogenes* up to 48 hours) enabling colony counting upon the incubation. This principle is time-consuming. Nowadays, a rapid detection of foodborne pathogens plays a crucial role in the food-processing industry. The methods of molecular biology (e.g. quantitative PCR) enable this parameter. Foodstuff, a priori a heterogeneous matrix, has to be purified to avoid inhibitory processes during PCR (Rossmanith and Wagner 2011). Many specific DNA and RNA sequences can be used as targets in

that way to investigate the microbiological quality of water and foodstuff. This is also the way how to improve the selectivity of identification.The detection of non-culturable species cannot be done by classical microbiological methods, but it can be detected using DNA or RNA sequences, as mentioned above (Egli et al. 2002). For the direct quantification of pathogenic bacteria in a food matrix, following requirements need to be fulfilled: (a) A sufficient sample volume has to be analysed, (b) recovery rates need to be reproducible and (c) putative inhibiting substances need to be removed to enable the use of alternative molecular methods (Mester et al. 2010).

1.3   The analytical chain

In food science, the contamination at very low levels and the complexity of food matrices are the major hurdles for an application of novel methods, developed for clinical investigations primarily. Microbiological methods can be classified as qualitative and quantitative. The former one answers the question if a pathogenic bacterium is present or not. The second one estimates the population of a detected pathogen. The methods for detecting pathogenic bacteria are qualitative in most cases – they detect the presence or absence of a target microorganism. To execute the qualitative detection, food matrices containing a putative pathogen are incubated in a nutrient medium for several hours to allow the growth of a target organism (enrichment culture), which is better detectable in subsequent analysis (Brehm-Stecher et al. 2009).

Further, Brehm-Stecher et al. emphasize that this detection, as a last step of the analytical chain, is of low significance if it is not fully in compliance with the previous steps, such as the isolation and the concentration of the sample. They suggest to develop a method, which is independent from the enrichment step and which delivers a sample of a sufficient size for a subsequent pathogen detection providing quantitative data (Brehm-Stecher et al. 2009).

1.3.1 Sample preparation

Separation of target organisms from the food matrix is an important step in the analytical chain, because food residues could inhibit the detection of microorganisms (mainly enzymatic reactions, such as qPCR). Separation and concentration lead to an improvement of sensitivity (lower detection threshold) and to a facilitated detection of pathogens (Brehm-Stecher et al. 2009). Nowadays, four categories of sample separation are known: physical, chemical, adsorptive and bioaffinity-based.

Physical separation is based on a centrifugation and subsequent filtration of samples. Adsorptive processes are based on applying absorbent material (resins, lectins) to cap-

ture the target organism and to remove the residual food matrix. Bacteria can be separated by exploiting their natural properties – this is the principle of a bioaffinity separation, when magnetic beads are coupled with antibodies, specific against antigen present on the surface of a distinct pathogen. A disadvantage of this method is that antibodies are only specific against single species (Brehm-Stecher et al. 2009).

To be able to detect pathogens in food matrices, a matrix volume has to be diminished (6.25-g or 12.5-g portions of matrix → 100 µL sample) (Mayrl et al. 2009; Rossmanith et al. 2007) and inhibitory substances need to be removed (e.g. many food ingredients inhibit the PCR) (Rossmanith et al. 2007). Removal of inhibitory substances is done by the sample preparation. The large heterogeneity of foodstuffs does not allow to develop an all-in-one separation method applicable for all food matrices as well as suitable for the detection of each pathogenic microorganism (Mester et al. 2014). A further requirement is the complete removal of fat-, carbohydrate- and protein-rich fractions from the food matrix, whereas special attention should be given to the removal of fat-rich fractions (Rossmanith et al. 2007). There was a need to develop a Matrix-Lysis protocol applicable on a wide range of food matrices (ranging from raw milk to carbohydrate-rich cooked products). The process of sample preparation results in a homogeneous sample, which can be further investigated either by microbiological methods (plate count method) or by methods of molecular biology (qPCR). The process of removal is also known as pre-PCR processing, described by Rådström et al., when complex food matrices are converted into samples amplifiable by PCR (Rådström et al. 2004).

Brief summary of the aim of the sample preparation:

- sufficient sample size upon procedure

- removal of inhibitors (e.g. qPCR inhibitors)

- complete removal of fat, carbohydrates and proteins

- enrichment of a potential pathogen (if present) in a small volume (≤ 200 µl)

1.3.2 **Matrix-Lysis**

A Matrix-Lysis protocol has been developed to solubilize and homogenize the food matrix prior to pathogen detection. To establish an appropriate protocol, combinations of detergents, buffer systems and hydrophilic substances were tested (Rossmanith et al. 2007). Further, pathogenic bacteria, if present in the matrix, have to stay alive to be detectable not only by methods of molecular biology, but also by microbiological methods (plate count methods). Upon solubilization and homogenization, bacteria needed to be separated unspecifically. Another challenge was to diminish a sample volume – conversion of 6.25-g or 12.5-g portions of heterogeneous foodstuff (Mayrl et al. 2009;

Rossmanith et al. 2007) into 100 μl of a homogeneous sample. Further, the process of Matrix-Lysis had to be an inexpensive rapid method (< 5 hours, according to Mester et al. 2010 or even 3 hours, according to Mester et al. 2014).

1.3.2.1 Development of Matrix-Lysis buffer systems

A milestone has been achieved with a successful establishment of the first Matrix-Lysis buffer, consisting of 1x PBS, 8 M urea and 1% SDS. Applying of this buffer solution caused a dissolution of fat residues and the subsequent washing step with 1x PBS enabled the collection of *L. monocytogenes*. Series of experiments have shown that maximal performance (in sense of pellet size reduction) was reached in a pH range of 7.0 to 9.5, 45°C to be the optimal temperature and 30 min. to be the optimal incubation duration (Rossmanith et al. 2007). A disadvantage of this buffer is that the viability of gram-positive bacteria (e.g. *L. monocytogenes*) was compromised strongly, which can be explained by a presence of the 1% SDS. The samples treated with this buffer could be examined by qPCR only, but not by a plate count method (Rossmanith et al. 2007). Another challenge under investigation was to establish a Matrix-Lysis protocol for gram-negative bacteria, which would be detectable by qPCR during the downstream investigation.

This has been achieved by replacing 1% sodium dodecyl sulphate by 1% Lutensol AO 7. Further, due to this modification the range of the food matrices could be broadened – this buffer system has shown high effectivity in solubilizing of UHT milk, raw milk, yoghurt, mozzarella and cottage cheese (Mayrl et al. 2009). Applying this buffer system has led to pellets of a reasonable size, containing intact bacteria (Mayrl et al. 2009). Similar to the first buffer, a temperature optimization had to be done to find an appropriate temperature if gram-negative bacteria (*E. coli*, *S.* Typhimurium) are targeted in the process of Matrix-Lysis. Series of experiments have revealed that 37°C is the appropriate temperature for *E. coli* and 45°C for *S.* Typhimurium (Mayrl et al. 2009). To demonstrate the potential of this novel buffer system to detect pathogenic bacteria in food matrices, quantitative PCR was done subsequently (Mayrl et al. 2009).

A major disadvantage of the previous two buffer systems was the reduction of the viability of target microorganisms due to the chemicals applied in the process of Matrix-Lysis. The development of the third buffer system was based on replacing urea (acting chaotropically) and detergents (dissolving the lipid-rich fraction of matrix) by ionic liquids (Mester et al. 2010). Ionic liquids are organic salts with a low melting point. Due to their negative Gibbs free energy they are in liquid state at room temperature. These non-flammable and highly stable chemicals (Endres and Zein El Abedin 2006) have been proved as effective solvents for solubilizing diverse biological molecules (Stevens and

Jaykus 2004). The Ionic liquid [emim]SCN (1-ethyl-3-methylimidazolium thiocyanate) combined with 1x PBS was revealed as a good solvent reducing a food matrix sufficiently if applied in a concentration range from 5 to 50% (v/v). The size of pellets at the end of the Matrix-Lysis process was ≤ 200 µl. The most probable explanation for a high efficiency of [emim]SCN as a solvent is based on both, a chaotropically acting thiocyanate anion solving protein-rich fractions of the matrix and the [emim] cation acting as detergent solving lipids (Mester et al. 2010). Further, [emim]SCN had to be tested for its ability to dissolve the food matrix and to protect the viability of the model organisms (*L. monocytogenes* and *S.* Typhimurium). Series of experiments have shown that [emim]SCN in a concentration range of 5 to 7.5% combined with 1x PBS fulfills both prerequisites. Disadvantageous was the diverging recovery rate of both model organisms, which can be explained by an injury of microorganisms during the Matrix-Lysis procedure (Mester et al. 2010).

Magnesium chloride is applied in many industrial branches, e.g. production of magnesium metal or applying as food additive (E511). Further, it is applied in in-vitro investigation of DNA and RNA molecules. The study done by Mester et al. 2014, shows that $MgCl_2$ can be applied in the process of Matrix-Lysis due to its ability to solubilize the protein in an aqueous solution. In this study, the lysis of foodstuff, (6.25-g or 12.5-g portions) has been provided in 1 M magnesium chloride solved in a tricine-buffered solution (50 mM) of neutral pH. The resulting pellet was re-suspended in washing buffer containing Lutensol AO 7 (0.35% v/v) and 1x PBS prior to pathogen detection. Series of experiments have proved this combination to be appropriate to dissolve the food matrices (mainly dairy products) (Mester et al. 2014). Compared to the previous three Matrix-Lysis buffers, the $MgCl_2$-based buffer has the following advantage: The average recovery rate upon artificial contamination was of 82% for *S.* Typhimurium and of 73% for *L. monocytogenes*, respectively. Based on these results, the $MgCl_2$-based buffer system seems to be an excellent tool for sample preparation, whereas pathogens are directly detectable by both, microbiological methods and methods of molecular biology (Mester et al. 2014). Further, the $MgCl_2$-based buffer system is advertised as non-toxic and environmentally friendly compared to ionic liquids used in the previous buffer system. Disadvantageous is the fact that the interaction of Mg^{2+} ions with proteins is highly dependent on the pH value. At low pH value, when the concentration of hydronium ions is high, proteins are positively charged, which in turn leads to electrostatic repulsion of bivalent magnesium ions. To circumvent the problem of insufficient lysis of acidic matrices, a high buffer concentration was applied (Mester et al. 2014).

Based on the knowledge mentioned above, it became clear that a detergent is necessary in the process of Matrix-Lysis due to its ability to dissolve the fat-rich fraction of the

food matrix. A strong ionic detergent (e.g. SDS) cannot be applied due to its high toxicity against *L. monocytogenes*. Further, combination of urea (8 M) with Lutensol AO 7 has led to a reduction of viability of target organisms. Application of ionic liquids caused diverging recovery rate of model organisms. Last but not least, $MgCl_2$ in combination with Lutensol AO 7 has shown a combinational effect (Kwisda 2012), which is the reason why the viability of target organisms has been compromised.

For a further improvement of the Matrix-Lysis approach, it was paramount to identify a Lutensol´s grade showing only a minimal combinational effect in combination with $MgCl_2$.

1.4 Surfactants

Surfactants (surface-active agents), also known as detergents, are compounds which lower the tension between two phases by altering the free interfacial energies of both surfaces. That is the reason for the modification of the interfacial properties of liquids (Rosen and Kunjappu 2012; Barel et al. 2001). The interfacial free energy is the energy (in form of work), which is necessary for the formation of an interface (Rosen and Kunjappu 2012). The structure of surfactants is asymmetric - divided into a hydrophobic (mostly an alkyl side chain) and a hydrophilic part (ionic head group or group with a free electron pair). In some cases, the hydrophobic moiety of surfactants can consist of polydimethylsiloxane or perfluorocarbon (Barel et al. 2001).

In aqueous solutions, the hydrophobic part shows a specific behavior, creating large aggregates, also known as micelles (Wennerström and Lindman 1979). Micellization is driven by hydrophobic interactions. Characteristics of these molecules are the rapid motion of side chains and the interaction of polar head groups with polar solvents (e.g. water) to establish water-amphiphil contact in micelles (Wennerström and Lindman 1979). The last one determines the interfacial activity of the surfactant. Both of them have to be present in distinct ratio to provide the correct function of the entire molecule (Wennerström and Lindman 1979). Surfactants used as a cleaner need to have good wetting properties and an ability to eliminate and solubilize the dirt preventing the redeposition (Dallmann 2011).

Surfactants are of great economic importance. In 2008, in Western Europe, the overall production of surfactants was about three million tons, whereas the anionic and non-ionic surfactants represented the biggest group with a market share of 41% and 47%, respectively (Dallmann 2011).

1.4.1 Classification of surfactants

From the chemical point of view, an appropriate classification is based on the presence of the solubilizing head group. All surfactants are divided into four primary classes, based on the nature of the main solubilizing group: anionic, cationic, non-ionic and amphoteric.

<div align="center">

Anionic: $^{(-)}$Head - Tail

Cationic: $^{(+)}$Head – Tail

Non-ionic: Head – Tail

Amphoteric: $^{(+)}$Head$^{(-)}$ – Tail

</div>

On the other hand, according to classification by application, surfactants are divided into the following groups: emulsifiers, wetting agents, foaming agents and dispersants. The classification according to physical characteristics is also possible – in this case, the solubility in water or in oil plays a key role. Another type of classification is based on the chemical structure of the material, e.g. the structure of the linking group between hydrophilic and hydrophobic part like (oxygen, nitrogen, amide, sulfonamide) (Myers 2006).

To imagine the process of the surfactant synthesis, its approximation to build a house with Lego building blocks is suitable. A large variety of building blocks with different colours and forms leads to a large diversity of house building. In case of surfactant synthesis a similar situation exists. A branched-chain alkyl groups, an unsaturated alkenyl chains, alkylbenzenes, alkylnapthalenes, fluoroalkylgroups, polydimethylsiloxanes, polyoxypropylene glycol derivatives, biosurfactants and derivatives of natural and synthetic polymers can be used as a hydrophobic tail of the surfactant. The hydrophobic tail is combined with head groups, acting either as a polar group containing free electron pairs or as a charged group. The netto charge of a head group depends on the presence or absence of the electron (Myers 2006). According to this, we distinguish negatively charged surfactants [e.g. sulfonates $(SO_3)^-$, sulfates $(-O-SO_3)^-$, carboxylates $(-COO)^-$ also known as soaps and phosphates $(PO4)^{3-}$], representing economically the most important group with 56% of the world production (Dallmann 2011). The other group comprises positively charged surfactants having an quarternary ammonium cation $[N^+(R)_4]$ as head group.

The charged head group provides an interaction with molecules of polar solvents. Their effectiveness in solubilizing of hydrocarbon side chains is very high – one sulfate or one quarternary ammonium is sufficient to solubilize the C_{12}-chain (Lomax 1996).

Large structural variety of hydrophobic side chains and their combination possibilities with different head groups lead to a diversity of end products on the one hand, but it might cause a problem to choose appropriate precursors for the surfactant synthesis in term of its further application on the other hand (Myers 2006).

Amphoteric surfactants have bivalent activities – they act either as cations or as anions depending on the pH-value of the solution. The wide-range solubility of amphotere surfactants is based on the presence of both – anionic and cationic groups. These are soluble even at low and high pH, in contrast to the non-ionic surfactants (Lomax 1996). Amphoteric surfactants are compatible with other classes of surfactants able to create mixed micelles (Dallmann 2011).

The characteristic feature of non-ionic surfactants is the presence of elements containing at least one free electron pair. In case of polyethers, the oxygen is the element with two free electron pairs. The effectiveness of the non-ionic surfactants is much smaller than the one of the ionic surfactants. Up to ten ethylene oxide units ($-CH_2-CH_2-O-$) are needed to reach the water-solubility comparable to those of one ionic group (Lomax 1996). Further, non-ionic surfactants are characterized by their acid resistance and their high tolerance against water hardness. Due to these features, non-ionic surfactants are successful products with a market share of 47% (Western Europe, 2008) (Dallmann 2011).

The water-solubilizing of the non-ionic surfactants is based on the intermolecular bonding of the hydrophilic part of surfactants and water molecules. The occurance of the counter-ions does not play any role, thus the non-ionic surfactants are dissolved over the entire pH range (Lomax 1996). This type of detergents breaks the lipid – lipid and lipid – protein interactions efficiently and is not suited for the interruption of protein - protein interaction (Bhairi 2001). Another characteristic feature of non-ionic detergents is the cloud point. This is a particular temperature, when non-ionic detergents become cloudy and undergo phase separation – a detergent-rich phase and an aqueous phase. The presumable reason for phase separation is the hydration decrease of the head group (Bhairi 2001).

1.4.2 **Lutensols**

As emulsifiers, wetting agents and dispersants in household and industry, Lutensols, as non-ionic detergens, have a wide range of application. They are produced on a large scale by BASF. Nowadays, there are about 250 Lutensol´s types available. Their effect is based on ethoxy groups ($-CH_2-CH_2-O-$) present in the side chain of molecules. The number of ethoxy groups characterizes physical and chemical properties of each Lutensol´s grade. Combination of Lutensol[®] with other surfactants (ionic or non-ionic) is possible and leads to a synergistic effect (Lutensol[®] AO types 2004, Lutensol[®] TO types 2004 and Lutensol[®] XP types 2003). This type of detergents is also compatible to sulfonates, sulfates and to sulfonated and sulfated detergents. Lutensols, as non-ionic detergents, seem to be a good compensation for pentasodium triphosphate ($Na_5P_3O_{10}$), whose widespread use has presumably led to eutrophication in the past. On the other

hand, the performance of Lutensols is much smaller than the performance of pentasodium triphosphate, thus the proportion of Lutensol in total surfactant content had to be increased (Lutensol® AO types 2004 and Lutensol® TO types 2004). Lutensol® AO types, as ethoxylated medium chain fatty alcohols, control the process of foaming in the household detergents, whereas the amount of produced foam depends also on the temperature. High performance and efficiency of liquid laundry detergents are achieved by non-ionic detergents with ethylene oxide in the side chain (Lutensol® AO types 2004). Lutensol® AO 3 is applied mainly as emulsifier and detergent. Lutensol® AO types of medium to high ethoxylation grade (e.g. AO 7) show excellent detergency and wetting power producing moderate amounts of foam. All Lutensol® AO types are up to 90% biodegradable (Lutensol® AO types 2004). In case of grades of Lutensol ® TO types, emulsifying effect becomes greater in combination with Emulan®.

Some grades of Lutensol® (TO types, XP types and AO types) are highly hygroscopic and must be stored in a dry place. The storage rooms do not have to be overheated, but the storage temperature should not drop below 20°C. Distinct types (e.g. TO 3, TO 6, TO 7, TO 8 and most of XP types) have cloudy appearance at room temperature and form sediments if they are stored over a long time period (Lutensol® AO types 2004, Lutensol® TO types 2004, and Lutensol® XP types 2003).

Grades of Lutensol® have found application also in other industrial branches – e.g. in preparation of the mesoporous TiO_2 samples (Kim et al. 2007). As reported by Eiden-Assmann, Lutensol® has an influence on size and size distribution of colloidal TiO_2 particles. The shorter the hydrophobic part of added Lutensol®, the larger TiO_2 colloids obtained during the synthesis. On the contrary, the length of hydrophilic part does not have any influence on the size of TiO_2 colloids (Eiden-Assmann et al. 2004).

1.5 Experimental approach

Based on this experience, the question has arisen as to whether all Lutensols show the same bactericidal effect in combination with $MgCl_2$ as AO 7. To answer this question, ten Lutensols (AO 7 including) were tested alone and in combination with $MgCl_2$.

E. coli and *L. monocytogenes* were chosen as model organisms, because their presence in dairy products is a major threat for public health.

S. Typhimurium, a gram-negative bacterium, was chosen as the third organism to extend the results. Together with *E. coli*, it has a tiny cell wall containing peptidoglycan positioned between plasma membrane and outer membranes. This anatomy does not provide efficient resistance against environmental stresses (e.g. high osmotic pressure or high temperature). Thus, they are being considered to be more sensitive than *L. monocytogenes* having a thick cell wall.

The major part of the investigation was dedicated to screening determining the influence of the selected Lutensols on the one hand and $MgCl_2$ on the other hand, on growth ability of model organisms.

Lutensols are biodegradable. They are not being considered to be a threat for the environment. Magnesium chloride is occurring naturally in the environment in form of hexahydrate known as bischofit. It is highly soluble in water, that´s why there is hypersaline brine of salt concentration higher than 6 M. In contrast to NaCl-rich environments, $MgCl_2$-rich environments seem to be sterile. This can be based on two major characteristics of magnesium chloride: either reduction of water activity or chaotropic activity (Hallsworth et al. 2007).

1.6 Main Goal

The aim of this study was to discover if the combinational effect is general characteristics of i) all Lutensols combined with $MgCl_2$, ii) other surfactants combined with $MgCl_2$ and iii) surfactants combined with other inorganic salts. Further questions were oriented toward the underlying principles of this combinational effect. Does the effect depend on the pH-value of the environment? What happens to bacteria? How does the way of killing look like (e.g. cell lysis, DNA damage, cell wall damage, protein synthesis inhibition)?

2 Material and Methods

2.1 Chemicals

Table 2.2 and Table 2.2 list all chemicals and equipment used in the experiments.

Table 2.1 Equipment

Equipment	Producer
16 ml tubes	Sarstedt (Nümbrecht-Rommeldorf, D)
50 ml Falcon-tubes	Greiner bio-one (Kremsmünster, AT)
96 well cell culture cluster Flat bottom with lid	Corning, Inc. (Corning, NY, USA)
Agilent HP 8452 A Diode Array Spectrophotometer	Hewlett Packard (Vienna, AT)
analytical balance Sartorius TE 214S	Sartorius (Göttingen, D)
ATP, Bac Titer-Glo^{TM} Microbial Cell Viability Assay	Promega Corporation (Madison, WI, USA)
camera Leica DFC300FX	Leica (Vienna, AT)
centrifuge 5424	Eppendorf (Hamburg, D)
centrifuge 5810R	Eppendorf (Hamburg, D)
Gilson pipettes (10 µl; 100 µl; 1000 µl) eppendorf research plus	Eppendorf (Hamburg, D)
inverse confocal laser scanning microscope Leica DM IRB	Leica (Vienna, AT)
microcentrifuge tubes 1.5 ml	Eppendorf (Hamburg, D)
microcentrifuge tubes 2.0 ml	Eppendorf (Hamburg, D)
multichannel pipette (100 µl) eppendorf research plus	Eppendorf (Hamburg, D)
NucleoSpin Tissue Kit	Macherey-Nagel (Düren, D)
Petri dish (9 cm)	Thermo Fischer Scientific (Waltham, USA)
pH-Meter Thermo Scientific Orion 3-Star benchtop	Thermo Fischer Scientific (Waltham, USA)
pipette boy	Integra Bioscience (Konstanz, D)
pipette tips	Biozym (Oldendorf, D)
semi-micro cuvettes	Greiner bio-one (Kremsmünster, AT)
TECAN infinite F200	TECAN (Männedorf, CH)
Thermomixer compact	Eppendorf (Hamburg, D)
Thermocycler Stratagene MX3000P	Life Technologies (A Thermo Fischer Scientific Brand, Fischer Scientific Austria, Vienna, A)
vortex Genius 3	IKA (Staufen, D)

Table 2.2 List of used chemicals

Chemical	Producer
Brij L23	Sigma Aldrich Chemie GmbH (Steinheim, D)
$CaCl_2$	Merck (Darmstadt, D)
EDTA	Merck (Darmstadt, D)
Emulan HE 51	BASF (Ludwigshafen, D)
Igepal CA-630	Sigma Aldrich Chemie GmbH (Steinheim, D)
KCl	Merck (Darmstadt, D)
K_2CO_3	Merck (Darmstadt, D)
KH_2PO_4	Merck (Darmstadt, D)
K_2HPO_4	Merck (Darmstadt, D)
$K_3PO_4*7H_2O$	Merck (Darmstadt, D)
K_2SO_4	Merck (Darmstadt, D)
LiCl	Merck (Darmstadt, D)
Lutensol (Types TO; AO; XP)	BASF (Ludwigshafen, D)
$MgCl_2*6H_2O$	Merck (Darmstadt, D)
$MgSO_4x7H_2O$	Merck (Darmstadt, D)
NaCl	Merck (Darmstadt, D)
Na_2CO_3	Merck (Darmstadt, D)
NaH_2PO_4	Merck (Darmstadt, D)
Na_2HPO_4	Merck (Darmstadt, D)
Na_2SO_4	Merck (Darmstadt, D)
N-Lauroylsarcosine	Sigma Aldrich Chemie GmbH (Steinheim, D)
NH_4Cl	Merck (Darmstadt, D)
$(NH_4)_2CO_3$	Merck (Darmstadt, D)
$NH_4H_2PO_4$	Merck (Darmstadt, D)
$(NH_4)_2HPO_4$	Riedel - De Haën (Seelze-Hannover, D)
$(NH_4)_2SO_4$	Merck (Darmstadt, D)
Plurafac LF 300	BASF (Ludwigshafen, D)
Plurafac LF 221	BASF (Ludwigshafen, D)
Plurafac LF 220	BASF (Ludwigshafen, D)
Pluronic PE 6100	BASF (Ludwigshafen, D)
Pluronic PE 6200	BASF (Ludwigshafen, D)
Pluronic PE 9200	BASF (Ludwigshafen, D)
SDS	Fischer Scientific (Loughborough, UK)
$SrCl_2$	Merck (Darmstadt, D)
Tris HCl	Calbiochem (La Jolla, USA)
Triton X-100	Merck (Darmstadt, D)
Tryptone soy agar granulated	Biokar diagnostics (Beauvais, F)
Tryptone soy broth	Oxoid (Basingstoke, UK)
Tween 20	Merck (Darmstadt, D)
Tween 80	Merck Schuchardt (Hohenbrunn, D)
Yeast extract	Biokar diagnostics (Beauvais, F)
$ZnCl_2$	Merck (Darmstadt, D)

2.1.1 **Solutions**

In order to prepare aqueous solutions of given molarity, a given volume or amount of the respective substance was solved in corresponding volume of deionized water. Solutions were sterilized by autoclaving at 121°C for 15 minutes, unless otherwise specified.

2.1.1.1 **PBS stock solution (10x concentrated)**

Phosphate buffered saline is used for maintaining the osmotic equilibrium and the pH-value of bacteria during experiments. A 10-times concentrated stock solution was prepared, from which an aliquot was taken and mixed with distilled water in ratio 1:9 to prepare 1x PBS. The pH-value was adjusted to 7.47 with hydrochloric acid.

Table 2.3 Composition of 10x PBS

substance	conc. (g/l)
NaCl	80
KCl	2
Na_2HPO_4	14.4
KH_2PO_4	2.4

2.1.1.2 **MgCl$_2$ stock solution (4 mol/l)**

Magnesium chloride hexahydrate, known as bischofite, is extremely soluble in water enabling a highly concentrated stock solution of 4 M.

Table 2.4 Composition of MgCl$_2$ stock solution

substance	conc. (mol/l)	volume (l)	weighing (g)
$MgCl_2*6H_2O$	4	0.5	406.6

2.1.1.3 Pre-lysis buffer

For an efficient DNA extraction, some bacterial strains, especially gram-positive bacteria, are more difficult to lyse, thus they have to be pre-incubated with lytic enzymes (lysozymes) solved in buffer, prepared according to the recommendation of the manufacturer (Genomic DNA from Tissue, User Manual, NucleoSpin® Tissue, Macherey-Nagel, Düren D). A lyophilised lysozyme was added immediately before the experiment.

Table 2.5 Composition of pre-lysis buffer

substance	concentration
Tris / HCl	20 mM
EDTA	2 mM
Triton X-100	1% (v/v)
pH	8

2.1.1.4 Salts

Table 2.6 lists all stock solutions of the various salts that were prepared in this project.

Table 2.6 Salt stock solutions

salts	mol/l	salts	mol/l
NH_4Cl	4	KH_2PO_4	1
$NH_4H_2PO_4$	2	K_2HPO_4	4
$(NH_4)_2HPO_4$	4	$K_3PO_4*7H_2O$	2
$(NH_4)_2CO_3$	2	K_2CO_3	2
$(NH_4)_2SO_4$	2	K_2SO_4	0.5
NaCl	2	NaH_2PO_4	4
KCl	2	Na_2CO_3	2
$CaCl_2$	2	Na_2SO_4	1
$ZnCl_2$	2	$MgSO_4*7H_2O$	1
$SrCl_2$	2	LiCl	1

2.1.2 **Surfactants**

The preparation of 10% solutions was done as follows: Liquid surfactants were mixed with distilled water in a ratio of 1:9. In case of solid surfactants, ten grams of each were solved in distilled water under constant stirring and heating and adjusted to a final volume of 100 ml. No surfactant´s solutions were autoclaved.

2.1.2.1 Lutensols

Lutensols are non-ionic surfactants. Because of their light sensitivity, both, the precursors and the aqueous solutions were stored in darkness protected from light. The numeric code in the product name indicates the total degree of ethoxylation.

Lutensol® TO types are made from saturated iso-C_{13} alcohol. They conform to the following structural formula:

R-O-$(CH_2$-CH_2-$O)_x$-H

R = iso-$C_{13}H_{27}$
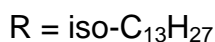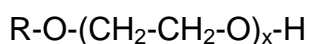
x is the number of ethoxy groups within one molecule (e.g. Lutensol TO 3 - has 3 ethoxy groups)

Figure 2.1 Structural formula

Table 2.7 Chemical and physical properties of 6 Lutensol® TO types, source: Lutensol® TO types, Technical information, BASF The chemical company, September 2003, ® = registered trademark of BASF Aktiengesellschaft

TO 3	molar mass (g/mol)	ca. 340
	physical form (23°C)	liquid
	pH (5% in water)*	ca. 7
	degree of ethoxylation	ca. 3

TO 6	molar mass (g/mol)	ca. 470
	physical form (23°C)	liquid
	pH (5% in water)*	ca.7
	degree of ethoxylation	ca. 6

TO 7	molar mass (g/mol)	ca. 500
	physical form (23°C)	liquid
	pH (5% in water)*	ca.7
	degree of ethoxylation	ca. 7

* The pH value of Lutensol TO types can decrease during storage, but this does not have any effect on their performance.

TO 8	molar mass (g/mol)	ca. 600
	physical form (23°C)	liquid
	pH (5% in water)*	ca.7
	degree of ethoxylation	ca. 8

TO 10	molar mass (g/mol)	ca. 630
	physical form (23°C)	liquid / paste
	pH (5% in water)*	ca.7
	degree of ethoxylation	ca. 10

TO 12	molar mass (g/mol)	ca. 750
	physical form (23°C)	paste
	pH (5% in water)*	ca.7
	degree of ethoxylation	ca. 12

* The pH value of Lutensol TO types can decrease during storage, but this does not have any effect on their performance.

The Lutensol® XP types:

- are cloudy liquids at room temperature,

- tend to form sediment,

- are alkyl polyethylene glycol ethers based on C_{10} – Guerbet alcohol and ethylene oxide:

$$R-O-(CH_2-CH_2-O)_x-H$$

$$R = C_{10}H_{21}$$
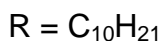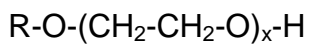
$$x = 3, 4, 5, 6, 7, 8, 9, 10, 14$$

Figure 2.2 Structural formula

Table 2.8 Chemical and physical properties of 2 Lutensol® XP types, source: Lutensol® XP types, Technical information, BASF The chemical company, September 2003, ® = registered trademark of BASF Aktiengesellschaft

XP 70	molar mass (g/mol)	ca. 445
	physical form (23°C)	liquid
	pH *	ca. 7
	degree of ethoxylation	ca. 7

XP 30	molar mass (g/mol)	ca. 290
	physical form (23°C)	liquid
	pH *	ca. 7
	degree of ethoxylation	ca. 3

* The pH value of Lutensol XP types can decrease during storage, but this does not have any effect on their performance.

The Lutensol® AO types are based on a saturated, predominantly unbranched $C_{13}C_{15}$ oxo alcohol that consists of 67% C_{13} and 33% C_{15}.

$$R\text{-}O\text{-}(CH_2\text{-}CH_2\text{-}O)_x\text{-}H$$
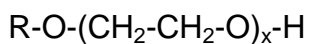
$$R = C_{13}C_{15} \text{ oxo alcohol}$$

$$x = 3, 4, 5, 7, 8, 10, 11 \text{ or } 30$$

Figure 2.3 Structural formula

Table 2.9 Chemical and physical properties of 2 Lutensol® AO types, source: Lutensol® AO types, Technical information, BASF The chemical company, September 2003, ® = registered trademark of BASF Aktiengesellschaft

AO 7	molar mass (g/mol)	ca. 520
	physical form (23°C)	liquid
	pH *	ca. 7
	degree of ethoxylation	ca. 7

AO 3	molar mass (g/mol)	ca.340
	physical form (23°C)	liquid
	pH *	ca. 7
	degree of ethoxylation	ca. 3

* The pH value of Lutensol AO types can decrease during storage, but this does not have any effect on their performance.

2.2 Microbiological methods

2.2.1 Strains and cultures

E. coli (ATCC 25922), *L. monocytogenes* EGDe (1/2a, internal number 2964) and *S. Typhimurium* (NCTC 12023), used as model organisms in this study, were part of the collection of bacterial strains at the Institute of Milk Hygiene, Milk technology and Food Science (University of Veterinary Medicine, Vienna). Their aliquots were kept at -15°C.

For all experiments, an overnight culture and a three-hour culture were prepared. The preparation of the first one was followed by an inoculation of a sterile non-selective medium (TSB + Y) with an aliquot of respective bacteria and a subsequent incubation at 37°C overnight in darkness under aerobic conditions, producing a high yield of respective model organisms. Bacteria of the overnight (O/N) culture were in the stationary phase. For reproducibility purposes, an aliquot (1 ml) of O/N culture was transferred into a sterile non-selective medium (TSB + Y) and incubated at 37°C in darkness under aerobic conditions for the next three hours.

2.2.2 Media and growth conditions

The detailed composition of the following media - Tryptone Soy Broth enriched with yeast extract and Tryptone Soy Agar (also known as Casein soya bean digest agar) enriched with yeast extract is shown in tables 2.10 and 2.11.

Table 2.10 Composition of TSB + Y

Glucose	Source of energy	2.5 g/l
NaCl	Maintenance of osmotic equilibrium	5 g/l
K_2HPO_4	Buffer to control the pH-value	2.5 g/l
Tryptone	Pancreatic digest of casein	17 g/l
Soytone	Peptic digest of soybean meal	3 g/l

Preparation according to the recommendation of the producer (Oxoid, UK): 30 g TSB and 6g of yeast extract (0.6% w/v) solved in 1L water and sterilized by autoclaving at 121°C for 15 minutes.

Table 2.11 Composition of TSA + Y

Tryptone	Pancreatic digest of Casein	15 g/l
Soytone	Papaic digest of soya bean meal	5 g/l
NaCl	Maintenance of osmotic equilibrium	5 g/l
Bacteriological agar	Solidifying agent and source of energy	15 g/l

Preparation of the media was performed according to the recommendation of the producer (Biokar diagnostics, France): 40 g TSA solved in 1L distilled water and brought to the boil to dissolve the substances completely. Six gram of yeast extract (Biokar diagnostics, France) was added per 1L. The final solution was sterilized by autoclaving at 121°C for 15 minutes.

2.2.3 Determination of total colony count

A three-hour culture with an OD_{600} of ~ 0.6, was diluted with fresh sterile TSB + Y in a ratio of 1:9. Based on this culture, 10-fold dilution series (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were prepared by diluting with 1x PBS in a ratio of 1:9. From the last three decimal dilutions (10^{-4}, 10^{-5} and 10^{-6}), 50 µl of each dilution were plated out twice, on both halves of non-selective agar (TSA + Y) using the Drigalski-triangle. Samples were incubated overnight at 37°C in darkness under aerobic conditions. On the next day, colonies were counted and the total cell count was calculated.

Following equation was used to determine the total cell count per ml:

$$weighted\ average\ of\ counted\ colonies\ \times 20 \times dilution\ factor = total\ cell\ count\ per\ ml$$

2.2.4 Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) is the distinct minimal concentration of an investigated substance inhibiting the bacterial growth under defined test conditions. This procedure, done with either agar or broth dilution methods, is important to determine the susceptibility of a respective bacterium to an investigated substance (Wiegand et al. 2008). It is based on applying different concentrations of the investigated substances into non-selective broth (e.g. TSB +Y) with a standardized number of bacteria (Wiegand et al. 2008). Subsequently the broth is evaluated with both OD-measurements and the determination of the total colony count (see chapter 2.2.3).

2.2.4.1 MIC determination by OD-measurement

To determine the minimal inhibitory concentration, the culture of respective model organism was incubated in a microtiter plate (96 well cell culture cluster Flat bottom with lid) with an investigated substance overnight at 37°C in darkness under aerobic conditions. For this purpose, a three-hour culture with an OD_{600} of ~ 0.6 was applied.

The maximal volume of one well is 200 µl. The bacterial culture (10-fold diluted three-hour culture) represents one eighth of the maximal volume (25 µl). The rest (175 µl) was filled up with salt solutions, detergents and fresh sterile TSB + Y (providing source of energy and nutrients) of variable volume.

The OD of each well was measured with the TECAN infinite F200 at 610nm – before and after an overnight incubation. Based on the difference between values of these measurements, the conclusion was reached as to whether bacteria have grown or not. The difference of OD-values equal or near zero refers to no evidence of growth – corresponding concentrations of investigated substances have been regarded as the minimal inhibitory concentration.

Each experiment included a positive control (containing 175 µl TSB + Y and 25 µl of respective bacteria) and a negative control (200 µl TSB + Y).

In addition to OD measurements, all plates were also inspected visually due to a possible sedimentation or growth of bacteria on the side of the well, which could lead to a false-negative OD measurement result.

2.2.5 Minimal bactericidal concentration

The minimal bactericidal concentration (MBC) is defined as the minimal concentration of an investigated substance necessary to kill 99.9% of the original inoculum. To establish the MBC-value, a ten-fold dilution series (10^0, 10^{-1} and 10^{-2}) of samples from the microtiter plate (96 well cell culture cluster Flat bottom with lid) had to be prepared and plated out (50 µl) twice on both halves of a non-selective agar (TSA + Y) and incubated overnight at 37°C in darkness under aerobic conditions. The next day the colonies were enumerated.

The percentage of surviving bacteria was calculated as follows, using the results of the respective total count (see section 2.2.3):

$$\frac{weighted\ average\ of\ colonies\ grown\ after\ replating\ \times 20 \times dilution\ factor}{total\ cell\ count\ per\ ml} = \%\ of\ surviving\ bacteria$$

$$100\% - \%\ of\ surviving\ bacteria = \%\ of\ killed\ bacteria$$

If the CFU reduction is equal or greater than 99.9%, the investigated substance of corresponding concentration is assumed to have a bactericidal effect.

2.3 Methods of molecular biology

2.3.1 BacLight^{TM}

To examine the integrity of bacterial plasma membrane after treatment with investigated substances, Bac Light^{TM} Kit was used. This method is based on the permeability of the cell membrane for distinct substances. An intact cell membrane is impermeable for the red fluorescent dye Propidium iodide, but permeable for green fluorescent dye SYTO 9.

Propidium iodide is a ready-to-use solution for the exclusion of the nonviable cells in flow cytometric analysis and for the examination of samples under fluorescent micro-scope using BacLight^{TM} kit. It intercalates between the base pairs of double-stranded DNA, but it cannot go through intact plasma membranes, thus it stains dead cells only. Propidium iodide is a membrane-impermeable anionic stain with an excitation maximum at 536 nm and an emission minimum at 620 nm (LIVE/DEAD$^®$ BacLight^{TM} Bacterial via-bility Kits, technical information, Invitrogen, © 2008 Invitrogen Corporation).

Syto 9, a lipophilic membrane-permeable cationic stain (Raad 2003), used for staining of nucleic acid with a high affinity to DNA, permeant through the intact plasma mem-brane, thus it can stain viable and dead cells. It exhibits enhanced fluorescence upon binding. Its excitation maximum lies at 483 nm, the emission maximum at 503 nm. It is used for the examination of bacteria under fluorescent microscope using BacLight^{TM} kit (LIVE/DEAD$^®$ BacLight^{TM} Bacterial viability Kits, technical information, Invitrogen, © 2008 Invitrogen Corporation).

Cells with an intact cell membrane appear green by examination under fluorescent mi-croscope. In contrast, cells with damaged cell membrane appear red. Both fluorescent dyes bind to the DNA. It is recommended to use a three-hour culture, when bacteria grow exponentially. They are small, the cytoplasma volume:DNA ratio is appropriate and applied dyes emit strong fluorescent signal. The cytoplasmic cell volume, coming from older cultures, is too big and the emitted signal would be weak due to the inappro-priate cytoplasma volume:DNA ratio.

The following procedure was done for all experiments:

An aliquot of a three-hour culture of respective bacterium was spinned down at 6010 g for 5 min., the supernatant was removed and the bacterial pellet resuspended in 1x PBS and spinned down at 6010 g for 5 min. These steps were repeated three times.

Washed cells were incubated with 1000 µl of the respective solution for one hour, at 30°C under constant shaking at 750 rpm in a thermomixer, followed by the next three washing steps and by the preparation of decimal dilution series (10^{-1}, 10^{-2} and 10^{-3}). The

1000-fold dilution was stained with two fluorescent dyes (Syto9 and Propidium iodide) and incubated in darkness for 15 minutes. The stained cell suspension (500 µl) was applied on a membrane, which in turn was put on glass slide (with samples towards the objective lenses) and covered with a mounting gel and cover slip. The mounting gel helps to create homogenous physical conditions necessary for observation under the fluorescent microscope. The samples were examined under the inverse confocal laser scanning microscope Leica DM IRB at magnification of 1000. The photography of the samples was taken with the camera Leica DFC300FX.

Subsequently, cells were counted by eye for further calculation of the average and total cell count per ml.

$$\frac{sum\ of\ counted\ cells}{number\ of\ samples} = average$$

$$average\ \times 2 \times 1000 \times 5230 = total\ cell\ count\ per\ ml$$

5230 is a factor which depends on the aperture of the objective.

The average and total cell count per ml was calculated for both, the green cells with an intact plasma membrane and the red cells with a damaged plasma membrane. The disadvantage of this method is the quick fade-out of both dyes.
L. monocytogenes is difficult to stain, even when twice as much dye was applied and the incubation time was prolonged up to 30 min.

2.3.2 Detection of ATP in supernatant

One of the most important molecules is adenosine triphosphate (ATP), a molecular unit of currency delivering energy to all processes occurring within cytosol. ATP is detectable by bioluminescence, a routine diagnostic method for detection of bacterial contamination (Squirell et al. 2002). This is based on the knowledge that every living organism does contain ATP and that the turnover of ADP into ATP is one of the characteristics of living organisms (Lundin et al. 1989).

In case of a plasma membrane disruption, ATP molecules can diffuse freely along the chemical gradient out of the cytoplasm into the supernatant, where they can be detected by bioluminescence. This method is based on the conversion of luciferin to oxyluciferin in the presence of oxygen, firefly luciferase and ATP. The latter one acts as the source of energy for the enzymatic reaction (Squirell et al. 2002). Simultaneously, the light of distinct wavelength will be emitted, whereas intensity of emitted light is proportional to the ATP, previously present in the bacterial cell (Venkateswaran et al. 2003; Griffiths 1996).

To determine the quantity of ATP in supernatant and to estimate the amount of damaged cells, an assay with Bac Titer-Glo™ Microbial Cell Viability Assay Kit (Promega, Madison, WI, USA) was carried out. The procedure involved the applying of a single reagent directly into the bacterial culture, whereas removal of the medium and washing the cells were not required. The reaction generated luminescence of glow-type detectable with spectrophotometer. Glow luminescence lasted for hours, but the emitted signal is not as bright as the emitted signal of the flash luminescence.

According to the manufacturer´s instruction, the reaction had a high sensitivity, whereas the signal from even ten bacterial cells in the medium is detectable. Moreover, the luminescent signal proportional to the amount of bacteria can be detected already 5 min. upon the mixture was added into the bacterial culture, and it is stable over 30 min. depending on the bacterium and the medium.

The experiment was carried out as follows: Equilibration of Bac Titer-Glo™ Buffer and Bac Titer-Glo™ reagent to RT. The buffer was added to the Bac Titer-Glo™ reagent to create reconstituted reagent.

A three-hour culture of the respective bacterium was washed in 1x PBS and spinned down at 6010 g for 5 min. for three times. The bacterial pellet was re-suspended in the respective solutions and incubated for 60 min. at 30°C under constant shaking (750 rpm). To be able to observe the ATP present in supernatant, the samples were spinned down at 6010 g for 5 min., and the supernatant was transferred into a new microcentrifuge tube and spinned down (6010 g for 5 min.) again. As this procedure has been finished, 100 µl of supernatant were taken up and injected into one well of microtiterplate. Each sample was treated with the reconstituted reagent (100 µl). Into the neighboring column, 200 µl of the respective investigated substance were put in as a negative control for detection of the background bioluminescence.

Reconstituted reagent BacTiter-Glo™ was added into each well in ratio 1:1 (standard dilution steps and a positive control). The samples were incubated at RT for 5 min. in darkness and examined in TECAN infinite F200 (Luminescence, Attenuation: NONE, Inegration time 1000 ms).

2.3.3 DNA extraction

The bacterial DNA was isolated with the NucleoSpin© Tissue Kit (Macherey-Nagel, Düren, Germany). The cells of the three-hour culture (*E. coli* or *S*. Typhimurium) were used. Before extraction, the cells had to be washed in 1x PBS and spinned down at 6010 g for 5 min. at RT. The supernatant was removed, the pellet resuspended in 1x

PBS (1 ml) and spinned down (6010 g; 5 min.; RT) again. This washing procedure was repeated three times.

Some bacteria are difficult to be lysed by a common procedure and require an additional lysis step, done with the Lysis buffer prepared according to the producers recommendation (Macherey – Nagel; Düren, Germany): 20 mM Tris/HCl, 2 mM EDTA, 1% Triton X -100 solved in distilled water. The pH-value was adjusted with NaOH to pH = 8 additionally. The ready-to-use solution was stored at room temperature. The lysozyme stored in a fridge was added immediately before use: 20 mg/ml lysis buffer. (according to: Genomic DNA from tissue, User manual, NucleoSpin® Tissue, June 2012/revision 012, Macherey-Nagel, Düren, Germany).

180 µl pre-lysis buffer containing lysozyme was added to one pelleted sample. The mixture was incubated for 60 min. at 37°C under constant shaking (750rpm). Subsequent steps were the addition of proteinase K (25 µl per sample) and an overnight incubation at 56°C under constant shaking (750 rpm).

The next day, cell lysis was achieved by applying of the B3 solution (containing chaotropic ions) which also improves the ability of DNA to bind to the silica membrane of a column. The sample needs to be vortexed to mix the content properly. In the next step, the sample was incubated at 70°C for 10 minutes. If some particles were visible, the sample was spinned down for 5 min. at 18407 g. Then, 96% ethanol was added (210 µl per sample) and the sample was vortexed to mix the content properly.

Further, the content was applied on the column and spinned down for 2 min. at 11363 g. DNA is bound to the column at this moment. The flow-through can be discarded. The addition of 500 µl washing buffer (BW) in the next step and a spinning down for 2 min. at 11363 g ensures the efficient removal of all contaminants.

After the application of B5 solution, the sample was spinned down twice (á 2 min. at 11363 g) to remove residual ethanol efficiently.

To elute the DNA from the silica membrane, pre-heated distilled water (50 µl; 70°C) was applied on the column and the sample was incubated for 1 min. at RT.

Finally, the sample was spinned down for 1min. at 11363 g. The elution step with pre-heated distilled water has been repeated twice. Eluted DNA of total volume of 100 µl can be used for PCR immediately or can be stored at -20°C for future use.

2.3.4 Quantitative PCR

Quantification of isolated bacterial DNA (see section 2.3.3) was performed by qPCR in which the amplification process of target DNA can be observed in real time, based on the use of a fluorescently labeled probe. On one end, the probe is flanked by fluorescent dye (= reporter), on the other end it is flanked by a quencher inhibiting the light

emission by a reporter. The labeled probe bound to the single stranded DNA template is degraded successively during the elongation process under the simultaneous release of the reporter and the quencher. The activity of the released reporter is no longer inhibited by the quencher, thus it starts to emit light of distinct wavelength upon excitation detectable by a machine. The unbound probes or a certain amount of already degraded ones emit also the fluorescent light of weak intensity causing a certain level of background fluorescence. The amplification of DNA is an exponential process multiplying one DNA template into two copies, whereas the target sequence to be multiplied is determined by bound primers. With growing amount of amplified DNA, the emitted fluorescent signal becomes stronger. At a distinct time point, known as threshold cycle (C_T), fluorescence of the multiplied DNA overcomes the signal of the background fluorescence. On the contrary, the plateau phase is characterized by the consumption of the majority of fluorescently labeled probes, thus the curve showing fluorescence becomes flatter. Due to both, the background fluorescence and the consumption of labeled probes, not the entire process can be used for evaluation, quite in contrast the period in between (two to three cycles).
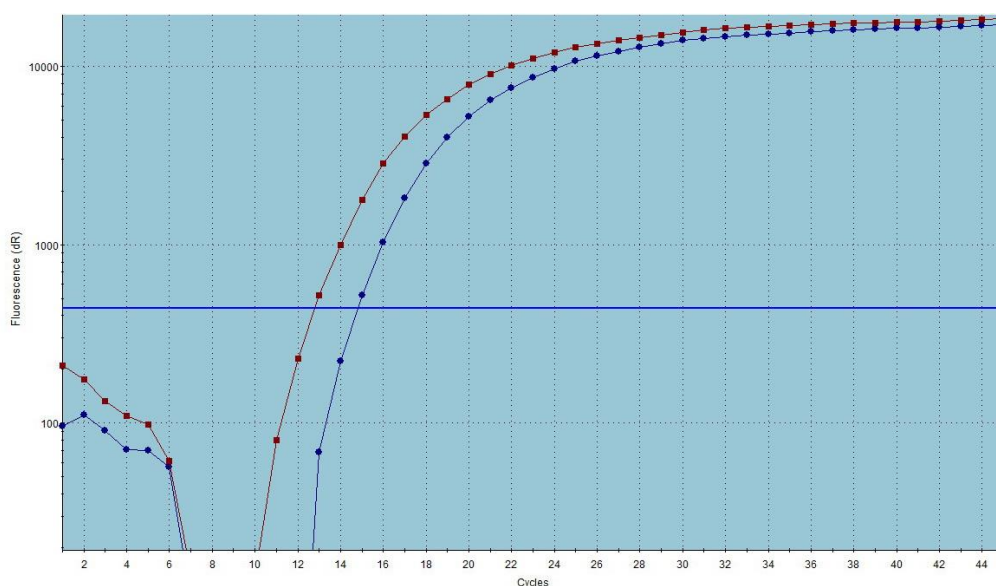
Figure 2.4 Visualisation of DNA amplification

In this study, the target was *sfmD* gene coding for putative outer membrane export usher protein, located in the genomic DNA of *E. coli* at the position 559697..562300 (Blattner et al. 1997). To amplify this gene, the primer sequences (Ert2F and Ert2R) have been used, according to Kaclíková et al. 2005.

To be able to run the qPCR, an Ert2 probe labeled with FAM (6-carboxyfluorescein) at the 5´end and with BHQ1 at the 3´end had to be added (according to Kaclíková et al. 2005).

Primers (Ert2F and Ert2R) and a labeled probe (Ert2 probe) were provided by Eurofins MWG Operon LLC, A Eurofins Genomic Company, Ebersberg, Germany.

The reactions were carried out in the Thermocycler Stratagene MX3000P (Life Technologies, A Thermo Fischer Scientific Brand, Fischer Scientific Austria, Vienna, Austria) using an modified programme (according to Kaclíková et al. 2005): initial denaturation at 94°C for 2 min., 50 cycles with denaturation at 94°C for 15 sec. and elongation at 60°C for 1 min.

The second target was *fimA*, a gene coding for the major component (fimbrin) of type 1 fimbriae, located within the *fim* gene cluster of the genomic DNA of *S*. Typhimurium (Rossolini et al. 1993), at the position 604115 … 604672 (McClelland et al. 2001). To amplify this gene, primer sequences fimAF1 and fimAR1 have been used (Cohen et al. 1996; Mann et al. 2013).

To be able to run the qPCR, the probe fimAS1, labeled with FAM (6-carboxyfluorescein) at the 5´end and with BHQ1 at the 3´end, had to be added (according to Mann et al. 2013).

The primers (fimAF1 and fimAR1) and the labeled probe (fimAS1 probe) were provided by Eurofins MWG Operon LLC, A Eurofins Genomic Company, Ebersberg, Germany.

The reactions were carried out in the Thermocycler Stratagene MX3000P (Life Technologies, A Thermo Fischer Scientific Brand, Fischer Scientific Austria, Vienna, Austria) using following programme according to Mann et al. (2013): initial denaturation at 94°C for 2 min., 45 cycles with denaturation at 94°C for 30 sec. and elongation at 60°C for 1 min.

Table 2.12 Primers and probes used in quantitative PCR

Organism	Name	Oligonucleotide	Function
	Ert2F	5´-ACT GGA ATA CTT CGG ATT CAG ATA CGT-3´	forward primer
E. coli	Ert2R	5´-ATC CCT ACA GAT TCA TTC CAC GAA A-3´	reverse primer
	Ert2 probe	5´-FAM-CAG CAG CTG GGT TGG CAT CAG TTA TTC G BHQ1-3´	probe
	fimAF1	5´CCT TTC TCC ATC GTC CTG AA 3´	forward primer
S. Typhimurium	fimAR1	5´TGG TGT TAT CTG CCT GA 3´	reverse primer
	fimAS1	5´FAM TGC GAT CCG AAA GTG GCG G BHQ1 3´	probe

3 Results

3.1 Screening of general characteristics of MgCl₂ and Lutensols

Detection and quantification of pathogenic microorganisms in foodstuffs is based on the methods of microbiology, such as selective enrichment and plating, or the methods of molecular biology, such as qPCR. Especially molecular biological methods require a prior sample preparation for concentration and purification of the target organism.

Being such a sample preparation method, Matrix-Lysis is based on the solubilization of the food matrix with appropriate solvents to subsequently concentrate the target organism by centrifugation.

Magnesium chloride solution acts as a good solvent of protein-rich matrices in the Matrix-Lysis procedure. To solve the lipid-rich fraction of matrix, detergents have to be applied, e.g. Lutensol AO 7. Unfortunately, the combination of MgCl₂ with Lutensol AO 7 has a bactericidal effect (Kwisda 2012), thus bacteria cannot be detected by conventional microbiological methods (e.g. by the plate count method). This has an influence on the detection of pathogenic bacteria in dairy products, where applying of Lutensol AO 7 in combination with MgCl₂ would lead to false negative results. The aim of this study was to find a grade of Lutensol, which would not show any combinational effect. The way to do this is to detect whether the combinational effect is a characteristic feature of other Lutensol´s grades and surfactants.

3.1.1 Lutensols

First, the ten different Lutensols were tested for their possible toxicity against bacteria without MgCl₂. Both model organisms (*L. monocytogenes* and *E. coli*) were incubated overnight in two-fold dilution series from 5% to 0.04% of each of the ten different Lutensols in TSB + Y. The results for respective bacteria are shown in tables 3.1 and 3.2 below.

Table 3.1 Determination of MIC and MBC (%) of ten Lutensol´s grades for *Listeria monocytogenes*

Lutensol	TO 3	TO 6	TO 7	TO 8	TO10	TO12	XP70	XP30	AO 7	AO 3
MIC	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
MBC	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

Table 3.2 Determination of MIC and MBC (%) of ten Lutensol´s grades for *Escherichia coli*

Lutensol	TO 3	TO 6	TO 7	TO 8	TO10	TO12	XP70	XP30	AO 7	AO 3
MIC	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5
MBC	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

As it is shown by the data in tables 3.1 and 3.2, none of the ten Lutensols inhibited the growth of *L. monocytogenes* and *E. coli* within the given experimental parameters. Due to the fact that the MIC was not reached within the experimental parameters, the MBC was not tested.

See table 3.3. to compare the activity of Lutensol´s grades with other renowned surfactants commonly used in the field of molecular biology. Low-level toxicity of Lutensol´s grades is comparable to that of other non-ionic detergents, Triton X-100 (MIC = 5%, MBC = >5%) and Tween 20 (MIC = 5%, MBC = 5%). On the other hand, in case of SDS, an anionic detergent, an interesting phenomenon has been observed, when *L. monocytogenes* was not able to survive even at a very low concentration (MBC = 0.01%).

Table 3.3 MBC-values (%) of chosen surfactants

		E. coli	*L. monocytogenes*
anionic	Sodium dodecyl sulphate	>5	0.01
non - ionic	Triton X-100	>5	>5
	Tween 20	5	5

3.1.2 **The influence of Lutensols and other surfactants on qPCR**

As Matrix-Lysis is also used as a sample preparation method for subsequent qPCR (Mester et al. 2014), a possible inhibitory effect of surfactant remnants has to be excluded to prevent false negative results. Therefore a possible inhibition of qPCR reaction for the 13 surfactants at different concentrations (final concentrations in the qPCR reaction of 5%, 1%, 0.1% and 0.01%), under constant amount of *E. coli* DNA, was tested. The results are shown in figures 3.1 and 3.2.

Table 3.4 qPCR with surfactant

Mastermix	end conc.	stock conc.	1x	20x
			µl	µl
Aqua dest. + DNA			8.2	164
10x buffer	1x		2.5	50
MgCl$_2$	3.5 mM	50 mM	1.75	35
Primer 1 = ert2F	500 nM	5 µM	2.5	50
Primer 2 = ert2R	500 nM	5 µM	2.5	50
probe = ert2 probe	250 nM	5 µM	1.25	25
dNTP`s	200 µM each	20 mM	1	20
Taq pol	1.5 U	5 U	0.3	6
surfactant as template			5	100
Total			25	500
Volume for 1 reaction			25	

Table 3.5 Lutensol mastermixes

Percentage (v/v) or (w/v)	chemical	µl	notice
25%	surfactant	500	prepared from pure substance
	H_2O	1500	
5%	surfactant	1000	prepared from 10% solution
	H_2O	1000	
0.50%	surfactant	100	prepared from 10% solution
	H_2O	1900	
0.05 %	surfactant	10	prepared from 10% solution
	H_2O	1990	
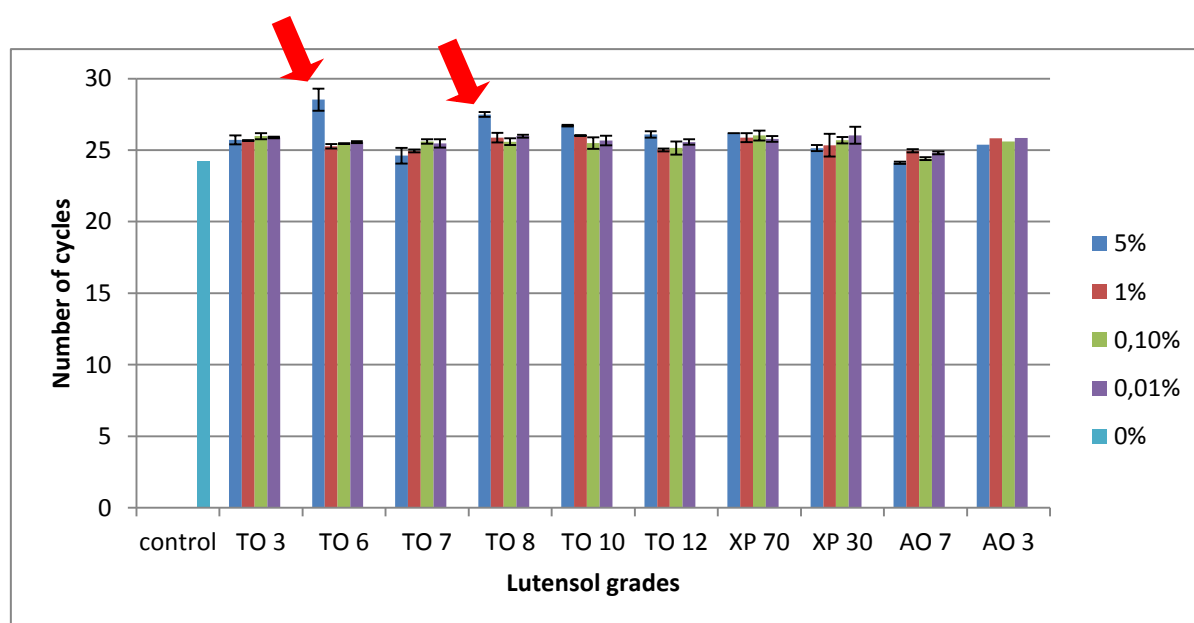
Figure 3.1 Influence of ten Lutensol´s grades on qPCR

As can be seen in figure 3.1, none of the ten Lutensols totally inhibited the qPCR, even at the highest concentration. Also for all ten Lutensols, at the concentrations <5% the C_T values were not significantly different from the control. Only for the concentrations of 5% a C_T shift was observed which could lead to false quantification results. This effect was most apparent for TO 6 and TO 8 but also detectable with TO 10 and TO 12. However, even at these extremely high concentrations, the actual amplification process has not been totally inhibited, and thus would not lead to false negative results.

Compared with the control sample (distilled water), a significant delay in achieving the threshold value is detectable, but it cannot be said that Lutensols would have considerable negative influence on the process of DNA amplification in the sense of the complete inhibition of the amplification process. Thus, the presence of residual volume of Lutensols does not constitute an obstacle in further detection of pathogenic microorganisms by qPCR.
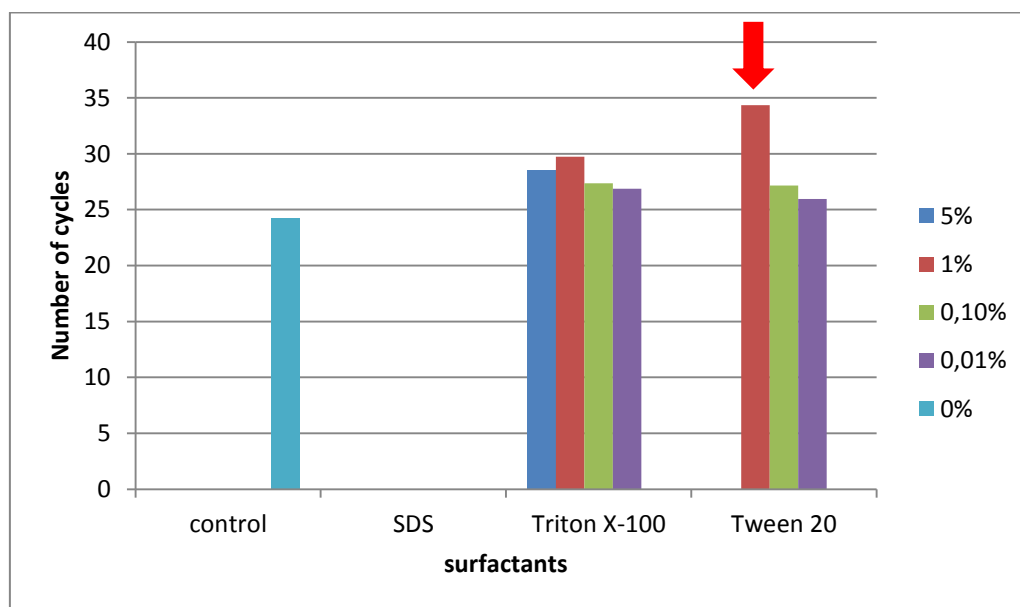
Figure 3.2 Influence of three chosen surfactants on qPCR

Sodium dodecyl sulphate, an anionic surfactant, applied in a broad range of concentrations (5% - 0.01%) and has a negative effect on the DNA amplification process in the sense of complete the inhibition of the reaction. This may be caused by binding of bivalent magnesium ions by dodecyl sulphate anions.

Application of Triton X-100, a non-ionic surfactant does not inhibit the process of DNA amplification independently from the concentration of an applied surfactant. The threshold value was reached between the 27th and 30th cycle, depending on the surfactant´s concentration.

Tween 20, another non-ionic surfactant, inhibits the process of DNA amplification completely, if applied in the concentration of 5%. The solution of Tween 20 (1%) has led to a delay in achieving of threshold cycle (C_T) value (34th cycle). Lower concentrations (0.1%, 0.01%) do not act inhibitory and the threshold value was reached in the 26th and the 27th cycle, respectively.

3.1.3 MgCl$_2$

Similar to Lutensol´s grades, the MIC and MBC of MgCl$_2$ against *L. monocytogenes* and *E. coli* was determined. MgCl$_2$ was applied in a concentration range 1 M – 0.1 M which is the optimal range for Matrix-Lysis, and the results are presented in table 3.6. According to these results, *L. monocytogenes*, a known halotolerant bacterium (Gandhi and Chikindas 2007), is more resistant against the osmotic stress induced by MgCl$_2$ than *E. coli*. The growth of *L. monocytogenes* was inhibited at 0.9 M MgCl$_2$ whereas the MBC was greater than 1 M MgCl$_2$. On the contrary, *E. coli* is much more sensitive against MgCl$_2$ and will be killed by 0.4 M MgCl$_2$.

Table 3.6 Determination of MIC and MBC of MgCl$_2$

	L. monocytogenes	*E. coli*
MIC [M]	0.9	0.4
MBC [M]	>1	0.4

3.1.4 MgCl$_2$ and Lutensols

After establishing the respective MIC- and MBC-values for the individual substances, the combinations of MgCl$_2$ and the ten Lutensols were tested to investigate a possible combinational effect as has been reported for MgCl$_2$ and Lutensol AO 7.

Three concentrations of MgCl$_2$ below the respective MIC for *E. coli* (0.3 M, 0.25 M and 0.2 M) and two for *L. monocytogenes* (0.5 M and 0.25 M) were investigated and combined with Lutensols to determine MIC and MBC.

The procedure was carried out, as described in chapter Materials and Methods, by incubation of the model organisms in two-fold dilution series of each of ten Lutensols (5% - 0.04%) combined with MgCl$_2$ of three (0.3 M; 0.25 M; 0.2 M) or two (0.5 M; 0.25 M) concentrations below MIC.

Some Lutensol´s grades show turbidity causing a problem during an evaluation based on the value of optical density (OD). Wells containing samples of high turbidity show a high OD-value already before the incubation. To solve this problem and to determine the MIC- and MBC-value, those samples were plated out on a non-selective agar (TSA + Y).

In the section below, exemplary results for Lutensols TO 10 and XP 30 are shown in detail, including the calculation of the original inoculum (table 3.7), and the MIC- and MBC-determination based on the OD-measurement (see table 3.8). The summarized results for all Lutensols are shown in table 3.7.

A three-hour culture of a model organism was incubated overnight with a mixture of Lutensol and MgCl$_2$. The OD was measured before and after the incubation. Dilution

series, done in parallel, were plated out on a non-selective agar (TSA + Y) to calculate the CFU per ml of a non-diluted untreated sample.

Table 3.7 CFU determined by plate count method

dilution	No.of colonies	No. of colonies
10^{-3}	144	73
10^{-4}	39	35
10^{-5}	9	14
10^{-6}	4	13

(144 + 73 + 39 + 35 + 9 + 14 + 13 + 4) / 2,222 * 20 * 10^3 = 2,98 * 10^6 CFU per 1 ml of non-diluted sample

The difference in OD-values is used for the determination of MIC. To determine the MBC-value, the samples were plated out (50 µl) twice on both halves of a non-selective agar (TSA + Y) and incubated overnight at 37°C. Next day, the colonies were counted and the percentage of CFU was calculated. Based on these results, the conclusion was reached as to whether the MBC was reached or not.

Table 3.8 Completed results for two chosen Lutensol´s grades

Bacterium	Lutensol	%	MgCl$_2$ [M]	OD-value before / after incubation		Diff.	MIC	No. of CFU	% CFU	MBC
E. coli	TO 10	2.5	0.25	0.10	0.18	0.08	YES	n.a.		NO
		5	0.25	0.10	0.10	0.00	YES	48	0.03	YES
L. mono-cytogenes	XP 30	0.16	0.5	0.11	0.19	0.08	YES	0	0	YES
		0.08	0.5	0.11	0.20	0.09	YES	0	0	YES

In this example, the MIC and MBC for the two Lutensols were shown. The combination of 0.25 M MgCl$_2$ with 2.5% Lutensol TO 10 has an inhibitory effect on the growth of *E. coli.* But the bacteria have not been killed, because they started to grow and to create colonies after re-plating on a non-selective agar (TSA + Y). First, 0.25 M MgCl$_2$ combined with 5% Lutensol TO 10 has shown a bactericidal effect, when a small number of colonies (48) was created after re-plating on a non-selective agar, and thus the MBC-value was reached.

Lutensol XP 30 has shown the bactericidal effect even at a very low concentration (0.08 – 0.16%), if it is combined with a half of the MIC of MgCl$_2$ (MIC = 1 M for *L. monocytogenes*).

Table 3.9 Combinational effect of MgCl$_2$ + Lutensols on *Escherichia coli*

% Lutensol	MgCl$_2$ [M]					
	0.3		0.25		0.2	
	MIC	MBC	MIC	MBC	MIC	MBC
TO 3	0.625	>5	1.25	>5	>5	>5
TO 6	0.625	>5	1.25	>5	>5	>5
TO 7	5	5	5	5	5	>5
TO 8	0.625	1.25	2.5	>5	>5	>5
TO 10	0.625	1.25	1.25	5	5	>5
TO 12	1.25	5	5	>5	5	>5
XP 70	1.25	5	5	>5	>5	>5
XP 30	1.25	5	>5	>5	>5	>5
AO 7	0.039	0.039	0.039	0.039	0.039	0.039
AO 3	0.3125	2.5	0.625	5	2.5	>5

toxic   non-toxic

AO 7 > AO 3 > TO 3; TO 6 > TO 10 > TO 8 > XP 70; XP 30; TO 12 > TO 7

Figure 3.3 Toxic effect of 10 Lutensol´s grades *Escherichia coli*

As can be seen in table 3.9, at half of the MIC of MgCl$_2$ (0.2 M), the combinational effect does not appear and *E. coli* grows unhindered. The only exception is Lutensol AO 7 showing bactericidal activity at the concentration of 0.039%.

In general, Lutensol AO 7 shows bactericidal activity only at a very low concentration (0.039%) at each of the tested salt concentrations.

At 0.3 M MgCl$_2$, Lutensols TO 12, XP 70 and XP 30 show a similar behavior – their MICs lie at 1.25% and their MBCs at 5%.

Comparable behavior was observed in the case of Lutensols TO 8 and TO 10 at 0.3 M MgCl$_2$ – their MICs and MBCs lie at 0.625% or 1.25%, respectively.

Last but not least, Lutensols TO 3 and TO 6 show similar properties – their MICs lie at 0.625% and MBCs at >5%, in the presence of 0.3 M MgCl$_2$.

Lutensol TO 7 shows the combinational effect first at 5% independently from the salt concentration. Thus, it can be used in the process of Matrix-Lysis and pathogen detection, if applied in the concentration lower than MIC, without killing the *E. coli*.

The following conclusions can be drawn: 5% Lutensol TO 12 combined with 0.25 M MgCl$_2$ can be used as a solvent in the process of Matrix-Lysis without killing *E. coli* what can be detected by the microbiological methods in subsequent steps.

The 5% solution of Lutensol TO 7 combined with 0.3 M MgCl$_2$ can be used also in the process of Matrix-Lysis for the detection of *E. coli*

Table 3.10 shows the respective results for *L. monocytogenes.* As the results clearly show, a strong combinational effect is found for most Lutensols.

Table 3.10 Combinational effect of MgCl$_2$ + Lutensols on *Listeria monocytogenes*

	MgCl$_2$ [M]			
	0.5		0.25	
% Lutensol	MIC	MBC	MIC	MBC
TO 3	0.039	0.039	0.039	0.039
TO 6	0.039	0.039	0.039	0.3125
TO 7	0.039	0.3125	0.039	2.5
TO 8	0.039	>0.156	>5	>5
TO 10	0.039	0.039	>5	>5
TO 12	>5	>5	>5	>5
XP 70	0.039	0.039	0.039	0.039
XP 30	0.039	0.039	0.039	0.039
AO 7	0.039	0.039	0.039	0.039
AO 3	0.039	0.039	0.078	0.078

toxic   non-toxic

AO 7; TO 3; XP 30; XP 70 >> AO 3 > TO 6 > TO 10 > TO 7 > TO 8 > TO 12

Figure 3.4 Toxic effect of 10 Lutensol´s grades on *Listeria monocytogenes*

The only exception is Lutensol TO 12, showing no combinational effect, independently from the salt concentration.

Lutensol AO 7 shows the bactericidal activity already at 0.039%, independently from the salt concentration. These results are in accordance with those of experiments done with *E. coli.*

Lutensols TO 8, TO 10 and TO 12 constitute a cluster of Lutensols showing similar behavior – their MICs and MBCs are higher than 5% if combined with 0.25 M MgCl$_2$.

The following conclusion can be drawn: The combination of 5% Lutensol TO 12 with 0.5 M MgCl$_2$ can be applied in the process of Matrix-Lysis due to its non-toxicity against *L. monocytogenes,* which can be detected in the subsequent pathogen detection.

The combination of 5% Lutensol TO 12 and 0.25 M $MgCl_2$ can be applied in the Matrix-Lysis procedure to allow the preparation and subsequent detection of both pathogens – *E. coli* and *L. monocytogenes*.

In contrast to *E. coli*, Lutensol TO 7 cannot be applied in the Matrix-Lysis procedure if *L. monocytogenes* is the target organism. This is because of the high toxicity of Lutensol TO 7 against *L. monocytogenes* if combined with $MgCl_2$.

Other Lutensol grades (AO 7 including) combined with 0.5 M $MgCl_2$ show the high toxicity against *L. monocytogenes* even at a very low concentration (0.039%) and cannot be used in the process of Matrix-Lysis.

3.2 Inorganic salts and their combination with Lutensols

The studies involving $MgCl_2$ and Lutensols have led to the question how the molecular mechanisms of this effect look like. Are chloride ions key players or even the valency of the cation? To answer these questions, inorganic salts alone had to be investigated at first to determine their MIC- and MBC-values. In the next step, inorganic salts were investigated in combination with two types of Lutensols (TO12; AO 7).

To choose appropriate salts, the following conditions had to be fulfilled: The salt should not be harmful to the personnel (e.g. radioactivity). Thus, radium chloride ($RaCl_2$) was not used in this study. The second condition was that the salt alone should not be classified as very toxic, otherwise the combinational effect would not be detectable. Therefore the MIC- and MBC-values for each salt were determined to be tested in advance together with Lutensols.

As mentioned in chapter 3.1.4, Lutensol TO 12 (5%) and $MgCl_2$ (0.25 M) is an appropriate combination applicable in the process of Matrix-Lysis allowing detection of both *E. coli* and *L. monocytogenes* by microbiological and molecular biological methods.

The results in chapter 3.1.4 also show that Lutensol AO 7 in combination with $MgCl_2$ kills bacteria effectively, even at a very low concentration (0.039%). Based on these results, Lutensol AO 7 was chosen as the second representative for further investigation of combinational effects.

The following results are based on the experiments, when chosen salts were combined with both Lutensols, each of two different concentrations (1% and 0.1%). The concentration of each salt was below the MIC.

3.2.1 Chloride-containing salts and valency

Based on the results shown in chapter 3.1.4, it was assumed that Cl-containing salts could contribute to the combinational effect. Additionally, the influence of heavy metal ions (Zn^{2+} and Sr^{2+}) and a chaotropically acting salt (LiCl) was tested.

First, all Cl-containing salts were tested separately to determine their MIC- and MBC-values against bacteria. Each salt was tested in 19 concentrations within the range 1M – 0.01M.

Further, the dilution series (1 M – 0.1 M) of each inorganic salt were investigated in combination with Lutensols (TO 12 and AO 7).

Table 3.11 MIC- and MBC-values of Cl-containing salts in combination with TO 12 and AO 7 for *Escherichia coli*

	MIC	MBC	MIC				MBC			
	without Lutensol		TO12		AO 7		TO12		AO 7	
			1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%
NaCl [M]	0.7	>0.9	1	1	1	1	>1	>1	>1	>1
KCl [M]	0.8	>1	1	1	1	1	>1	>1	>1	>1
ZnCl$_2$ [M]	1	1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
CaCl$_2$ [M]	>1	>1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
SrCl$_2$ [M]	1	1	0.1	0.1	0.1	0.1	0.5	0.5	0.5	0.5
LiCl [M]	0.7	0.9	0.5	0.5	0.9	0.9	0.9	0.9	0.9	0.9

Heavy metal salts (ZnCl$_2$ and SrCl$_2$) themselves do not have a negative effect on bacterial growth. The MIC- and MBC-values of these salts, without Lutensols, are quite high (up to 1 M). But the combination of heavy metal salts (ZnCl$_2$ and SrCl$_2$) with Lutensols has led to the decrease of MIC- and MBC-values. Further, *E. coli* has shown high susceptibility against the salts of bivalent cations, if combined with one of both Lutensols. The MIC- and MBC-values were reduced from 2- to 10-fold.

Lithium chloride is a good example of the salt, showing an ambivalent effect on two different model organisms. *E. coli* shows the resistance against chaotropically acting lithium chloride and its growth will be inhibited first in 0.7 M solution of LiCl. The addition of Lutensol TO 12 has led to a reduction of the MIC-value (0.5 M), but the MBC remained unchanged at 0.9 M LiCl in combination with both Lutensols.

On the other hand, *L. monocytogenes* is extremely sensitive against lithium chloride (MBC = 0.1 M), and so the combinational effect could not be examined.

Table 3.12 MIC- and MBC-values of Cl-containing salts in combination with TO12 and AO 7 for *Listeria monocytogenes*

	MIC	MBC	MIC				MBC			
	without Lutensol		TO12		AO 7		TO12		AO 7	
			1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%
NaCl [M]	>1	>1	1	1	1	1	1	1	1	1
KCl [M]	>1	>1	>1	>1	>1	>1	>1	>1	1	1
ZnCl$_2$ [M]	>1	>1	1	1	1	1	>1	>1	>1	>1
CaCl$_2$ [M]	>1	>1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
SrCl$_2$ [M]	>1	>1	1	1	1	1	>1	>1	>1	>1

In case of *L. monocytogenes*, the reduction of MIC and MBC is less evident in comparison to *E. coli*. The MIC- and MBC-values for four of five salts were maintained. Only calcium chloride combined with Lutensol has led to more than a 2-fold reduction for MIC- and MBC-values. *L. monocytogenes*, able to survive in the solution of CaCl$_2$-concentration greater than 1 M, will be eliminated in the mixture of Lutensol (1%; 0.1%) and 0.5 M CaCl$_2$.

3.2.2 Impact of various anions on the combinationanl effect

Next, the role of different (non-chloride) anions with K$^+$, Na$^+$ or Mg^{2+} as cation was investigated, respecting a possible combinational effect. The anions can be composed either by one or more chemical elements. Depending on chemical compounds, their chemical and physical characteristics can vary, e.g. solubility or toxicity. Some of the chosen salts [K$_2$CO$_3$; K$_3$PO$_4$; Na$_2$CO$_3$] has shown the strong toxic effect even at a low concentration (MIC = 0.1 mol/l), and could not be used for an investigation of the combinational effect. Dipotassium sulphate (K$_2$SO$_4$) was completely insoluble in TSB + Y, and thus it was also excluded from the experiments.

Table 3.13 MIC- and MBC-values of selected inorganic salts in combination with two representatives of Lutensols for *Escherichia coli*

	MIC	MBC	MIC				MBC			
	without Lutensol		TO12		AO 7		TO12		AO 7	
			1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%
K_2HPO_4 [M]	0.3	>0.5	0.5	0.5	0.5	0.5	1	1	1	1
NaH_2PO_4 [M]	0.5	>0.8	1	1	1	1	1	1	1	1
KH_2PO_4 [M]	0.8	>1	1	1	1	1	>1	>1	>1	>1
Na_2SO_4 [M]	0.4	>0.6	1	1	1	1	>1	1	>1	1
$MgSO_4$ [M]	1	>1	0.5	0.5	0.5	0.5	>1	>1	>1	>1

Table 3.13 shows the results for *E. coli*. In combination with Lutensol, three of five tested salts (K_2HPO_4; NaH_2PO_4 and Na_2SO_4) show even higher MBC-values than without it. The MIC-values of KH_2PO_4 were higher, if combined with Lutensols, whereas MBC-values of this salt remained unchanged.

$MgSO_4$ is an exception – if combined with one of both Lutensol types, its MIC-values were reduced by 50%. Its MBC-values remained unchanged.

On the contrary, treatment of *L. monocytogenes* with the same mixtures has led to a decrease of MIC- and MBC-values in three of five chosen representatives. The results are shown in the table 3.14.

Table 3.14 MIC- and MBC-values of selected inorganic salts in combination with two representatives of Lutensols for *Listeria monocytogenes*

	MIC	MBC	MIC				MBC			
	without Lutensol		TO12		AO 7		TO12		AO 7	
			1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%
K_2HPO_4 [M]	0.9	0.9	0.5	0.5	0.1	0.1	0.5	0.5	0.1	0.1
NaH_2PO_4 [M]	0.7	0.7	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
KH_2PO_4 [M]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Na_2SO_4 [M]	0.7	0.7	0.5	0.5	0.5	0.5	0.5	0.5	>0.5	>0.5
$MgSO_4$ [M]	0.5	0.5	0.8	0.8	>1	>1	0.8	0.8	>1	>1

For two of the tested salts, no combinational effect was observed. The MIC- and MBC-values of the first one, potassium dihydrogenphosphate (KH_2PO_4), remained un-

changed, independently of the presence or absence of Lutensols. The second one, $MgSO_4$, shows higher MIC- and MBC-values in combination with Lutensols.

3.2.3 The impact of NH_4-containing salts on the combinational effect

The behavior of the ions and the presence of the combinational effect can be explained by the Hofmeister series. According to these series, ammonium ions show stronger chaotropic effect than the magnesium ions showing combinational effect. Based on this knowledge, ammonium ions were assumed to show the combinational effect in the presence of Lutensols. To confirm this assumption, the following ammonium-containing salts had to be investigated: NH_4Cl; $NH_4H_2PO_4$; $(NH_4)_2SO_4$; $(NH_4)_2HPO_4$ and $(NH_4)_2CO_3$.

Diammonium carbonate - $(NH_4)_2CO_3$ – shows a high toxicity against *E. coli* (MIC = 0.1 M; MBC = 0.2 M), thus it had to be excluded from further investigations.

Table 3.15 MIC- and MBC-values of selected inorganic salts in combination with two representatives of Lutensols for *Escherichia coli*

	MIC	MBC	MIC				MBC			
	without Lutensol		TO 12		AO 7		TO 12		AO 7	
			1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%
NH_4Cl [M]	0.7	>0.9	1	1	1	1	>1	>1	>1	>1
$NH_4H_2PO_4$ [M]	0.6	>0.8	0.5	0.5	0.5	0.5	1	1	1	1
$(NH_4)_2SO_4$ [M]	0.5	>0.7	0.5	0.5	0.5	0.5	1	1	1	1
$(NH_4)_2HPO_4$ [M]	0.4	0.6	>0.6	>0.6	>0.6	>0.6	>0.6	>0.6	>0.6	>0.6

Ammonium chloride (NH_4Cl) does not show combinational effects – an increase of the MIC- and MBC-values was observed if combined with one of both Lutensols. The MIC-values of ammonium dihydrogenphosphate ($NH_4H_2PO_4$) combined with one of the Lutensols have been reduced, but the MBC-values increased in comparison to the MBC-values of pure ammonium dihydrogenphosphate. Diammonium sulphate - $(NH_4)_2SO_4$ – the MIC-values remained unchanged and the MBC-values increased if combined with Lutensols. Diammonium hydrogenphosphate - $(NH_4)_2HPO_4$ – shows an increase of both – the MIC- and MBC-values if combined with Lutensols.

Table 3.16 MIC- and MBC-values of selected inorganic salts in combination with two representatives of Lutensols for *Listeria monocytogenes*

	MIC	MBC	MIC				MBC			
	without Lutensol		TO 12		AO 7		TO12		AO 7	
			1%	0.1%	1%	0.1%	1%	0.1%	1%	0.1%
NH_4Cl [M]	1	1	1	1	1	1	1	1	1	1
$NH_4H_2PO_4$ [M]	0.9	0.9	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$(NH_4)_2SO_4$ [M]	0.5	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$(NH_4)_2HPO_4$ [M]	0.5	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$(NH_4)_2CO_3$ [M]	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

In contrast to *E. coli*, NH_4Cl leads neither to a reduction nor to an increase of the MIC- and MBC-values in case of *L. monocytogenes*. The MIC- and MBC-values of ammonium dihydrogenphosphate ($NH_4H_2PO_4$), diammonium sulphate ($(NH_4)_2SO_4$), diammonium hydrogenphosphate ($(NH_4)_2HPO_4$) and diammonium carbonate ($(NH_4)_2CO_3$) were reduced if combined with Lutensols.

These series of experiments have shown that the combinational effect cannot be explained by the Hofmeister series.

3.3 The pH value and its influence on combinational effects

So far all the previous experiments were performed in a non-selective medium of neutral pH-value (pH~7). As many ion-detergent interactions are influenced through the pH, in the following experiments the combinational effect was investigated at different pH values. First, it was necessary to determine the window of life for *L. monocytogenes* depending on the pH value. Only *L. monocytogenes* has been selected for these series of experiments due to its general higher resistance compared to *E. coli*.

Table 3.17 Viability of *Listeria monocytogenes* in dependency on pH-value

	changed pH-value				
	4	5	6	7	8
CFU	0	n.a.	n.a.	n.a.	n.a.
growth	-	+	+	+	+

+ growth   - no growth

As shown by the result in table 3.17, *L. monocytogenes* was effectively killed at a pH < 5 and thus this condition was not included in the following experiments. On the other hand, media of pH > 8 were not investigated, based on the experience that the combinational effect does not appear due to the strong precipitation of Mg-ions as insoluble $Mg(OH)_2$. Further, *L. monocytogenes* was examined under a different pH-value (within the range 5 – 8) combined with descending concentration of $MgCl_2$ (1 M to 0.008 M in 2-fold dilution steps).

Table 3.18 Viability of bacterial cell in dependency on pH-value and salt concentration

	5	6	7	8	pH
MIC	0.5	0.5	0.9	>1	$MgCl_2$ [M]
MBC	0.5	1	>1	>1	

Finally, the combinations of different $MgCl_2$ concentrations and Lutensols (1% and 0.1%) were tested at four different pH values. The results for all combinations with 1% Lutensol are shown in table 3.19 and for 0.1% in table 3.20.

Table 3.19 Dependency of the combinational effect on the pH-value, part I

Lutensol	MgCl$_2$ [M]	changed pH-value				unchanged pH-value
		5	6	7	8	
1% TO 3	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	-	-		-
	0.0625	-				
1% TO 6	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	+	+		+
	0.0625	-				
1% TO 7	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	-	+
	0.125	-	-	+		+
	0.0625	-				
1% TO 8	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	-	+
	0.125	-	-	+		+
	0.0625	-				
1% TO10	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	+	+
	0.125	-	+	+		+
	0.0625	-				
1% TO12	1				-	
	0.5		-	+	-	+
	0.25	-	+	+	+	+
	0.125	+	+	+		+
	0.0625	+				
1% XP 70	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	-	-		-
	0.0625	-				
1% XP 30	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	-	-		-
	0.0625	-				
1% AO 7	1				-	
	0.5		-	-	-	-
	0.25	-	+	+	+	+
	0.125	+	+	+		+
	0.0625	+				
1% AO 3	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	-	+
	0.125	-	+	+		+
	0.0625	-				

Legend:
- (green) non tested
- (gray) growth impossible due to pH-value and salt concentration
- (+) growth
- (-) no growth

Lutensol	MgCl$_2$ [M]	changed pH-value				unchanged pH-value
		5	6	7	8	
0.1% TO 3	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	-	-		-
	0.0625	-				
0.1% TO 6	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	-	+
	0.125	-	+	+		+
	0.0625	-				
0.1% TO 7	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	-	+
	0.125	-	+	+		+
	0.0625	-				
0.1% TO 8	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	+	+
	0.125	-	-	+		+
	0.0625	-				
0.1% TO10	1				-	
	0.5		-	-	-	-
	0.25	-	+	+	+	+
	0.125	-	+	+		+
	0.0625	-				
0.1% TO12	1				-	
	0.5		-	+	+	+
	0.25	+	+	+	+	+
	0.125	+	+	+		+
	0.0625	+				
0.1% XP 70	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	-	-		-
	0.0625	-				
0.1% XP 30	1				-	
	0.5		-	-	-	-
	0.25	-	-	-	-	-
	0.125	-	-	-		-
	0.0625	-				
0.1% AO 7	1				-	
	0.5		-	-	-	-
	0.25	-	+	+	+	+
	0.125	+	+	+		+
	0.0625	+				
0.1% AO 3	1				-	
	0.5		-	-	-	-
	0.25	-	-	+	-	+
	0.125	-	+	+		+
	0.0625	-				

Legend:
- (green) non tested
- (light gray) growth impossible due to pH-value and salt concentration
- (+) growth
- (-) no growth

acidic	changed	TO 6, TO 7, TO 8, TO 10, TO 12, AO 7, AO 3
	unchanged	TO 3, XP 70, XP 30

basic	changed	TO 7, TO 8 (1% only), TO12 (1% only), AO 3
	unchanged	TO 3, TO 6, TO10, XP70, XP30, AO 7

The combinational effect of TO 3, TO 6, XP 70 and XP 30 remained unchanged even under acidic conditions. Compared to the medium of neutral pH-value, behavior of six Lutensol ´s grades have changed in the acidic environment not allowing the bacterial growth – thus the combinational effect was expressed even under a changed pH-value.

Under slightly basic conditions, the combinational effects have remained unchanged in six of ten Lutensol´s grades and bacteria could not grow. Other four grades have shown changed behavior in the sense of growth inhibition (-) compared to the medium of neutral pH-value.

Interestingly, Lutensol TO 12 has shown the combinational effect in combination with 0.5 M $MgCl_2$, if pH = 8.

In summary the combinational effect is independent from the pH-value.

3.4 Combinational effects of further surfactants

As mentioned in chapter 1.4.1., detergents are subdivided into four groups according to their structure: non-ionic, amphoteric, cationic and anionic. Lutensols, as non-ionic surfactants, are only one group of the large family of detergents. It may be interesting to investigate the behavior of detergents from other groups. To answer the question if the combinational effect is a characteristic feature of other surfactants, further detergents (anionic and non-ionic) were investigated. First, they were investigated separately to determine their toxicity against bacteria (Table 3.21). Afterwards a possible combinational effect of the detergents was tested with $MgCl_2$, NH_4Cl and $CaCl_2$. In both cases qualitative tests have been carried out to determine if the model organism is able to grow and create colonies under certain conditions or not.

Table 3.21 MBC-values (%) of chosen surfactants

		E.coli	L. monocytogenes
anionic	N-Lauroylsarcosine	>5	0.01
	Sodium dodecyl sulphate	>5	0.01
non - ionic	Brij L23	>5	>5
	Igepal CA-630	>5	>5
	Triton X-100	>5	>5
	Tween 20	5	5
	Tween 80	>5	>5
	Plurafac LF 300	>5	>5
	Plurafac LF 221	>5	>5
	Plurafac LF 220	>5	>5
	Emulan HE 51	5	>5
	Pluronic PE 6100	>5	>5
	Pluronic PE 6200	>5	>5
	Pluronic PE 9200	>5	>5

Based on the MBC-values of surfactants, one interesting phenomenon was observed – *L. monocytogenes* is highly susceptible against both anionic detergents, N-Lauroylsarcosine and SDS (MBC = 0.01% w/v).

Non-ionic detergents do not have a strong negative effect on the viability and growth capability of model organisms. Only two detergents have bactericidal activity at 5% (v/v) - Tween 20 (both model organisms) and Emulan HE 51 (*E. coli*).

In the next step each surfactant was combined with three inorganic chloride-containing salts of different concentrations to detect the combinational effect.

3.4.1 **Combination of various surfactants with MgCl$_2$**

The combination of MgCl$_2$ with 14 detergents has shown diverse results, depending on all the three conditions – model organism, type of surfactant and salt concentration.

Different MgCl$_2$-concentrations, below the MIC-value of respective bacterium, were applied (0.3 M, 0.1 M MgCl$_2$ for *E. coli* and 1 M, 0.5 M; 0.1 M for *L. monocytogenes*) in combination with 0.1% and 1% of each surfactant. The results are shown in table 3.22.

If the salt concentration was 0.1 M, the combinational effect did not appear and both model organisms have grown after re-plating on a non-selective agar (TSA + Y).

According to the results shown in table 3.22, no combinational effect was observed in case of *E. coli*. Re-plating on a non-selective agar after an overnight incubation has led to the growth within the majority of samples, thus MBC was not reached. Growth of *E. coli* was inhibited by 1% NLS combined with 0.3 M MgCl$_2$ only.

L. monocytogenes is sensitive against 1 M MgCl$_2$ combined with grades of Plurafac (LF 300, LF 221, LF 220), Emulan HE 51, Triton X-100, Tween 20 and Igepal CA-630, independently from the detergent´s concentration.

If the salt concentration was 0.5 M, Emulan HE 51, Igepal CA-630 and Tween 20 (each of them in concentration of 1%) have shown the combinational effect and bacteria did not grow after replating on a non-selective agar. Plurafac LF 300 combined with 0.5 M MgCl$_2$ has shown the combinational effect, independently of surfactant´s concentration.

Table 3.22 Growth ability after treatment with surfactant + $MgCl_2$ combination

		E. coli		L. monocytogenes		
		$MgCl_2$ [M]		$MgCl_2$ [M]		
		0.3	0.1	1	0.5	0.1
Brij L23 (%)	1	+	+	+	+	+
	0.1	+	+	+	+	+
Emulan HE 51 (%)	1	+	+	-	-	+
	0.1	+	+	-	+	+
N-Lauroylsarcosine (%)	1	-	+	n.t.	n.t.	n.t.
	0.1	+	+	n.t.	n.t.	n.t.
Igepal CA-630 (%)	1	+	+	-	-	+
	0.1	+	+	-	+	+
Sodium dodecyl sulphate (%)	1	+	+	n.t.	n.t.	n.t.
	0.1	+	+	n.t.	n.t.	n.t.
Triton X-100 (%)	1	+	+	-	+	+
	0.1	+	+	-	+	+
Tween 20 (%)	1	+	+	-	-	+
	0.1	+	+	-	+	+
Tween 80 (%)	1	+	+	+	+	+
	0.1	+	+	+	+	+
Plurafac LF 300 (%)	1	+	+	-	-	+
	0.1	+	+	-	-	+
Plurafac LF 221 (%)	1	+	+	-	+	+
	0.1	+	+	-	+	+
Plurafac LF 220 (%)	1	+	+	-	+	+
	0.1	+	+	-	+	+
Pluronic PE 6100 (%)	1	+	+	+	+	+
	0.1	+	+	+	+	+
Pluronic PE 6200 (%)	1	+	+	+	+	+
	0.1	+	+	+	+	+
Pluronic PE 9200 (%)	1	+	+	+	+	+
	0.1	+	+	+	+	+

+ growth

- no growth

n.t. not tested - those substances show high toxicity at very low concentration (0.01%)

3.4.2 Combination of various surfactants with NH$_4$Cl

Ammonium chloride was tested in three different concentrations (1 M, 0.3 M and 0.1 M for *E.coli* and 1 M, 0.5 M and 0.1 M for *L. monocytogenes*). If the salt concentration was 0.1 M, the combinational effect did not appear and bacteria started to grow and to create colonies after replating on a non-selective agar.

The results show that the combination of detergents with 1 M ammonium chloride seems to have no negative effect on the growth ability of *E. coli*. Three exceptions were observed, when bacterial growth was inhibited – Emulan HE 51 (1%), SDS (1%; 0.1%) and Pluronic PE 6200 (1%). Emulan HE 51 (1%) has shown the combinational effect in combination with 0.3 M NH$_4$Cl as well.

Directly compared to *E. coli*, *L. monocytogenes* has died due to the combinational effect in several cases (e.g. 1 M NH$_4$Cl combined with the following detergents: Igepal CA-630, Tween 20, Triton X-100 and all three grades of Plurafac). Further, bactericidal effect of Emulan HE 51 (1%) combined with NH$_4$Cl (1 M and 0.5 M), and of Igepal CA-630 (1% and 0.1%) and 1 M NH$_4$Cl was detected for *L. monocytogenes* as well.

If the salt concentration was 0.5 M, the following surfactants have shown the combinational effect: Emulan HE 51 (1%), Triton X-100 (1%), Tween 20 (1%; 0.1%) and Plurafac LF 300 (1%; 0.1%).

Table 3.23 Growth ability after treatment with surfactant + NH$_4$Cl combination

		E. coli			L. monocytogenes		
		NH$_4$Cl [M]			NH$_4$Cl [M]		
		1	0.3	0.1	1	0.5	0.1
Brij L23 (%)	1	+	+	+	+	+	+
	0.1	+	+	+	+	+	+
Emulan HE 51 (%)	1	-	-	+	-	-	+
	0.1	+	+	+	+	+	+
N-Lauroylsarcosine (%)	1	+	+	+	n.t.	n.t.	n.t.
	0.1	+	+	+	n.t.	n.t.	n.t.
Igepal CA-630 (%)	1	+	+	+	-	+	+
	0.1	+	+	+	-	+	+
Sodium dodecyl sulphate (%)	1	-	+	+	n.t.	n.t.	n.t.
	0.1	-	+	+	n.t.	n.t.	n.t.
Triton X-100 (%)	1	+	+	+	-	-	+
	0.1	+	+	+	-	+	+
Tween 20 (%)	1	+	+	+	-	-	+
	0.1	+	+	+	-	-	+
Tween 80 (%)	1	+	+	+	+	+	+
	0.1	+	+	+	+	+	+
Plurafac LF 300 (%)	1	+	+	+	-	-	+
	0.1	+	+	+	-	-	+
Plurafac LF 221 (%)	1	+	+	+	-	+	+
	0.1	+	+	+	-	+	+
Plurafac LF 220 (%)	1	+	+	+	-	+	+
	0.1	+	+	+	-	+	+
Pluronic PE6100 (%)	1	+	+	+	+	+	+
	0.1	+	+	+	+	+	+
Pluronic PE 6200 (%)	1	-	+	+	+	+	+
	0.1	+	+	+	+	+	+
Pluronic PE 9200 (%)	1	+	+	+	+	+	+
	0.1	+	+	+	+	+	+

+ growth

- no growth

n.t. not tested - those substances show high toxicity at very low concentration (0.01%)

3.4.3 **Combination of various surfactants with CaCl$_2$**

Calcium chloride was tested in three different concentrations (1 M, 0.3 M and 0.1 M for *E. coli* and 1 M, 0.5 M and 0.1 M for *L. monocytogenes*).

The combination of CaCl$_2$ (1 M) with each of 14 detergents has led to growth inhibition – *E. coli* was not able to grow after re-plating on a non-selective agar (TSA + Y). Further, in the presence of 0.3 M CaCl$_2$, all three grades of Plurafac and two of three Pluronic ´s grades have shown the combinational effect. The same situation was observed in case of Igepal CA-630 and Triton X-100. Last but not least, 0.1% N-Lauroylsarcosine com-bined with 0.3 M CaCl$_2$ has shown the combinational effect.

Contrary, *L. monocytogenes* has shown higher resistance against these combinations. The majority of samples plated out on a non-selective agar have created colonies. The combination of 1 M CaCl$_2$ with grades of Plurafac (1%; 0.1%), Tween 20 (1%; 0.1%), Triton X-100 (1%; 0.1%), Igepal CA-630 (1%; 0.1%) and Emulan HE 51 (1%; 0.1%) has a negative effect on the growth ability of *L. monocytogenes*. Additionally, 1% Emulan HE 51 had bactericidal activity, if the salt concentration was 0.5 M or 0.1 M, respective-ly. If the salt concentration was 0.5 M, Igepal CA-630, Triton X-100 and Plurafac LF 300 have shown bactericidal activity independently of detergent´s concentration. Tween 20 (1%) combined with 0.5 M CaCl$_2$ has a bactericidal effect as well.

Table 3.24 Growth ability after treatment with surfactant + $CaCl_2$ combination

		E. coli			L. monocytogenes		
		$CaCl_2$ [M]			$CaCl_2$ [M]		
		1	0.3	0.1	1	0.5	0.1
Brij L23 (%)	1	-	+	+	+	+	+
	0.1	-	+	+	+	+	+
Emulan HE 51 (%)	1	-	+	+	-	-	-
	0.1	-	+	+	-	+	+
N-Lauroylsarcosine (%)	1	-	+	+	n.t.	n.t.	n.t.
	0.1	-	-	+	n.t.	n.t.	n.t.
Igepal Ca-630 (%)	1	-	-	+	-	-	+
	0.1	-	-	+	-	-	+
Sodium dodecyl sulphate (%)	1	-	-	+	n.t.	n.t.	n.t.
	0.1	-	+	+	n.t.	n.t.	n.t.
Triton X-100 (%)	1	-	-	+	-	-	+
	0.1	-	-	+	-	-	+
Tween 20 (%)	1	-	+	+	-	-	+
	0.1	-	+	+	-	+	+
Tween 80 (%)	1	-	+	+	+	+	+
	0.1	-	+	+	+	+	+
Plurafac LF 300 (%)	1	-	-	+	-	-	+
	0.1	-	-	+	-	-	+
Plurafac LF 221 (%)	1	-	-	+	-	+	+
	0.1	-	-	+	-	+	+
Plurafac LF 220(%)	1	-	-	+	-	+	+
	0.1	-	-	+	-	+	+
Pluronic PE 6100 (%)	1	-	-	+	+	+	+
	0.1	-	-	+	+	+	+
Pluronic PE 6200 (%)	1	-	-	+	+	+	+
	0.1	-	-	+	+	+	+
Pluronic PE 9200 (%)	1	-	+	+	+	+	+
	0.1	-	+	+	+	+	+

+ growth

- no growth

n.t. not tested - those substances show high toxicity at very low concentration (0.01%)

3.5 Mode of action

The results of the screening have shown that bacteria die due to the combinational effect. How does the mechanism of combinational effect work? Does it lead to the disintegration of a plasma membrane? To investigate the integrity of a plasma membrane, the cells were investigated under a fluorescence microscope after a treatment with a substance of interest. In case of the disintegration of a plasma membrane, cytoplasmic molecules were released into supernatant. Small molecules (e.g. ATP) diffuse rapidly and freely through the damaged plasma membrane and can be detected easily. On the other hand, DNA, as a large molecule, should not be released immediately upon membrane disruption, and its amount depends on the used substance and the time of incubation. The DNA remained in the pellet was extracted and detected by qPCR.

3.5.1 Plasma membrane as target

A plasma membrane is a phospholipid bilayer containing integral and superficial proteins. From the chemical point of view, phospholipids are the esters of fatty acids and glycerol, having a polar head group in the form of phosphate residue. Phospholipids aggregate in an aqueous environment building micelles (Alberts et al. 2002). Micelles are spherical structures of glycerol monoester having a polar hydrophilic head oriented towards the aqueous environment, whereas hydrophobic side chains are hidden within the spherical structure (Latscha and Kazmaier 2008). As soon as two alcohol residues of glycerol are replaced by a fatty acid chain, there is not enough space to create a micelle. Because of this steric hindrance, glycerol diester molecules start to create bilayer. The edges of some bilayers are energetically unfavorable because of the exposure of hydrophobic side chains of fatty acid to the aqueous environment (Alberts et al. 2002). Thus, the phospholipid bilayer starts to build a closed structure, having hydrophilic head groups on both sides enabling interaction with an aqueous environment on both sides, outside and inside the sphere (Alberts et al. 2002). This compact structure can be destroyed by surfactants reducing the surface tension – a well known example of a disintegration of a plasma membrane and the removal of integral proteins from plasma membrane by adding sodium dodecyl sulphate (Alberts et al. 2002). This is based on the fact that detergents can mimic the lipid-bilayer environment. Proteins removed from the membrane bilayer contained hydrophobic parts, interacting with a hydrophobic part of the detergent. The hydrophilic part of a detergent interacts with water and thus the protein is completely solved in an aqueous solution (Bhairi 2001).

The similar mechanism is expected in case of Lutensol combined with chaotropic inorganic salts. In contrast to sodium dodecyl sulphate, Lutensols, as non-ionic detergents, have a much smaller surfactant´s activity than sodium dodecyl sulphate.

The integrity of the plasma membrane of *E. coli* and *L. monocytogenes* was investigated after the treatment with five different conditions – 1x PBS, 1% SDS, 1 M $MgCl_2$ + 1% Lutensol TO 12, 1 M $MgCl_2$ + 1% Lutensol AO 7 and 1 M $MgCl_2$ incubated for 60 min. at 37°C under constant shaking (750 rpm). The sample treated with 1x PBS was used as a negative control showing the major population of viable cells.

This experiment was extended by the third model organism – *S.* Typhimurium – to emphasize the differences between gram-positive and gram-negative bacteria. Together with *E. coli*, *S.* Typhimurium, as a gram-negative bacterium, shows resistance against sodium dodecyl sulphate.

On the contrary, *L. monocytogenes*, a halotolerant bacterium (Gandhi and Chikindas 2007), exhibits a high resistance against $MgCl_2$.

3.5.1.1 Escherichia coli

Figure 3.5 shows the BacLight results of the five conditions on *E. coli* and Figure 3.6 depicts the respective plate counts of each experiment.

According to the examination under fluorescence microscope (images shown in figure 3.7), the sample of *E. coli* treated with buffer solution (1x PBS) also contains damaged bacteria (46.20% of total cell count, red in diagram). These are the dying cells at the end of their life span.

Using the same method to compare the effect of 1% SDS on viability of *E. coli*, it was observed that more than six tenths of the total cell count have shown a damaged cell membrane. This is quite in contrast to the results of screening, showing that *E. coli* was able to survive even in 5% SDS during an overnight incubation. It might be that the population of cells with a damaged cell membrane contains mainly elderly cells at the end of the life span. Another explanation could be that *E. coli* has induced protecting mechanisms against SDS during the overnight incubation. On the contrary, the three tenths were able to grow and create colonies detectable by a plate count method.

The sensitivity of *E. coli* against $MgCl_2$ was confirmed by viable cell counting, when only one fifth of the total cell count has survived and was able to create colonies after re-plating on a fresh non-selective agar (TSA + Y). Compared to 1x PBS, approximately 64% of cells were killed after the treatment with 1 M $MgCl_2$.

After the treatment with the combination of 1 M $MgCl_2$ and one of both Lutensols, the number of damaged cells has doubled compared to the sample treated with 1x PBS. Additionally, a picture of the sample treated with 0.2 M $MgCl_2$ has been added to emphasize the different influence of variable salt concentrations on bacteria.
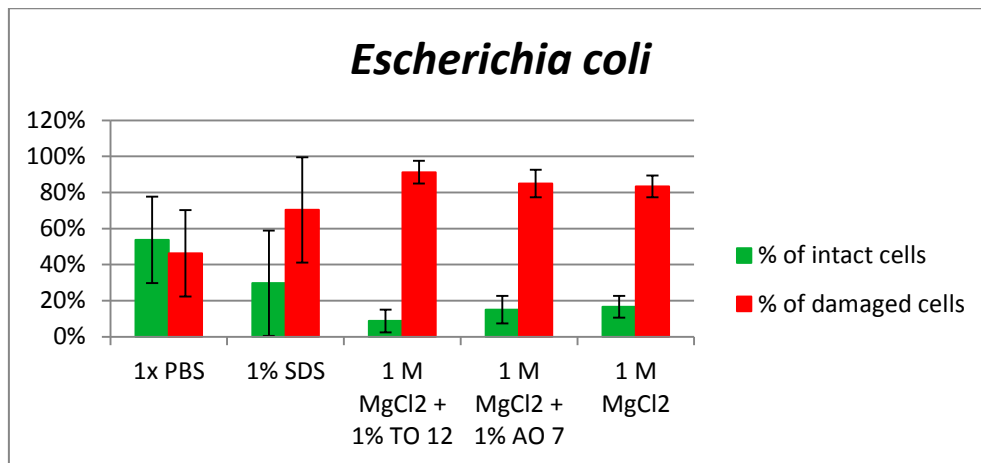
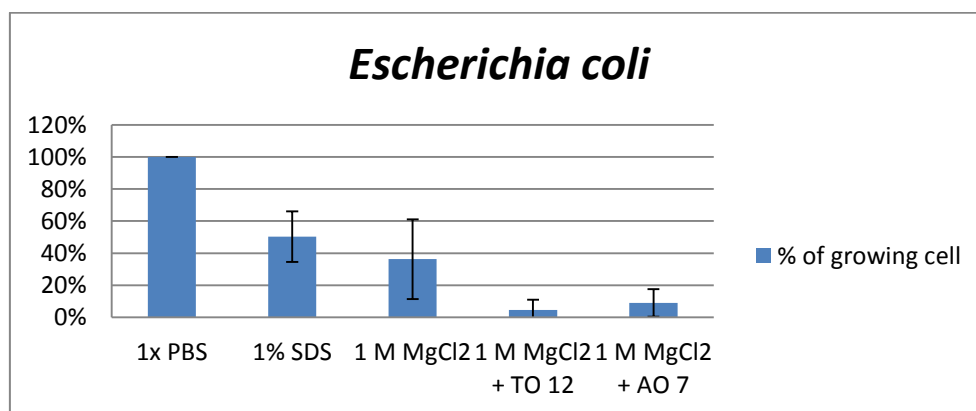Figure 3.5 Viable cell counting after an incubation with five different conditions

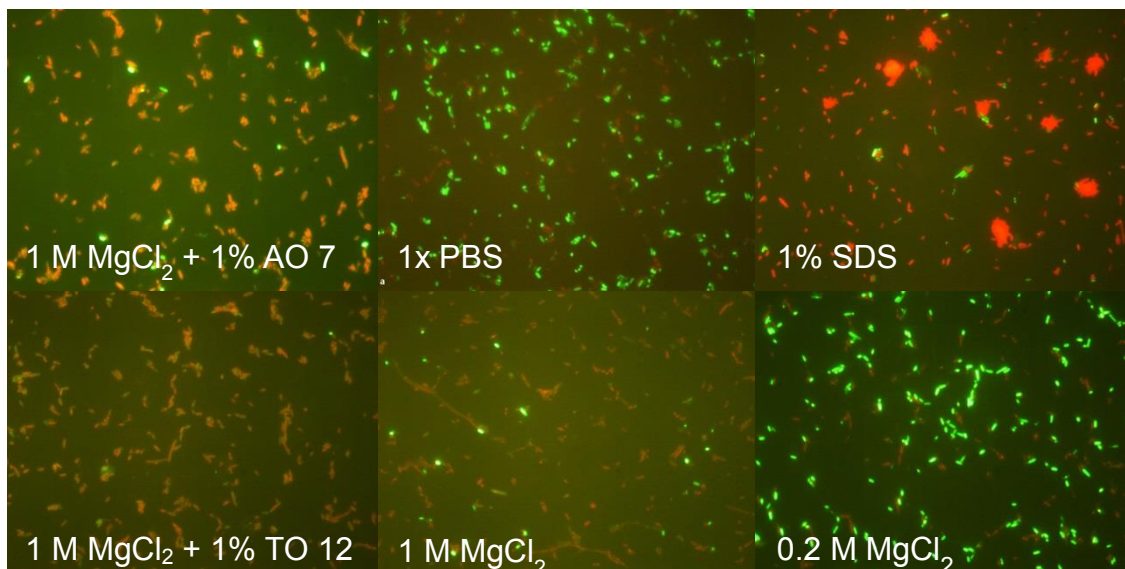Figure 3.6 Percentage of growing cells after incubation with five different conditions

Figure 3.7 Appearance of *Escherichia coli* under fluorescence
microscope after treatment

3.5.1.2 Listeria monocytogenes

Figure 3.8 shows the BacLight results of the five conditions on *L. monocytogenes*, figure 3.9 depicts the respective plate counts of each experiment, and figure 3.10 elucidates the appearance of *L. monocytogenes* under the fluorescence microscope after the treatment with substances of interest. Additionally, a picture of sample treated with 0.2 M $MgCl_2$ has been added to emphasize the different influence of variable salt concentrations on bacteria.

As for E. coli, the samples treated with 1x PBS contain about 30% of damaged cells. Treatment with 1% SDS has led to the increase of damaged cells: About four fifth of the total cell count have shown a damaged cell membrane. The one fifth of the total cell count examined as a population with intact plasma membrane was no longer able to grow and to create colonies after replating on a fresh non-selective agar (TSA + Y). A possible explanation for this phenomenon is that those cells persist in VBNC state unable to react on the environment.

Interestingly, the treatment with 1 M $MgCl_2$ caused cell membrane damage in one half of the total cell count only. The other half, showing an intact membrane, has grown and created colonies detectable by a plate count method.

After the treatment with 1M $MgCl_2$ combined with one of both Lutensols, about six tenths of total cell count have been damaged. The rest of the total cell count (about four tenths), showing an intact cell membrane, was unable to grow and to create colonies.

A high standard deviation was observed in total. This might be because of the different fitness of three-hour cultures used in the series of experiments.

Results of the plate count method are in accordance with the BacLight results, showing that *L. monocytogenes* is resistant against 1 M $MgCl_2$. Further, the plate count method has confirmed the sensitivity of *L. monocytogenes* against SDS. The combination of 1 M $MgCl_2$ and one of both Lutensols has killed up to 100% of the total cell count, in comparison to the cells treated with 1x PBS. Bactericidal activity of these combinations is comparable to that of 1% SDS.

L. monocytogenes is difficult to stain, even when both fluorescent dyes were used in excess (2 µl of each dye per 1000 µl cell suspension) and the incubation time was doubled (30 min.).
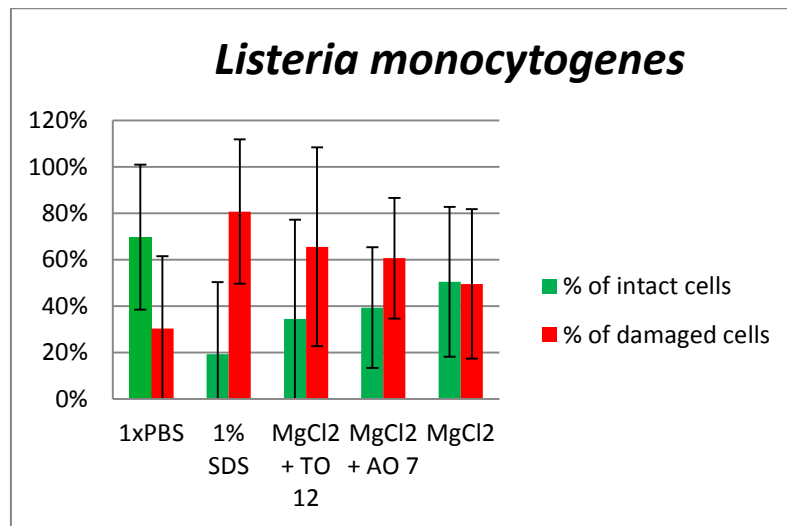
Figure 3.8 Viable cell counting after the incubation with five different conditions
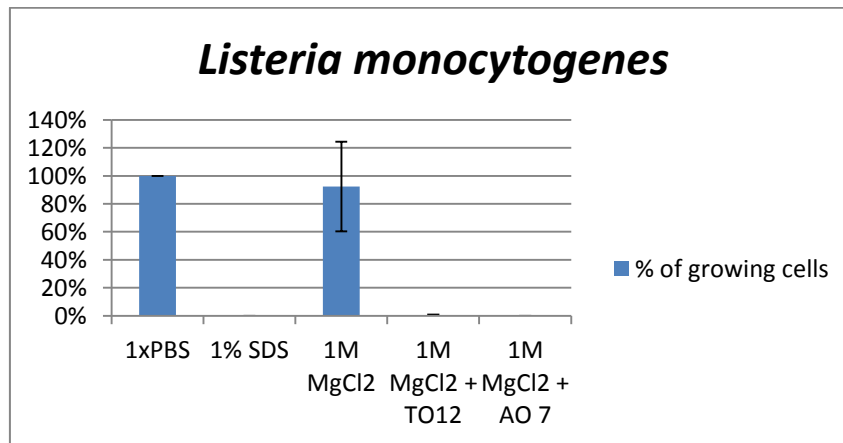

Figure 3.9 Percentage of growing cells after the incubation with five different conditions
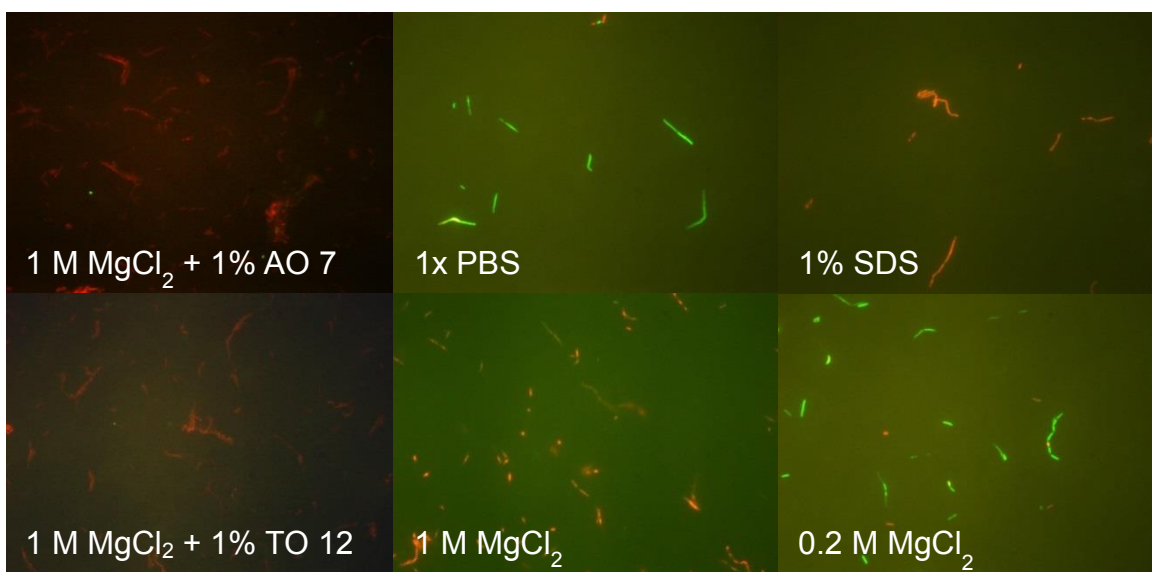

Figure 3.10 Appearance of *Listeria monocytogenes* under fluorescence microscope after treatment

3.5.1.3 *Salmonella enterica*, ssp*. enterica*, serovar Typhimurium

Figure 3.11 shows the BacLight results of the five conditions on *S.* Typhimurium, figure 3.12 depicts the respective plate counts of each experiment, and figure 3.13 elucidates appearance of *L. monocytogenes* under the fluorescence microscope after the treatment with substances of interest. Additionally, a picture of sample treated with 0.2 M $MgCl_2$ has been added to emphasize the different influence of variable salt concentrations on bacteria.

The major population of sample treated with 1x PBS has shown a damaged plasma membrane. This can be explained by the fact that these cells are at the end of their life span.

Treatment of *S.* Typhimurium with 1% SDS has led to an interesting phenomenon – cells with the damaged plasma membrane have represented a small population. A possible explanation is that dead and dying cells were no longer able to withstand the stress induced by the detergent, and were lysed.

Interestingly, the number of cells with a damaged plasma membrane after treatment with 1 M $MgCl_2$ is smaller than the sample treated with 1x PBS. But the results of a plate count method have shown that one fifth of the total cell count is able to grow and create colonies in comparison to 1x PBS.

In case of treatment with the combination of 1 M $MgCl_2$ with one of both Lutensols, more than six tenths of the total cell count have shown a damaged cell membrane (AO 7) and in case of TO 12 more than seven tenths have shown a damaged cell membrane.

The comparison of effect of 1x PBS and 1 M $MgCl_2$ shows that nearly 18% of cells were able to grow and to create colonies after the treatment with magnesium chloride.

Less than 2% of cells created colonies after the treatment with 1 M $MgCl_2$ combined with one of both Lutensols.
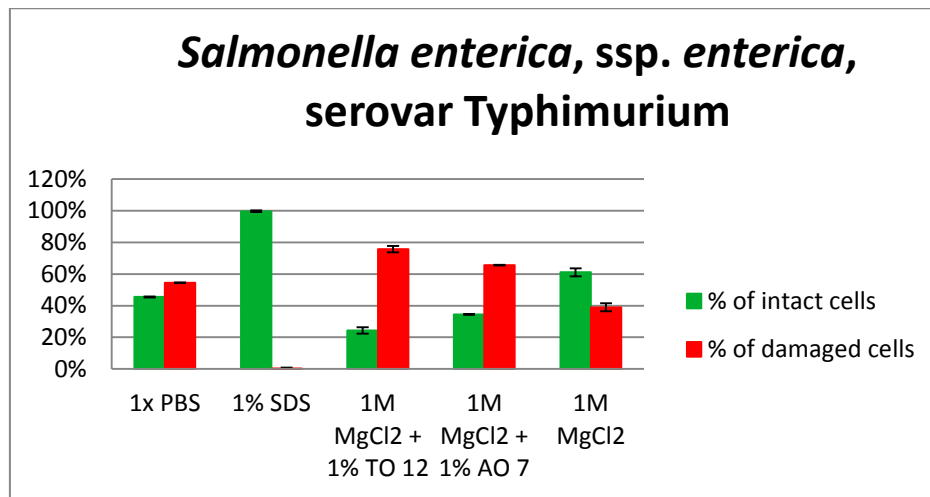
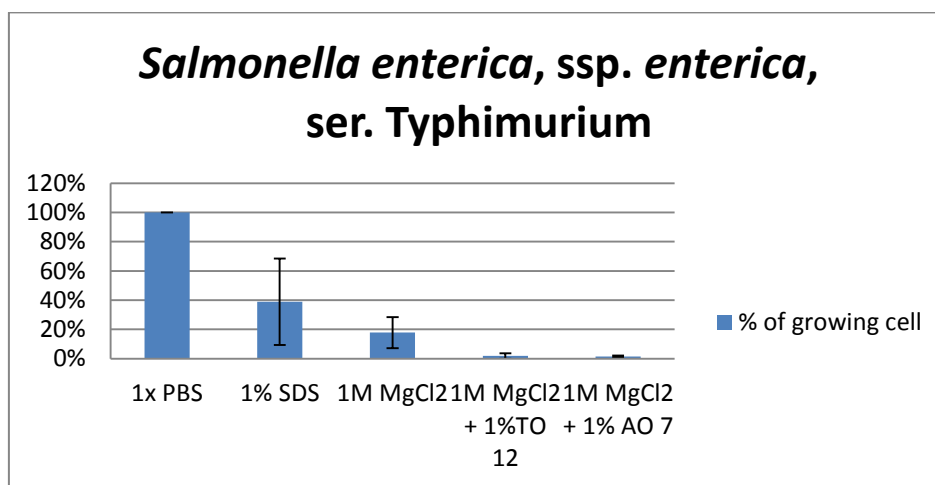Figure 3.11 Viable cell counting after the incubation with different conditions

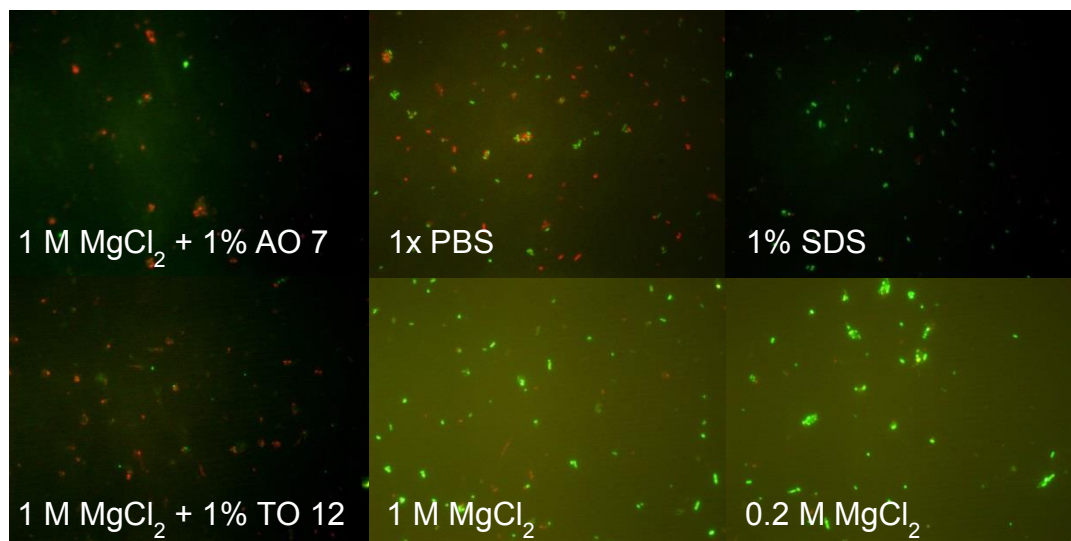Figure 3.12 Percentage of growing cells after the incubation with five different conditions

Figure 3.13 Appearance of *Salmonella enterica*, ssp. *enterica*, serovar Typhimurium under fluorescence microscope after treatment

3.5.2 **ATP – bioluminescence**

The results of BacLight have shown that the treatment with 1 M $MgCl_2$ combined either with 1% Lutensol TO 12 or with 1% Lutensol AO 7 has led to a plasma membrane damage. Cell content (e.g. ATP) can diffuse freely and can be detected in supernatant. To conduct this experiment, a three-hour culture of *L. monocytogenes* has been used. The procedure described in Materials and Methods was carried out.

To have a benchmark, a standard curve of ATP bioluminescence is necessary. Its preparation has been done as follows: ATP stock solution (10 mM) of a final volume of 2 ml was prepared by solving the ATP powder (12.104 mg) in distilled water. An aliquot of ATP stock solution was 10000-times diluted to create the solution of concentration of 1 µM. The ATP stock solution (10 mM) was stored in a refrigerator for future reuse. Using 1 µM ATP solution, seven 10-fold dilution steps (10^{-6} M – 10^{-12} M) were prepared by the addition of distilled water in a ratio of 1:9. As it can be seen, the standard curve is linear within the concentrations range from 10^{-10} M to 10^{-6} M. Within this range it can be used for the purpose of measurement. R^2 = 0.9897 (R^2 is the coefficient of determination indicating how well the data fit a statistical model).
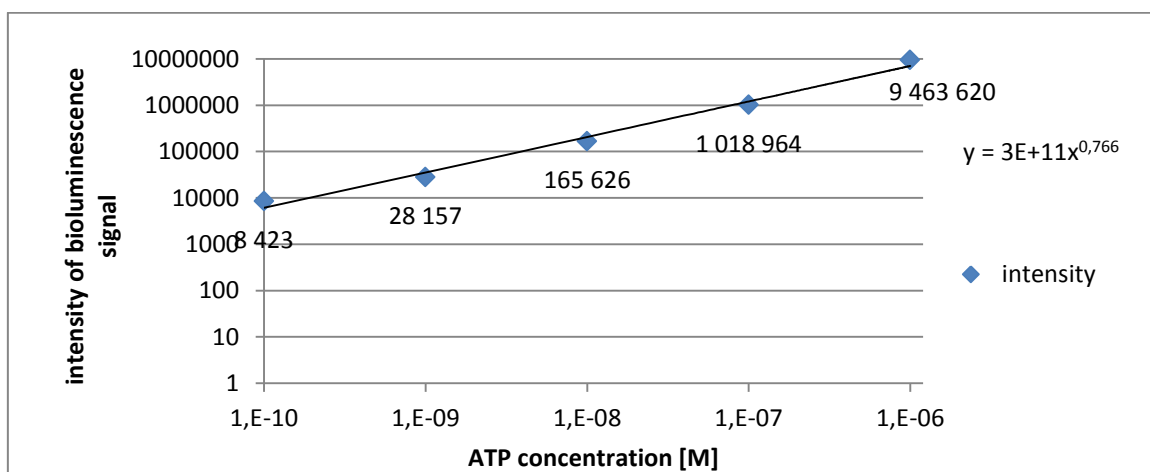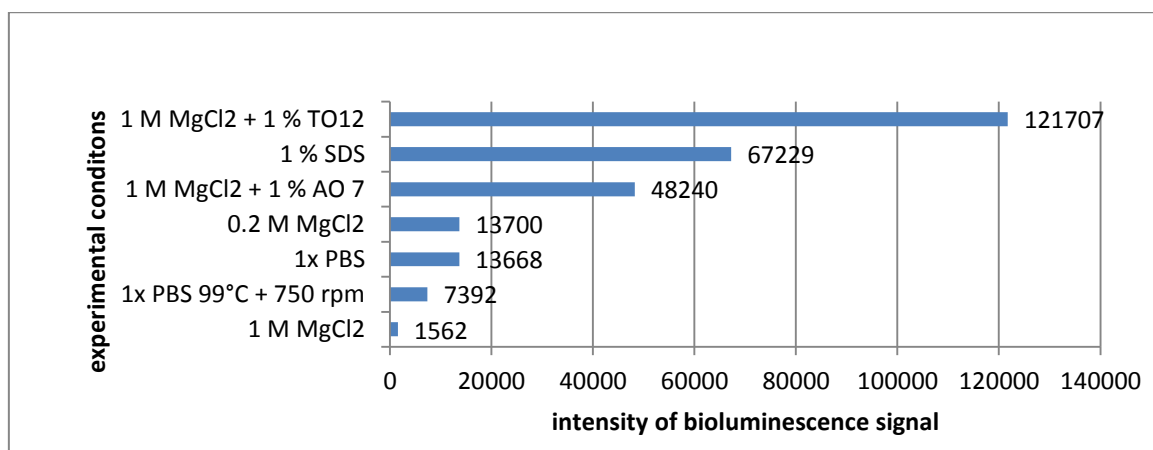

Figure 3.14 Standard curve

Figure 3.15 ATP bioluminescence after the treatment with substances of interest

To have results from different points of view, the samples were not only investigated in the spectrophotometer (TECAN infinite F200), but also plated out on a non-selective agar (TSA + Y). The number of colonies, counted after an overnight incubation at 37°C, was compared with the intensity of the bioluminescence signal of each sample.
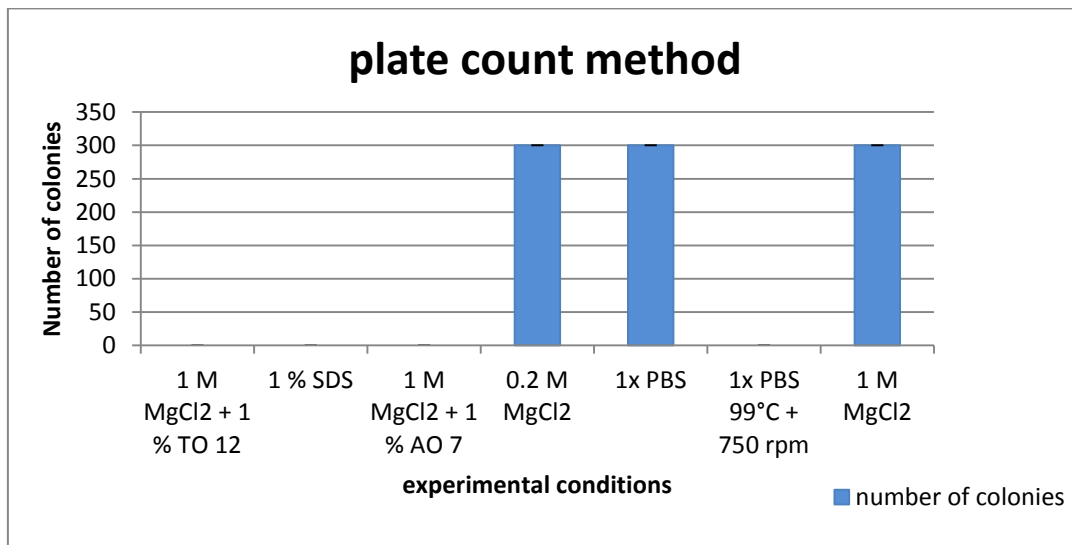

plate count method

Figure 3.16 Plate count method after the treatment with substances of interest

Comparing the bioluminescence and the plate count method, the results correspond to each other – treatment with 1x PBS, 0.2 M $MgCl_2$ or 1 M $MgCl_2$ has no bactericidal effect and the cells were able to grow and to create colonies after re-plating on a fresh non-selective agar (TSA + Y). The bioluminescence signal of these samples was of low intensity, indicating viable cells with an intact plasma membrane, and ATP molecules detectable by bioluminescence at very low rate have originated from dead cells at the end of their life span.

Phosphate buffered saline (PBS) has had no lytic effect on cells of the three-hour culture. Thus, the low amount of released ATP (and the low strengths of bioluminescence signal) is the consequence of cells died because of their restricted life span and not because of the incubation conditions. The amount of ATP corresponds to the concentration of 10^{-9} to 10^{-10} M. This result is in accordance with the results of plate count method, when cells, treated with 1x PBS, has grown and created colonies (>300) after re-plating on a fresh non-selective agar (TSA + Y).

Interestingly, treatment with 1x PBS at 99°C for 60 min. under constant shaking (750rpm) has led to the reduction of the bioluminescence intensity in comparison to the intensity of the sample treated with 1x PBS at 30°C. This decrease can be explained by the fact that ATP is heat-labile. The bactericidal effect of these conditions was confirmed by the plate count method, when samples plated out on a non-selective agar (TSA + Y) did not grow.

L. monocytogenes, known as a halotolerant bacterium, is able to survive in the salt solution, and thus it was viable after treatment with 0.2 M $MgCl_2$ and 1 M $MgCl_2$. The cells of those samples were able to grow and to create colonies (>300) after re-plating on a fresh non-selective agar (TSA + Y). The weak intensity of the bioluminescence signal is in accordance with the results of the plate count method and it points out that the majority of bacteria stay unaffected after the treatment.

Treatment with 1% SDS has led to a ~ 5-fold increase of bioluminescence signal intensity in comparison to samples treated with 1x PBS, indicating membrane disintegration as the main bactericidal effect of 1% SDS. These results were confirmed by the results of the plate count method (no colonies detectable).

Magnesium ions are necessary in enzymatic conversion of luciferin to oxyluciferin, but magnesium chloride has clearly inhibited the bioluminescence.

Treatment with 1 M $MgCl_2$ has led to the signal of the lowest intensity from all three samples (1x PBS; 0.2 M $MgCl_2$; 1 M $MgCl_2$) incubated under non-toxic conditions.

The inhibitory activity of $MgCl_2$ was experimentally proven when bioluminescence signal intensity emitted from 10^{-6} M ATP solution was 24.5-times weaker in presence of 1 M $MgCl_2$ than the 10^{-6} M ATP solution without $MgCl_2$. Proportionally, 0.5 M $MgCl_2$ has led to 12.2-fold reduction of bioluminescence and 4.9-fold reduction was observed when 0.2M $MgCl_2$ was present in the sample. This fact has to be taken into account when evaluating samples treated with 1 M $MgCl_2$ + 1 % Lutensol.

The lytic effect of 1 M $MgCl_2$ combined with 1% TO 12 was confirmed by both methods – no colonies were detected by a plate count method. The luminescence signal intensity was 9-times stronger than the intensity of 1x PBS sample. However, taking into account the inhibitory activity of 1 M $MgCl_2$, an even higher ATP release is probable but could not be exactly quantified with this experimental approach. A similar situation was observed in treatment with the combination 1 M $MgCl_2$ + 1% AO 7 showing a 4-fold increase of bioluminescence signal intensity, in comparison to the sample treated with 1x PBS.

3.5.3 DNA – extraction + qPCR

As the results of fluorescence microscopy have shown, combinational effect is based on the disintegration of a plasma membrane and a subsequent release of the cell content. Procedure of quantitative PCR has been applied for the detection of DNA remained within the pellet. To conduct this experiment, a three-hour-culture of *S.* Typhimurium was treated with chosen chemicals for 60 min. at 30°C under constant shaking at 750 rpm, and DNA was extracted subsequently as described in chapter 2.3.3.

The stronger the disintegration of a plasma membrane due to treatment, the stronger is the release of cell content into supernatant, and the amount of DNA remained within the

pellet is proportionally smaller. Thus, the threshold value is achieved later in comparison to the untreated sample. An untreated sample, named as an input, was used as a positive control containing the maximal amount of DNA within the pellet.

Resistance of *S*. Typhimurium against ionic stress induced by SDS was detected during screening (data not shown) and confirmed by DNA amplification experiments, where the threshold value of the sample treated with 1% SDS was reached between the 17th and the 18th cycle. In comparison, the threshold value of the untreated sample was achieved in the 16th cycle.

After application of 1 M $MgCl_2$, the threshold value was reached between the 18th and the 19th cycle. Contrary, the combination of 1 M $MgCl_2$ with 1% Lutensol TO 12 has shown a strong impact on the plasma membrane enabling the release of DNA into supernatant. Thus, only a small amount of DNA remained in the pellet, which in turn has led to a late achievement of the threshold value (21st cycle). This is the evidence of the combinational effect.

Interestingly, combination of inorganic salt with Lutensol TO 12 has had a stronger negative effect on the integrity of a plasma membrane than the combination of salt with Lutensol AO 7.

Table 3.255 qPCR for the detection of plasma membrane damage

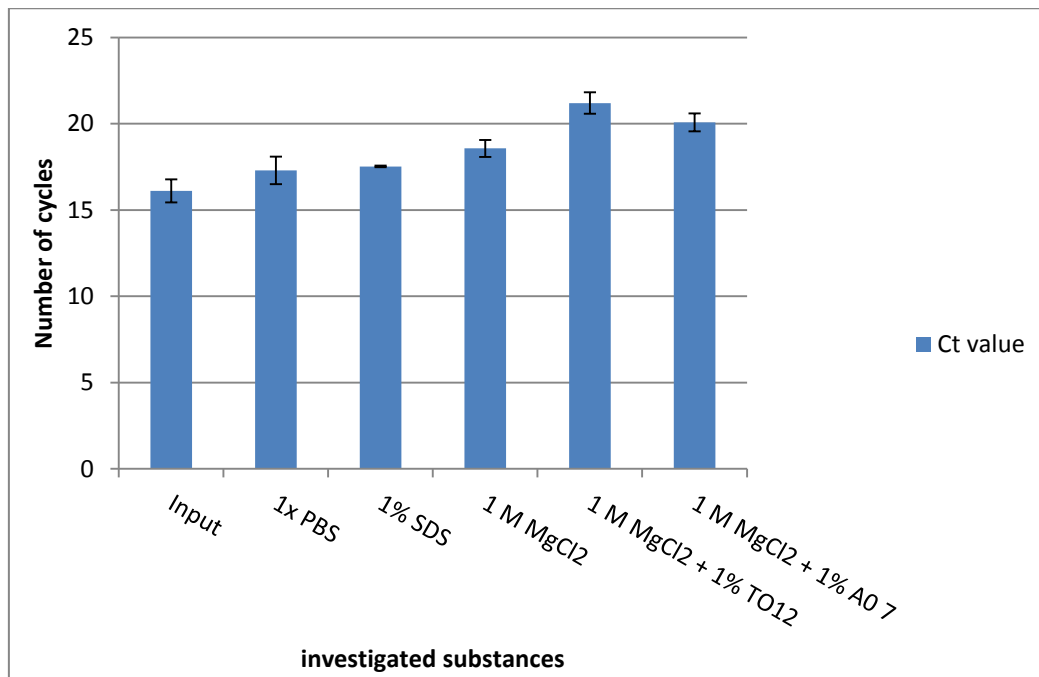| Mastermix	end conc.	stock conc.	1x	48x
			µl	µl
Aqua dest.			9.95	477.6
10x puffer	1x		2.5	120
MgCl2	3.5 mM	50 mM	1.75	84
fim AF1	300 nM	5 µM	1.5	72
fim AR1	300 nM	5 µM	1.5	72
Probe AS1	300 nM	5 µM	1.5	72
Probe2	100 nM	5 µM	0	0
dNTP`s	200 µM each	20 mM	1	48
Taq pol	1.5U		0.3	14.4
IPC	50 copies	2e2 copies/µl	0	0
Template			5	240
Total			25	1200
1x volume			25	

Figure 3.17 qPCR results after the treatment with substances of interest

4 Discussion

The aim of this study was to discover if the combinational effect is a general characteristic for i) all Lutensols combined with $MgCl_2$, ii) other surfactants combined with $MgCl_2$ and iii) surfactants combined with other inorganic salts. Another question was how the mechanism of combinational effect works. Three model organisms were used to answer these questions and to elucidate the basic principles of the combinational effect: *Listeria monocytogenes* representing the major threat for the dairy product industry. The second model organism was *Escherichia coli*, a gram-negative commensal living in human gut, and the third model organism was *Salmonella* Typhimurium.

4.1 Screening

Lutensol AO 7 in combination with $MgCl_2$ is toxic to bacteria (Kwisda 2012), thus it could be applied in the process of Matrix-Lysis with restrictions only (detection of bacteria by the methods of molecular biology). It was necessary to find a grade of Lutensol with minimal combinational effect, and thus it can be applied in the process of the Matrix-Lysis. The following screening has been done to answer this question: Ten Lutensol´s grades (AO 7 including) belonging to three different classes (TO; XP; AO) were investigated separately to determine their toxicity against two model organisms (*E. coli*, *L. monocytogenes*). The results of the screening have shown that none of the tested Lutensol´s grades is toxic – bacteria have survived even in 5% Lutensol solution.

Next, toxicity of $MgCl_2$ was investigated to determine the window of survival for both model organisms. The results have shown that *L. monocytogenes* is more resistant against the osmotic stress induced by $MgCl_2$ (MBC > 1 M $MgCl_2$) than the *E. coli* (MBC = 0.4 M $MgCl_2$).

Based on these results, the following experiments have been done investigating bacterial growth in the presence of $MgCl_2$ combined with one of ten Lutensol´s grades. The results have shown that Lutensol AO 7 is highly toxic (MBC = 0.039 %) for both model organisms independently of the salt concentration.

Interestingly, in case of *E. coli*, no combinational effect was observed in nine of ten Lutensol´s grades if the salt was applied at half of the MIC. This phenomenon was not observed in *L. monocytogenes*. A further screening done with *E. coli* has revealed Lutensol TO 7 as the most appropriate substitute for the Lutensol AO 7. The MIC of Lutensol TO 7 was 5% if combined with 0.3 M $MgCl_2$. The concentration below 5% can be applied in the process of Matrix-Lysis without compromising *E. coli*. The second most appropriate substitute was Lutensol TO 12 with a MIC of 1.25% if combined with 0.3 M $MgCl_2$ and 5% in combination with 0.25 M $MgCl_2$, respectively.

The results of the screening done with *L. monocytogenes* have shown that Lutensol TO 12, with a MIC > 5% independently of salt concentration, was the most appropriate detergent applicable in the process of Matrix-Lysis without a negative influence on the viability of bacterial cells.

Comparing these results with the results of screening done with *E. coli*, the conclusion can be drawn that 5% Lutensol TO 12 is applicable in the process of Matrix-Lysis without affecting the viability of bacteria, if applied in combination with 0.25M $MgCl_2$.

Summarizing, the strength of the combinational effect depends on the variable molecular structure of Lutensol containing a variable number of ethoxy groups within the side chain and diverse radices as backbone.

Moreover, the detection of possible inhibitory effects of Lutensol grades and other detergents has shown that none of the Lutensol grades within the concentration range 5% - 0.01% leads to the complete inhibition of the reaction. A distinct shift of the C_T-value has only been observed after applying a 5% solution of Lutensol TO 6 and Lutensol TO 8, respectively.

This is an advantageous property in comparison to SDS, leading to a complete inhibition of DNA amplification, independently of the detergent concentration. A negative influence on the DNA amplification has also been observed if 5% Tween 20 (complete inhibition) and 1% Tween 20 (shift of C_T-value) applied.

The application of Triton X-100 within the range from 5% to 0.01% has no negative influence on the DNA amplification, thus its properties are comparable to those of Lutensol grades.

4.2 Molecular principles of combinational effects

Further investigation was dedicated to determinate the role of the salt in the combinational effect.

4.2.1 Effect of inorganic salts

The first hypothesis was that chloride anion (Cl⁻) could have an influence on the combinational effect. Thus, six chloride-containing salts were investigated. This set of experiments comprised also the investigation of the effect of heavy metal ions (Sr^{2+}) and LiCl, acting chaotropically. The results have shown that neither Cl-ions, nor heavy metal ions (Sr^{2+}) have a negative influence on viability and growth ability. The absence of the toxicity of heavy metal ions can be explained by efflux pumps, positioned in the bacterial plasma membrane, transporting poisonous ions outside of the cell with a very high rate and efficiency.

Chaotropically acting LiCl has shown an ambivalent effect on both model organisms – *E. coli* has shown a relatively high resistance (MIC = 0.7 M; MBC = 0.9 M), thus the combinational effect could be investigated (MIC = 0.5 M and MBC = 0.9 M in combination with TO 12, MIC = 0.9 M and MBC = 0.9 M in combination with AO 7). On the contrary, *L. monocytogenes* was highly susceptible to LiCl (MBC = 0.1 M) and the combinational effect could not be investigated. During the experimental work, no satisfying explanation was found for this phenomenon.

Applying chloride-containing salts having monovalent (e.g. Na^+) or bivalent (Ca^{2+}) cations. The question whether valency is the factor affecting the combinational effect has been answered. *E. coli* has shown high susceptibility against the salts of bivalent cations ($ZnCl_2$; $CaCl_2$; $SrCl_2$) combined with one of both Lutensols (TO 12 and AO 7). The MIC- and MBC-values have been reduced 2- to 10-fold. Contrary, susceptibility of *L. monocytogenes* has not been as clear as in case of *E. coli*. Applying one ($CaCl_2$) of three salts has led to a 2-fold decrease of the MIC- and MBC-values in combination with one of both Lutensol grades. The MIC- and MBC-values of other two salts ($ZnCl_2$, $SrCl_2$) remained unchanged. Based on these results, a conclusion can be drawn that the valency and cations do play a distinct role in the combinational effect, but the strength of this effect depends on the target organism as well. Maybe various bacteria react to a different valency by altering their behavior, e.g. their growth will be inhibited in the presence of ions of higher valency.

Another hypothesis has suggested an important effect of various anions. The investigation has led to highly heterogeneous results, when *E. coli* was able to survive in three (K_2HPO_4, NaH_2PO_4 and Na_2SO_4) of five salts combined with one of both Lutensols, and the MBC-values were even higher in comparison to situations when salts were applied separately. MIC-value of potassium dihydrogenphosphate (KH_2PO_4) has become higher if combined with grades of Lutensols, but the MBC-values have been maintained.

Magnesium sulphate ($MgSO_4$) was the only exception showing a reduction of the MIC-values if combined with one of both Lutensols.

The experiments done with *L. monocytogenes* have revealed a decrease of the MIC- and MBC-values in three of five representatives, whereas the combinational effect was not detected in case of two salts.

All these results seem to be highly heterogeneous to make a clear conclusion that the contribution of anions to the combinational effect plays a crucial role.

A lot of phenomena in physics (conductivity), chemistry (solubility of salts) and biochemistry (e.g. solubility of proteins), can be explained with the Hofmeister series, described by Franz Hofmeister who studied the solubility of proteins in the presence of distinct anions and cations. This could have been a possible explanation of the combinational

effect. To prove this hypothesis, five ammonium-contaning salts were investigated (see chapter 3.2.3). In case of *E. coli*, ammonium chloride (NH_4Cl) has not shown the combinational effect at all – its MIC- and MBC-values were higher when combined with one of both Lutensols. Ammonium dihydrogenphosphate ($NH_4H_2PO_4$) had lower MIC-values when combined with one of both Lutensols. On the contrary, its MBC-values have been increased. The MIC-values of diammonium sulphate ($(NH_4)_2SO_4$ combined with Lutensols remained unchanged, but an increase has been observed regarding its MBC-values. An increase of both – the MIC- and the MBC-values has been detected, if diammonium hydrogenphosphate ($(NH_4)_2HPO_4$ was combined with one of both Lutensols.

In case of *L. monocytogenes*, the results were more uniform – four of five ammonium-containing salts - $NH_4H_2PO_4$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $(NH_4)_2CO_3$ - have shown a reduction of their MIC- and MBC-values in combination with Lutensols.

The only exception was ammonium chloride (NH_4Cl) showing neither reduction nor increase of the MIC- and MBC-values.

The high heterogeneity of the results shows that the combinational effect cannot be explained by the Hofmeister series.

It seems that the combinational effect depends on cations, anions and model organisms.

4.2.2 The effect of pH-values

In contrast to the Matrix-Lysis, the combinational effect is independent of the pH-value and is detectable over the entire pH range determined as a window of bacterial life (pH = 5 – 8). At a pH-value < 5 bacterial growth was already inhibited, independently from salt and detergent concentrations.

In the acidic environment, superficial proteins are positively charged due to their high hydronium concentration. The hypothesis indicates that the combinational effect is pH-dependent, due to the electrostatic repulsion of magnesium ions.

In slightly basic environment (pH = 8), bivalent magnesium ions start to precipitate in form of highly insoluble $Mg(OH)_2$, thus the MIC- and MBC-values for *L. monocytogenes* were higher than 1 M. The higher is pH-value, the stronger is the precipitation, and the combinational effect could not be detectable. Thus, pH-values > 8 were not investigated.

4.2.3 Effects of further surfactants

Based on the results of previous experiment series, the conclusion can be drawn, that combinational effect depends on three variables – the Lutensol grade, the inorganic salt (its concentration) and the model organism. In contrast, it is independent from the pH

value. The questions, if the combinational effect is a characteristic feature of Lutensol´s grades combined with inorganic salt has arisen. To answer this question 14 diverse surfactants (2 anionic and 12 non-ionic) have been investigated, first alone to test their toxicity against model organisms and then in combination with three diverse inorganic salts.

The screening of 14 surfactants has revealed a phenomenon of high susceptibility of *L. monocytogenes* against anionic surfactants (SDS, NLS) – MBC = 0.01% v/v. Contrary, *E. coli* was able to survive even in 5% solution of one of both surfactants.

The other 12 surfactants did not show toxicity (MBC > 5%) neither against *E. coli* nor against *L. monocytogenes*. The only two exceptions are Tween 20, a non-ionic surfactant, of MBC = 5% for both model organisms and Emulan HE 51, a non-ionic surfactant, of MBC = 5% for *E. coli*.

In further experiments, surfactants have been combined with one of three inorganic salts ($MgCl_2$; NH_4Cl; $CaCl_2$).

Applying of $MgCl_2$ in two concentrations (0.1 M and 0.3 M), combinational effect did not appear and bacteria grew. The only exception was a combination of 0.3 M $MgCl_2$ with 1% NLS leading to growth inhibition of *E. coli*.

L. monocytogenes is sensitive against 1 M $MgCl_2$ combined with seven of 12 non-ionic surfactants [grades of Plurafac LF (300, 221 and 220), Emulan HE 51, Triton X-100, Tween 20 and Igepal CA-630]. At a $MgCl_2$ concentration of 0.5 M, four of 12 non-ionic surfactants (Emulan HE 51, Igepal CA-630, Plurafac LF 300 and Tween 20, each of 1% v/v) have caused growth inhibition whereas the toxicity of Plurafac LF 300 was independent form its concentration.

In the next step, following concentrations of ammonium chloride have been tested: 1 M, 0.5 M and 0.1 M for *L. monocytogenes* and 0.3 M and 0.1 M for *E. coli*. The combinational effect was not expressed, if salt concentration was 0.1 M – the growth of both model organisms was detectable after re-plating on fresh non-selective agar (TSA +Y).

Treatment with 1 M NH_4Cl did not have a negative effect on the viability of *E. coli*, except for Emulan HE 51 (1%), SDS (1%; 0.1%) and Pluronic PE 6200 (1%). Additionally, 1% Emulan HE 51 has shown combinational effect in combination with 0.3M NH_4Cl.

In contrast to *E. coli*, *L. monocytogenes* is much more sensitive and dies due to combinational effect in several cases (1 M NH_4Cl combined with all three grades of Plurafac, Igepal CA-630, Tween 20, Triton X-100 and Emulan HE 51). Following detergents have shown combinational effect, if combined with 0.5 M NH_4Cl: Emulan HE 51 (1%); Triton X-100 (1%), Tween 20 (1%; 0.1%) and Plurafac LF 300 (1%; 0.1%).

In contrast to the previous two inorganic salts, $CaCl_2$ is positioned on the other end of the Hofmeister series and should act more cosmotropically, thus the combinational ef-

fect should be expressed minimally. On the other hand, valency and cations, summarized in chapter 4.2.1, might play a crucial role regarding combinational effect. Thus, $CaCl_2$ has been combined with 14 diverse surfactants. According to the results, *E. coli* shows a strong susceptibility against the combination of 1 M $CaCl_2$ with each of 14 surfactants – bacteria have been killed. This fact was detectable after re-plating on a fresh non-selective agar (TSA + Y). If salt concentration was lowered to 0.3M, the combinational effect was still remained at all three grades of Plurafac LF (300, 221 and 220), Igepal CA-630, Triton X-100 and Lauroylsarcosine (1%).

Comparing the results, *L. monocytogenes* shows a higher resistance against the combination of $CaCl_2$ and the detergent. This was confirmed by the detection of colonies grown after re-plating on a fresh non-selective agar (TSA + Y). If salt concentration was 1 M $CaCl_2$, the combinational effect has been observed in the following detergents: all three grades of Plurafac LF (300, 221, 220), Tween 20, Triton X-100, Igepal CA-630 and Emulan HE 51. Additionally, 1% Emulan HE 51 had bactericidal activity, if salt concentration was 0.5 M or 0.1 M, respectively.

Bactericidal activity has been observed in the following detergents: Igepal CA-630, Triton X-100 and Plurafac LF 300, independent from the concentration of the detergent, if salt concentration was 0.5 M $CaCl_2$.

Based on these heterogeneous results, the previous statement regarding the dependence of combinational effect on Lutensol´s grades, salts (their concentration) and model organisms need to be modified: It is dependent on the type of the surfactant (Lutensol or others), inorganic salts (their concentration) and on model organisms (*E. coli* and *L. monocytogenes*). Further, the valency of inorganic salts seems to be an important factor. These results point out that the combinational effect is not a characteristic feature of Lutensol´s grades combined with inorganic salts.

In summary, the combinational effect seems to vary significantly depending on different cations, anions and model organisms. So far, no universal pattern or underlying principle could be identified which would explain the results obtained in this study and would allow us to make predictions. Given the fact that information about Lutensols is very sparse, much more detailed experiments studying these effects need to be done in the future.

4.3 Mode of action

Investigation has revealed that bacteria die due to combinational effect. This raised the question how the underlying principle looks like. Do the bacteria burst due to combina-

tional effect? Is the integrity of plasma membrane maintained? The subsequent investigation has answered these questions.

4.3.1 Plasma membrane as target

Lutensols, as non-ionic detergents, disintegrate the structure of plasma membrane in combination with $MgCl_2$. Integrity of plasma membrane was investigated indirectly under fluorescent microscope using DNA-binding fluorescent dyes. The integrity of the bacterial plasma membrane was examined upon incubation under six different conditions (1xPBS, 1% SDS, 0.2M $MgCl_2$, 1M $MgCl_2$, 1M $MgCl_2$ + 1% TO12 and 1M $MgCl_2$ + 1% AO 7).

Treatment with 1x PBS, a buffer solution commonly used to maintain the osmotic conditions, should not lead to death. But samples of *E. coli* and *S.* Typhimurium used for examination under fluorescent microscope, treated with 1x PBS, contained a high number of dead (red fluorescent) cells. This can be explained by the fact that samples are a highly dynamic system containing bacteria in different states of life span. In this case, the percentage of damaged cells should be named as percentage of dead cells, which have died due to their limited life span and not due to environmental conditions.

Solution of 0.2 M $MgCl_2$ did not have any negative impact on the viability and the growth ability of bacteria. The treatment with this chemical was used to illustrate the situation when bacteria suffer under salt stress, but when the stress is not high enough to kill them. Cells have not been counted due to the assumption that such samples contain only viable (green fluorescent) cells. If red cells have appeared, it is due to their limited life span and not due to the conditions. Pictures of such samples have been done to visualize the differences between low and high salt stress.

The treatment of *E. coli* with 1 M $MgCl_2$ has revealed interesting results - more than one-third of original inoculum has grown and created colonies. It was surprising that this art of treatment did not kill more than 99.9 % of the original inoculum. Based on the screening results, it is known that the minimal bactericidal concentration lies at 0.4 M $MgCl_2$. A possible explanation for this phenomenon could be that samples, intended for investigation under fluorescence microscope, were treated at 30°C for 60 min., only. Contrary, samples used for screening, were treated with magnesium chloride over a longer period of time (overnight) and at a higher temperature (37°C).

In case of *L. monocytogenes*, an equal percentage of damaged and intact cells could refer to dual activity of 1 M $MgCl_2$. This is inconsistent with screening results (see chapter 1.2) showing that the growth of *L. monocytogenes* is inhibited at 1 M $MgCl_2$, while the cells are not killed. In this case, the bactericidal effect of $MgCl_2$ can be explained with the fact that a bacterial culture is a highly dynamic system, containing cells of variable fitness, responding differently to the same stress inducer.

Treatment with 1 M $MgCl_2$ has an influence on the small extent on *S.* Typhimurium. More than 60 % cells of original inoculum show an intact plasma membrane. The reason for this resistance might be interesting to figure out. Both, Escherichia and Salmonella are gram-negative bacteria having thin cell walls, thus the cell wall cannot be the crucial factor. Quite in contrast, the protection has to be based on other principles – e.g. on the accumulation of substances acting antagonistically to $MgCl_2$.

L. monocytogenes samples treated with 1% SDS or 1 M $MgCl_2$ combined with Lutensol TO 12 or AO 7, respectively, show a distinct amount of intact cells (20% to 40%) detectable by the examination under fluorescence microscope, but not by the plate count method. These cells may have persisted in a state, when they are unable to grow or to divide and when their metabolism is strongly reduced. This phenomenon is known as viable but nonculturable state. It would mean that the mixture of Lutensol and $MgCl_2$ has caused such changes with the consequence that bacteria are still present in the sample, their plasma membrane is intact but they cannot grow under laboratory conditions.

Growth of *S.* Typhimurium was compromised due to the combined treatment with $MgCl_2$ and one of both Lutensols. Treatment with 1% SDS has led to an interesting phenomenon – the majority (>99%) of cells, observed under fluorescent microscope, has a green appearance – living cells. The number of red fluorescent cells was reduced (< 1%), what can be explained by the fact that older or damaged cells could not withstand the stress induced by 1% SDS and were lysed. Intact cells were able to grow and to create colonies after re-plating on fresh non-selective agar (TSA + Y).

Due to the treatment with 1% SDS, the majority of *E. coli* has been killed – red fluorescent cells under fluorescent microscope. The minor population (~ 30%), showing an intact cell membrane, was able to grow and to create colonies after re-plating on fresh non-selective agar (TSA + Y).

In case of both gram-negative bacteria - *E. coli* and *S.* Typhimurium - the treatment with combination of 1% Lutensol (TO 12 or AO 7) and 1 M $MgCl_2$ has led to diminishing of cell population able to grow and create colonies, detectable by the plate count method.

The investigation of *E. coli*, upon treatment, has revealed the majority of red fluorescent cells (~ 80 – 90%). In contrast to *L. monocytogenes*, a small population of green cells, detected by the fluorescent microscope, has grown and created colonies after re-plating on fresh non-selective agar (up to 10%).

S. Typhimurium has shown a higher resistance against this type of treatment than E. *coli*. The population of green fluorescent cells was greater, in case of *S.* Typhimurium, but after re-plating on fresh non-selective agar, only a few colonies have been detected. This may indicate that treatment with 1% Lutensol and 1 M $MgCl_2$ induces VBNC state,

when bacteria are present, their plasma membrane is intact, but they are not able to grow under laboratory conditions.

Based on the results of the plate count method, one can say that the combination of Lutensol and $MgCl_2$ might have a dual effect – it kills bacteria or it induces changes in their physiology and metabolism leading to the VBNC state.

Finally, it should to be argued that due to the rapid bleaching of fluorescent dyes, in particular Propidium iodide, viable cell counting is less reliable than the plate count method. On the other hand, the plate count method is based on the growth of bacteria, plated on non-selective agar and incubated in darkness at stable temperature (here 37°C).

4.3.2 Detection of DNA

Due to its size DNA is released with another rate than ATP. The amount of released DNA is proportional to the damage of plasma membrane and the amount of DNA remaining in the pellet is proportionally smaller. Compared to untreated samples, known as the input, each kind of treatment has led to delay regarding the achievement of the C_T-value. This is clearly pictured in the sample treated with a combination of salt and Lutensol grade. This finding is in accordance with both results of the fluorescent microscopy, when cells having damaged plasma membrane emit red fluorescent light and results of ATP bioluminescence assay when samples containing cells with damaged plasma membrane emit bioluminescence signal of strong intensity.

Quantitative PCR, as a method of DNA detection, has been used for the estimation of the plasma membrane damage. This was done based on the fact that the cell content is released upon membrane disintegration to the treatment with the combination of Lutensol and $MgCl_2$. Due to damaged plasma membrane particles can diffuse freely along the chemical gradient out of the cell into the supernatant. DNA, as a large molecule, needs more time to be released. The stronger the disintegration of the plasma membrane due to the treatment, the stronger is the release of cell content into the supernatant and the amount of DNA; remained within the pellet, proportionally smaller. Thus, the threshold value (C_T) during qPCR is achieved later, compared to the untreated sample. For purposes of investigation, *S*. Typhimurium, a gram-negative bacterium, was used. According to the results of qPCR, *S*. Typhimurium is resistant against 1% SDS, when the threshold value was reached between the 17th and the 18th cycle. This is in accordance with the BacLight result showing a majority (>99.9 %) of green cells. This can be explained by the fact that elderly and damaged cells cannot withstand the treatment with 1% SDS and will be lysed. The phenomenon that treatment with 1% SDS and 1x PBS has led to the same CT-values (17th – 18th cycle) could be explained by the fact that any type of treatment leads to a shift in the C_T-value in comparison to untreated sample (C_T of untreated sample: 16th cycle). Due to applying of 1 M $MgCl_2$ achievement of C_T value

was shifted slightly – between the 18th and the 19th cycle. Larger shift in achievement of C_T value has been observed after applying of 1 M $MgCl_2$ combined with 1% Lutensol TO 12 (21st cycle). Higher C_T-value indicates plasma membrane damage and DNA release into supernatant. The phenomenon of plasma membrane damage is supported by results of the BacLight approach.

Interestingly, the combination of $MgCl_2$ with Lutensol TO 12 has caused a later achievement of the C_T value (21st cycle) than the combination with Lutensol AO 7 (19th and 20th cycle). First, the difference is not very enormous. Second, screening has revealed that the combinational effect depends on all three conditions – the model organism, the detergent and on the inorganic salt. It might be that *S*. Typhimurium is more sensitive against the combination of Lutensol TO 12 and the inorganic salt than the *E. coli*.

4.3.3 **Detection of ATP**

Due to the damage of the plasma membrane, cell content is released into the supernatant and can be detected (e.g. ATP by bioluminescence). The greater the plasma membrane damage, the larger the amount of released ATP into the supernatant and thus the bioluminescence signal intensity is stronger.

In this set of experiments, the influence of different conditions has been investigated. The samples were examined by both, plate count method and by the ATP bioluminescence assay. Both methods have shown that treatment with 1x PBS, 0.2 M $MgCl_2$ and 1 M $MgCl_2$ does not have any negative influence on the membrane integrity of cells of the three-hour culture of *L. monocytogenes*.

The sample treated with 1x PBS at 99°C under constant shaking (750 rpm) was used as a negative control containing no viable bacteria (no colonies after re-plating on a fresh non-selective agar). Interestingly, according to the results of the ATP bioluminescence assay, the signal intensity of this sample was about 50 % weaker than the intensity of the sample treated with 1x PBS at 30°C. This phenomenon can be explained by the fact that ATP is heat-labile, as many organic molecules.

Treatment with 1 % SDS has led to a bioluminescence signal of strong intensity. Additionally, no colonies were detected after re-plating on a fresh non-selective agar (TSA + Y). These results are an experimental evidence for the sensitivity of *L. monocytogenes* against SDS, when bacteria have died due to the plasma membrane damage and the release of cell content (e.g. ATP) into the supernatant.

The combination of 1 M $MgCl_2$ with one of both Lutensol´s grades (TO 12 or AO 7) has a bactericidal effect confirmed by the plate count method, when no colonies were detected on a fresh non-selective agar after re-plating and an overnight incubation at 37°C. To evaluate the results of ATP bioluminescence assay, the inhibitory activity of 1

M MgCl$_2$ must be taken into account. The presence of 1 M MgCl$_2$ has had a negative influence on the bioluminescence signal intensity, but it could not be exactly quantified with this experimental approach.

Due to the plasma membrane damage caused by the combinational effect, the ATP is released into the supernatant. On the other hand, the plasma membrane damage did not lead to complete cell lysis.

5 Conclusion

The main aim of this study has been fulfilled – The screening of ten Lutensol grades has revealed an appropriate substitute for Lutensol AO 7, which can be applied in the process of Matrix-Lysis procedure without impairing the viability of pathogens. The viable pathogenic microorganisms can be detected by both methods of microbiology (plate count method) and methods of molecular biology (qPCR).

Further investigations have revealed that the combinational effect is not a unique feature of the Lutensol grades combined with inorganic salts. Quite in contrast, the combinational effect has been detected also by applying other detergents (anionic and non-ionic) in combination with diverse inorganic salts. According to the results of the screening, the combinational effect depends on all three factors: the detergent, the inorganic salt and the model organism. It does not depend on the pH value.

The investigations of molecular mechanisms of the combinational effect have revealed the plasma membrane damage as the cause of death. Due to plasma membrane damage, an increased permeability for substances of various types has been detected (e.g. DNA fluorescent dyes – propidium iodide and Syto 9 – diffuse freely into the cytoplasm and stain the DNA; ATP, a small molecule, is released out of the cell much faster than the DNA). These phenomena were documented by procedures, such as fluorescent microscopy, ATP bioluminescence and quantitative PCR.

Lutensol grades alone do not show a toxic effect against bacteria. It could be interesting to investigate why the combinational effect appears in combination with distinct salts and how the detailed process of plasma membrane disintegration looks like?

Overall, the findings of this thesis have contributed to the improvement of the Matrix-Lysis procedure and to the elucidation of the molecular mechanisms of the combinational effect.

Distinct parts of this thesis (e.g. the investigation of the combinational effect in presence of various ions) have been presented in form of poster at the conference "55. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene 2014, Garmisch-Partenkirchen, Germany."

6 References

- Genomic DNA from Tissue, User Manual, NucleoSpin® Tissue, Macherey-Nagel

- LIVE/DEAD® BacLight™ Bacterial viability Kits, technical information, Invitrogen, © 2008 Invitrogen Corporation

- Lutensol® AO types, 2004, Technical information, BASF The Chemical Company

- Lutensol® TO types, 2004, Technical information, BASF The Chemical Company

- Lutensol® XP types, 2003, Technical information, BASF The Chemical Company

- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002) Molecular biology of the cell, 4th edition, Garland Science, a member of the Taylor & Francis Group, 270 Madison Avenue, New York, NY 10016

- Allerberger, F. and Wagner, M. (2010) Listeriosis: a resurgent foodborne infection, Clinical Microbiology and Infection, Volume 16, Issue 1, pp. 16 -23

- Barel, A. O., Paye, M., Maibach H. I. (2001) Handbook of cosmetic science and technology, Marcel Dekker, Inc.

- Bassler, B.L. (2002) Small Talk. Cell-to-cell communication in bacteria, Cell, Volume 109, Issue 4, pp. 421 - 424

- Bhairi, S. M. (2001) Detergents, A guide to the properties and uses of detergents in biological systems, Calbiochem-Novabiochem Corporation

- Blattner, F.R., Plunket III, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B. and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12 (Review), Science, Volume 277, Issue 5331, pp. 1453 - 1462

- Boehme, S., Werner, G., Klare, I., Reissbrodt, R. and Witte, W. (2004) Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs, Molecular Nutrition & Food Research, Volume 48, Issue 7, pp. 522 - 531

- Brehm-Stecher, B., Young, Ch., Jaykus, L.-N.; Tortorello, M.L. (2009) Sample preparation: The forgotten beginning, Journal of Food Protection, Volume 72, Number 8, pp. 1774 – 1789

- Bremer, P.J., Monk, I., Osborne, C.M. (2001) Survival of *Listeria monocytogenes* attached to stainless steel surfaces in the presence or absence of *Flavobacterium spp.*, Journal of Food Protection, Volume 64, Issue 9, pp. 1369 - 1376

- Cohen, H. J., Mechanda, S. M. and Lin, W. (1996) PCR amplification of the fimA gene sequence of *Salmonella* Typhimurium, a specific method for detection of *Salmonella* spp., Applied and Environmental Microbiology, Volume 62, No. 12, pp. 4303 - 4308

- Cotter, P.D., Emerson, N., Gahan, C.G.M. and Hill C. (1999) Identification and disruption of lisRK, a genetic locus encoding a two-component signal transduction system involved in stress tolerance and virulence in *Listeria monocytogenes*, Journal of Bacteriology, Volume 181, Issue 21, pp. 6840 - 6843

- Cotter, P.D., Gahan, C.G.M. and Hill, C. (2001) A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid, Molecular Microbiology, Volume 40, Issue 2, pp. 465 – 475

- Dallmann, S. (2011) Reinigung superhydrophober Oberflächen, Dissertation, Technische Universität Dortmund, 1.Gutachter: Prof. Dr. Heinz Rehage, 2. Gutachter: Prof. Dr. Roland Winter

- Egli, T., Köster, W. and Meile, L. (2002) Pathogenic microbes in water and food: changes and challenges, FEMS Microbiology reviews, Volume 26, Issue 2, pp. 111 - 112

- Eiden-Assmann, S., Widoniak, J. and Maret, G. (2004) Synthesis and Characterization of porous and nonporous monodisperse colloidal TiO_2 particles, Chemistry of Materials, Volume 16, Issue 1, pp. 6 - 11

- Endres, F. and El Abedin, S. Z. (2006) Air and water stable ionic liquids in physical chemistry, Physical Chemistry Chemical Physics, Issue 8, pp. 2101 – 2116

- Ethelberg, S., Lisby, M., Torpdahl, M., Sørensen, G., Neimann, J., Rasmussen, P., Bang, S., Stamer, U., Bertil Hansson, H., Nygård, K., Lau Baggesen, D., Møller Nielsen, E., Mølbak, K. and Helms, M. (2004), Prolonged restaurant-associated outbreak of multidrug-resistant *Salmonella* Typhimurium among patients from several European countries, Clinical Microbiology and Infection, Volume 10, Issue 10, pp. 904 - 910

- Fretz, R., Sagel, U., Ruppitsch, W., Pietzka, A. T., Stöger, A., Huhulescu, S., Heuberger, S., Pichler, J., Much, P., Pfaff, G., Stark, K., Prager, R., Flieger, A., Feenstra, O. and Allerberger, F. (2010) Listeriosis outbreak caused by acid curd cheese ´Quargel´, Austria and Germany 2009; Eurosurveillance, Volume 15, Issue 5

- Fukushima, H., Hoshina, K., Gomyoda, M. (1999) Long-term survival of Shiga toxin-producing *Escherichia coli* O26, O111 and O157 in bovine feces, Applied and Environmental Microbiology, Volume 65, No. 11, pp. 5177-5181

- Gandhi, M. and Chikindas, M.L. (2007) *Listeria*: A foodborne pathogen that knows how to survive, Review, International Journal of Food Microbiology, Volume 113, Issue 1, pp. 1 - 15

- Gaastra, W. and Svennerholm, A.M. (1996) Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC), Trends in Microbiology, Volume 4, Issue 11, pp. 444 - 452

- Geue, L. and Löschner, U. (2002) *Salmonella enterica* in reptiles of German and Austrian origin, Veterinary Microbiology, Volume 84, Issue 1 -2, pp.79 – 91

- Graziani, C., Busani, L., Dionisi, A. M., Lucarelli, C., Owczarek, S., Ricci, A., Mancin, M., Caprioli, A. and Luzzi, I. (2008) Antimicrobial resistance in *Salmonella enterica* serovar Typhimurium from human and animal sources in Italy, Veterinary Microbiology, Volume 128, Issue 3 - 4, pp. 414 - 418

- Griffiths, M. (1996) The role of ATP bioluminescence in the food industry: new light on old problems, Food Technology, Volume 50, Issue 6, pages 62–72

- Hallsworth,J.E., Yakimov, M.M., Golyshin, P.N., Gillion, J. L. M., D´Auria, G., Alves, F.d. L., La Cono, V., Genovese, M., McKew, B.A., Hayes, S. L., Harris, G., Giuliano, L., Timmis, K. N. and McGenity, T. J. (2007) Limits of life in $MgCl_2$-containing environments: chaotropicity defines the window, Environmental Microbiology, Volume 9, Issue 3, pp. 801-813

- Hefford, M.A., D´Aoust, S., Cyr, T.D., Austin, J.W., Sanders, G., Kheradpir, E., Kalmokoff, M.L. (2005) Proteomic and microscopic analysis of biofilms formed by *Listeria monocytogenes* 568, Canadian Journal of Microbiology, Volume 51, Number 3, pp. 197 - 208

- Helms, M., Ethelberg, S., MØlbak, K. and the DT104 study group (2005) International *Salmonella* Typhimurium DT104 Infections, 1992 – 2001, Emerging Infectious Diseases, Volume 11, Number 6

- Hofstra, H., van der Vossen, J.M.B.M. and van der Plas, J (1994) Microbes in food processing technology, FEMS Microbiology Reviews, Volume 15, Issue 2 - 3, pp. 175 – 183

- Kaclíková, E., Pangallo, D., Oravcová, K., Drahovská, H. and Kuchta, T. (2005) Quantification of *Escherichia coli* by kinetic 5´-nuclease polymerase chain reac-

tion (real-time PCR) oriented to smfD gene, Letters in Applied Microbiology, Volume 41, Issue 2, pp. 132 – 135

- Kahn, L. K. (2006) Confronting zoonoses, linking human and veterinary medicine, Emerging infectious diseases, Volume 12, Number 4, pp. 556 - 561

- Kemper, M.J. (2012) Outbreak of hemolytic uremic syndrome caused by *E. coli* O104:H4 in Germany: a pediatric perspective, Pediatric Nephrology, Volume 27, Issue 2, pp. 161 - 164

- Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A. and Finlay, B. B. (1997) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells, Cell, Volume 91, Issue 4, pp. 511 - 520

- Kim, D. S., Han, S. J., Kwak, S.-Y. (2007) Synthesis and photocatalytic activity of mesoporous TiO_2 with the surface area, crystallite size, and pore size, Journal of Colloid and Interface Science, Volume 316, Issue 1, pp. 85 - 91

- Kwisda, Koko (2012) Method development for the molecular biological detection of food pathogens, Diplomarbeit, Universität Wien, Zentrum für molekulare Biologie, Betreuer: Seipelt, Joachim

- Latscha, H. P. and Kazmaier, U. (2008) Chemie für Biologen, 3. Auflage, Springer-Verlag Berlin Heidelberg

- Leroy, F. and De Vuyst, L. (2004) Lactic acid bacteria as functional starter cultures for the food fermentation industry, Trends in Food Science & Technology, Volume 15, Issue 2, pp. 67 – 78

- Lomax, E.G. (1996) Amphoteric surfactants, Second edition, Marcel Dekker, Inc. 270 Madison Avenue, New York, New York, 10016

- Long, S.M., Adak, G.K., O´Brien, S.J., Gillespie, I.A. (2002) General outbreaks of infectious intestinal disease linked with salad vegetables and fruit, England and Wales, 1992 – 2000, Communicable disease and public health / PHLS, Volume 5, Issue 2, pp. 101 - 105

- Lou Y. and Yousef A. E. (1999) Characteristics of *L. monocytogenes*. In *Listeria*, Listeriosis and Food Safety, 2nd edn ed. Ryser, E.T. and Marth E.H., pp. 131 – 224, New York: Marcel Dekker

- Lundin, A., Stanley, P.E., McCarthy, B.J., Smither, R. (1989) Rapid Methods in Microbiology, Blackwell Scientific, Oxford, UK, pp. 11–27

- Madigan, M.T., Martinko, J.M., (2006) Brock Biology of Microorganisms, 11th edition, Pearson Prentice Hall, Pearson Education, Inc. Upper Saddle River, NJ 07458

- Mann, E., Hein, I., Mester, P., Stessl, B., Rossmanith, P., Wagner, P. and Dzieciol, M. (2013) A robust and poisson validated quantitative 5´nucleaseTaqMan® Real-Time PCR assay targeting fimA for the rapid detection of *Salmonella* spp. In food, Food Analytical Methods, Volume 6, Issue 4, pp. 991 – 995

- Mayrl, E., Roeder, B., Mester, P., Wagner, M. and Rossmanith, P. (2009) Broad range evaluation of the matrix solubilization (Matrix-Lysis) strategy for direct enumeration of foodborne pathogens by nucleic acids technologies, Journal of Food Protection, Volume 72, Number 6, pp. 1225 – 1233

- McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, Ph., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, Ch., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R. and Wilson R. K. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2, Nature, Volume 413, Issue 6858, pp. 852 – 856

- Mellmann, A., Harmsen, D., Cummings, C. A., Zentz, E. B., Leopold, S. R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S. F., Henkhaus, J. K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P. M., Moore, R. L., Guenther, S., Rothberg, J.M., Karch, H. (2011) Prospective genomic characterization of the German Enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology, PLoS ONE, Volume 6, Issue 7, artical number e22751

- Meslin, F.-X., Stöhr, K. and Heymann, D. (2000) Public health implications of emerging zoonoses, Scientific and Technical review of the Office International des Epizooties, Volume 19, Number 1, pp. 310 - 317

- Mester, P., Schoder, D., Wagner, M. and Rossmanith, P. (2014) Rapid sample preparation for molecular biological food analysis based on magnesium chloride, Food Analytical Methods, Volume 7, Number 4, pp. 926 - 934

- Mester, P., Wagner, M. and Rossmanith, P. (2010) Use of ionic liquid-based extraction for recovery of *Salmonella* Typhimurium and *Listeria monocytogenes* from Food Matrices, Journal of Food Protection, Number 4, pp. 680 - 687

- Morrissey, J.P., Dow, J. M., Mark, G.L., O´Gara, F. (2004) Are microbes at the root of a solution to world food production? EMBO reports, Volume 5, Issue 10, pp. 922 – 926

- Myers, D. (2006) Surfactant Science and technology, published by John Wiley&Sons, Inc., Hoboken, New Jersey

- Naqid, I.A., Owen, J.P., Maddison, B.C., Gardner, D.S., Foster, N., Tchórzewska, M.A., La Ragione, R.M., Gough, K.C. (2015) Prebiotic and probiotic agents enhance antibody-based immune responses to *Salmonella* Typhimurium infection in pigs, Animal Feed Science and Technology, Volume 201, pp. 57 - 65

- Neidhardt, F.C. (Ed. in Chief), Curtis III, R., Ingraham, J. L., Lin, E. C. C., Low K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E. (eds) (1996) *Escherichia coli* and *Salmonella*: Cellular and molecular biology, American Society for Microbiology, 2 vols, 2898 pages

- O´Driscoll, B., Gahan, C.G. and Hill, C. (1996) Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence, Applied and Environmental Microbiology, Volume 62, Issue 5, pp. 1693 - 1698

- Pereira, A.C.M., Britto-Filho, J.D., de Carvalho, J.J., de Luna, M.d.G. and Rosa, A.C.P. (2008) Enteroaggregative *Escherichia coli* (EAEC) strains enter and survive within cultured intestinal epithelial cells, Microbial pathogenesis, Volume 45, Issue 5 – 6, pp. 310 - 314

- Rapid detection, characterization and enumeration of foodborne pathogens (2011) edited by J. Hoorfar, ASM Press, American Society for Microbiology,

- Raad, I., Chatzinikolaou, I., Chaiban, G., Hanna, H., Hachem, R., Dvorak, T., Cook, G. and Costerton, W. (2003) In Vitro and Ex Vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces, Antimicrobial agents and chemotherapy, Volume 47, Issue 11, pp. 3580 – 3585

- Rabsch, W., Andrews H. L., Kingsley R. A., Prager, R., Tschäpe, H., Adams, L. G. and Bäumler, A. J. (2002) *Salmonela enterica* serotype Typhimurium and its host-adapted variants, Infection and Immunity, Volume 70, Number 5, pp. 2249 – 2255

- Rådström, P., Knutsson, R., Wolffs, P., Lövenklev, M., Löfström, Ch. (2004) Pre-PCR processing, Molecular Biotechnology, Volume 26, Issue 2, pp. 133 – 146

- Rocourt, J. and Bille, J. (1997) Foodborne listeriosis (Review), World Health Statistics Quarterly, Volume 50, Issue 1 -2, pp. 67 – 73, incorporated in Bulletin of World Health Organization

- Rosen, M.J. and Kunjappu, J.T. (2012) Surfactans and interfacial phenomena, Fourth Edition, John Wiley&Sons, Inc., Published 2012 by John Wiley&Sons, Inc., Hoboken, New Jersey

- Rossmanith, P., Süß, B., Wagner, M., Hein, I. (2007) Development of matrix lysis for concentration of gram positive bacteria from food and blood, Journal of Microbiological Methods, Volume 69, Issue 3, pp. 504 – 511

- Rossmanith, P. and Wagner, M. (2011) Aspects of systems theory in the analysis and validation of innovative molecular-biological based food pathogen detection methods, Trends in Food Science &Technology, Volume 22, Issue: 2 -3, pp. 61 – 71, special issue: SI

- Rossolini, G. M., Muscas, P., Chiesurin, A. and Satta, G. (1993) Analysis of the Salmonella fim gene cluster: identification of the new gene (fimI) encoding a fimbrin-like protein and located downstream from the fimA gene, FEMS Microbiology Letters, Volume 114, Issue 3, pp. 259 - 265

- Schoder, D., Rossmanith, P., Glaser, K. and Wagner, M. (2012) Fluctuation in contamination dynamics of *L. monocytogenes* in quargel (acid curd cheese) lots recalled during multinational listeriosis outbreak 2009/2010, International Journal of Food Microbiology, Volume 157, Issue 3, pp. 326 - 331

- Shabala, L., Budde, B., Ross, T., Siegumfeldt, H. and McMeekin, T. (2002) Responses of *Listeria monocytogenes* to acid stress and glucose availability monitored by measurements of intracellular pH and viable counts, International Journal of Food Microbiology, Volume 75, Issue 1-2, pp. 89 – 97

- Sikorska, B., Knight, R., Ironside J.W. and Liberski P.P. (2012) Creutzfeldt-Jakob Disease, Neurodegenerative Diseases, Advances in Experimental Medicine and Biology, Volume 724, pp. 76 - 90

- Slutsker, L. and Schuchat, A. (1999) Listeriosis in humans. In *Listeria*, Listeriosis and Food Safety, 2nd edn ed. Ryser, E.T.

- Small, P.L.C. and Waterman, S.R. (1998) Acid stress, anaerobiosis and gadCB lessons from *Lactococcus lactis* and *Escherichia coli*, Trends in Microbiology, Volume 6, Issue 6, pp. 214 - 216

- Squirell, D.J., Price, R.L. and Murphy, M.J. (2002) Rapid and specific detection of bacteria using bioluminescence, Analytica chimica acta, Volume 457, Issue 1, 15 April 2002, pp. 109 – 114

- Stevens, K. A. and Jaykus L. - A. (2004) Bacterial separation and concentration from complex sample matrices: a review, Critical Reviews in Microbiology, Volume 30, Number 1, pp. 7 – 24

- Venkateswaran, K., Hattori, N., La Duc, M. T., Kern, R. (2003) ATP as a biomarker of viable microorganisms in clean-room facilities, Journal of Microbiological Methods, Volume 52, Issue 3, March 2003, pp. 367 – 377

- Xavier, K.B. and Bassler, B.L. (2003) LuxS quorum sensing: More than just a numbers game (review), Current Opinion in Microbiology, Volume 6, Issue 2, pp. 191 - 197

- Wennerström, H., Lindman, B. (1979): Micelles. Physical chemistry of surfactant association, Physics reports, Volume 52, Issue 1, pp. 1-86

- Wiegand, I., Hilpert, K. and Hancock, R. E. W. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances, Nature Protocols, Volume 3, Issue 2, pp. 163 - 175

- Woolhouse, M.E.J., Haydon, D.T., Antia, R. (2005) Emerging pathogens: the epidemiology and evolution of species jumps, trends in Ecology &Evolution, Volume 20, Issue 5, pp. 238 – 244

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Career objective: I am a master student studying molecular microbiology and immunobiology at the University of Vienna. I want to complete my study in the next future and then start with the PhD study at a research institute focusing on immunobiology because I would like to become active in research and science.

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