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detection of food pathogens

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1. Introduction

1.1 Food safety in general

Food safety is a topic that concerns everyone. The more globalisation of food production has progressed over the past years, the more difficult it has become to retrace and control every step of any given food product. Several food scandals in the 1990's, e.g. bovine spongiform encephalopathy (BSE), have prompted the European Union (EU) to set up the European food safety authority (EFSA): this independent agency's primary concern is monitoring and assessment of risks associated with the food chain and advising politicians scientifically. In 2002, the EU made controls for every processing step of foods mandatory: Creation, production, transport and distribution of animals for slaughter as well as feeding stuff [1].

The main concerns of food safety are as follows [98]:

- Microbiological hazards, such as pathogenic microorganisms, parasites and prions
- Chemical hazards, such as toxins secreted by microorganisms, environmental toxins like lead and mercury, food additives and pesticides
- Other hazards, including irradiation of food and genetic engineering

Since this work is focused on improving surveillance techniques for microorganisms, only the first point will be further addressed.

1.2 Food safety regarding microbiological hazards

Food-borne infections are caused pathogens that have either been directly transmitted from an infected animal to a human or have contaminated foodstuffs anywhere within the chain of production, processing, transport and distribution. Often, pathogens use animals as vector, leaving the animal itself without symptoms of an infection. This makes eradication of certain pathogens from livestock a challenge [4, 43]. The EU made surveillance of the following food-borne pathogens mandatory: *Brucella*, *Campylobacter*, *Listeria*, *Salmonella*, *Mycobacterium bovis* and verotoxin-forming *Escherichia coli* [2]. Consequences of food-borne infections can range from a mild flu to meningitis and death, depending on the pathogen and the immune status of the infected person [23, 48, 57, 63, 82, 127]. Table 1.1 summarises outbreaks due to food-borne pathogens.

Table 1.1: Outbreaks due to food-borne pathogens in the European Union in 2010, according to EFSA [34]

			Human Cases		
Pathogen	Notifications rate per 100000 population	%	Cases	Hospitalised	Deaths
<i>Salmonella</i> spp.	341	48.9	5212	944	9
<i>Campylobacter</i>	27	3.9	398	10	0
<i>Escherichia coli</i> , <i>pathogenic</i>	2	0.3	58	2	0
Bacterial toxins ¹	87	12.5	2297	215	1
Other bacterial agents ²	19	2.7	473	40	4
Viruses ³	87	12.5	2441	17	0
Parasites ⁴	15	2.1	360	74	0
Other causative agents ⁵	61	8.7	334	49	1
Unknown	59	8.5	836	21	0
EU total⁶	698	100	12409	1442	15

¹Bacterial toxins include toxins produced by *Bacillus*, *Clostridium* and *Staphylococcus*.

²Other bacterial agents include *Brucella*, *Listeria*, *Shigella* and other unspecified bacterial agents.

³Food-borne viruses include calicivirus, flavivirus, rotavirus and hepatitis A virus.

⁴Parasites include primarily *Trichinella*, but also *Anisakis*, and *Cryptosporidium*.

⁵Other causative agents include mushroom toxins, marine biotoxins, histamine, mycotoxins, wax esters and other unspecified agents.

⁶Data from 698 outbreaks are included: Austria (10), Belgium (16), Denmark (48), Estonia (2), Finland (24), France (75), Germany (40), Hungary (30), Ireland (3), Latvia (7), Lithuania (7), Netherlands (13), Poland (118), Portugal (4), Romania (19), Slovakia (20), Slovenia (3), Spain (196), Sweden (13) and United Kingdom (50).

An outbreak is defined as any illness that affects more than two people at the same time and that can be traced back to the same source, e.g., contaminated food. Recent outbreaks of haemolytic-uremic syndrome (HUS) in Germany and Listeriosis in Austria and Germany were both caused

by food-borne pathogens and vividly demonstrated the difficulties of tracing the pathogens back to their source [126, 147]. Such outbreaks have raised fears with the public and as a result consumers, non-governmental organisations (NGOs) and international trading partners have demanded more information about and better surveillance of food products. The key to that lies in regular monitoring of food products, which the EU made the food businesses duty [1]. The interest in food safety for businesses is also an economical one, although the costs of such steady surveillance are enormous [10]. But spending that money is worth it considering that unrecognised contaminations might drive companies to insolvency [96].

Establishing a number for all incidents related to food-borne pathogens proves to be difficult for several reasons: first of all, food can be contaminated with several agents. Second, not all infected people seek medical care and third, even if they do, only a fraction of pathogens are verified by laboratories, which in turn report their data to health agencies [123]. The EFSA and her US counterpart Food borne Diseases Active Surveillance Network (FoodNet) gather this information and support the World Health Organisation's (WHO) effort to establish the number of food-borne diseases worldwide [64]. From 2000 to 2008, 31 pathogens were responsible for 9.4 million episodes of food-borne illnesses, 55.961 hospitalizations and 1.351 deaths in the USA alone [123]. Since *Listeria monocytogenes* was responsible for 19% of deaths due to food-borne infectious diseases during that time and that the main part of this work was based on this pathogen it shall be further characterised.

1.2.1 *Listeria monocytogenes*

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive, facultative anaerobic bacterium which is quite common in nature: its sources include mud, carcasses, water and human faeces. In 1983, *L. monocytogenes* was shown to be mainly transmitted over food [125]. It has since been associated with outbreaks of listeriosis due to various contaminated foodstuffs like seafood [27], meat [40] and dairy products [31, 74]. It represents a major health risk to infants, immunocompromised and old people as well as pregnant women. An infection with *L. monocytogenes* can lead to severe consequences, including meningitis, septicaemia, peritonitis, endocarditis, conjunctivitis, arthritis and cutaneous lesions [44, 92, 94, 150]. In pregnant women an infection can induce spontaneous abortions, stillborn children or severely ill babies [94]. From 1996 to 2009, the incidence was between 0.1 and 0.56 per 100.000 inhabitants per year in Austria, the case fatality rate in 2010 was at 12 % (AGES Annual report, 2010; http://www.ages.at/uploads/media/jb_listeriose_2010_final-1.pdf). The high mortality rate and

the possibly severe consequences of an infection with *L. monocytogenes* call for fast and efficient detection methods.

1.2.2 *Salmonella* Typhimurium

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S. Typhimurium*) is a gram-negative, facultative anaerobe, rod-shaped Enterobacterium. It is the cause of non-bloody diarrhoea, mostly due to eggs from contaminated laying hens, slaughter and breeding pigs as well as turkeys [35]. *Salmonella* spp. rank among the most prominent food-borne pathogens, being responsible for 26 % of hospitalisations due to food-borne transmission and 31% of food-related deaths in the USA alone [78].

Since *S. Typhimurium* only served as a model organism for gram-negative bacteria in this study, it shall not be described further.

1.3 Detection Methods for food pathogens

Traditionally, there used to be a zero tolerance policy against food-borne pathogens. In 2005 however, particular limiting values for respective pathogens in foods were defined, as contamination below these values are not considered to be dangerous for humans [3]. For example, the limiting value for *L. monocytogenes* has been set at 100 CFU/g. These values call for exact determination of any number of contaminating agents within a sample.

1.3.1 Traditional detection methods of pathogens (microbiology)

Traditionally, pathogens are identified via microbiological methods. But food or environmental analysts often have to decide whether they want to identify or enumerate their target organisms, i.e., use qualitative or quantitative methods. Both methods depend on a microorganism's ability to grow though. This is in accordance with microbiological pathogen diagnostics, stating that only a cell capable of replication poses a potential threat.

Most qualitative methods start with enrichment culture in a nutritional medium that usually favours the proliferation of the target organism while suppressing the growth of secondary flora as much as possible at the same time. The sample gets incubated for many hours, thereby allowing as little contamination as a single bacterial cell to proliferate to levels at which the pathogens become detectable ($>10^3$ CFU/ml [25]). Incubation is typically followed by plating on selective agar which allows for easy identification of any particular target organism. Quantitative

methods are also based on incubating the sample in highly nutritional medium before selective plating, albeit for less time. Therefore, the retrieved number of target organisms is only an approximation.

1.3.1.1 Standard method for detecting *Listeria monocytogenes*

The standard analytical methods for *L. monocytogenes* according to ISO 11290-1 and -2 are good examples for qualitative and quantitative microbiological assays.

The *qualitative* method according to ISO 11290-1 [6] adds 25g of food sample to 225ml of half-Fraser broth and incubates for 24h/30°C. Half-Fraser broth is a pre-enrichment medium for different *Listeria* strains, supplying their growth with essential nutrients while certain antibiotics suppress the growth of common bacteria, e.g. enterocci. After 24h there is a first round of selective plating on Oxoid chromogenic *Listeria* Agar (OCLA) with incubation for 48h/37°C and 0.1ml of the pre-enriched sample is put under further selective pressure in 10ml full Fraser broth for another 48h/37°C. Then, a second round of selective plating occurs. It needs another 48h/37°C to develop colonies on the plates, which in turn can be further analysed biochemically [9]. All in all, it takes a week to scan a food sample for the presence of *Listeria* this way.

The *quantitative* method according to ISO 11290-2 [7] adds 10g sample to 90ml peptone water, which provides basic nutrients for the growth of any microorganism. After an hour of rest, the sample gets diluted in ringer's solution 1:10 steps and plated on OCLA. Again, one needs 48h/37°C before first results can be obtained, which in turn need confirmation again.

1.3.2 Pro and con of microbiological methods

One major advantage of microbiological methods is that they are single-step applications, which makes handling of the samples easy. Also, the fact that enrichment culture can promote the growth of a single cell makes it a very sensitive method [113]. Another advantage is the possibility of stimulating stressed or injured cells into growth, thereby making them detectable. Also, molecular biological methods are relatively new whereas microbiologists can rely on over 100 years of experience. They remain the gold standard till today and all laws concerning food safety are based on them.

The downside of these methods is that they are very time-consuming, taking up to a week for the result. Also, as mentioned before, even the quantitative assays only estimate the number of

pathogens and are only able to detect 10^3 - 10^4 colony forming units (CFU) per g of the sample [25]. However, bacteria have a tendency to clump together or form chains, which can distort the counting. Therefore the CFUs counted will not depict the real number of bacteria.

Microbiological methods allow for no direct quantification and consequently the initial contamination numbers can only be estimated. Another drawback is the necessity to again confirm allegedly positive results biochemically or with molecular biological methods.

Microbiological methods depend on growth and therefore mostly mirror the quality of the enrichment broth and so interfere with information on how many pathogens originally resided in the sample [121]. Also, they cannot detect dead or viable but non-culturable cells (VNBCs) that may still pose a threat to human health.

1.3.3 Alternative detection methods: Molecular biological methods

Considering the limits of microbiological methods it seems reasonable to look for alternative detection methods. Since Matrix-Lysis is coupled with qPCR, it will be the only molecular biological method described. An overview over other methods is provided by Rossmanith *et al.* [115].

PCR was first introduced in 1983 and published 1986 [84] and has become a standard laboratory procedure. It allows the amplification of targets in low concentrations on a logarithmic scale. The reaction relies on the basic cellular mechanisms of DNA amplification: a DNA polymerase connects nucleotides to form a new DNA strand using any given target DNA as template. The reaction occurs in three steps: first, the double-stranded (ds) DNA is denaturised. Then specific primers anneal to the single stranded (ss) DNA and finally, the DNA gets elongated by the polymerase. The different reaction steps are regulated via temperature adjustment. Originally, the end products of this amplification reaction were analysed: this included their separation by size on an agarose gel and comparing the different bands with each other or some reference gene of known size [14, 69, 141].

PCR is an assay sensitive enough to detect and amplify a single DNA molecule within a couple of hours [15, 85, 120]. Nonetheless, the necessary post PCR processing procedures are lengthy and expose the sample to possible contamination [32, 79].

The contamination limits for pathogens set up by the EU, e.g. 100 CFU/g for *L. monocytogenes* [3], require a detection method that provides reliable results for low levels of contamination.

PCR makes amplification of the littlest contamination possible and therefore seems to be the perfect method. Also, the reduced detection time and the high specificity of detection make PCR a desirable detection assay. The downside of it is that by law large food sample volumes have to be processed because typically low concentrations of pathogens are unequally distributed across the foodstuffs. Because of its specificity as well as small amplification volumes (10-50 µl), PCR has to be preceded by preparation methods that will separate the target organisms from a food sample and purify and concentrate them subsequently; if samples are directly subjected to PCR, inhibitory substances might interfere with the reaction; the surrounding flora may also conceal target organisms, which are usually present at low concentrations [106, 131, 145]. Also, the sample has to be confirmed, e.g., by hybridising with an additional probe. A real drawback of any PCR method is that it does not take into account whether the target DNA originated from a pathogen that had been alive or dead.

1.3.3.1 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) is based on conventional PCR, but qPCR circumvents many of its problems. QPCR allows for amplification and quantification of the sample during the PCR reaction, i.e., in “real time” [49]. The system is based on determining DNA amounts during the exponential amplification phase [47, 49]. This PCR format utilises either an additional fluorescence based binding probe or a DNA binding dye to measure fluorescence changes. Through the amplification cycles and a dilution series of a standard with known target DNA concentration, the concentration of the unknown samples can be quantified [59, 107]. In both cases, the fluorescent signal increases proportionally to the number of target DNA molecules, mirroring the amount of organisms [47]. During the exponential phase the increase of DNA equals the increase of fluorescence but obviously there is a lot of background fluorescence, which will conceal the initial increase of DNA. Once the amount of newly synthesized DNA exceeds that background a threshold cycle (Ct) can be defined. By detecting the level of fluorescence after every amplification circle and comparing the Ct-values of the samples to a standard of defined DNA amounts, the number of DNA copies within any sample can be determined by means of a calibration line. Since this work is based on a probe based system, it will be further elaborated.

The TaqMan system was developed by Holland *et al.* [50]. It was the first qPCR assay containing the *Thermus aquaticus* DNA polymerase. This polymerase possesses a 5' to 3' exonuclease

activity and cleaves only double-stranded DNA. The novelty of this assay included an oligonucleotide probe complementary to a DNA sequence lying somewhere in between the two primers. The probe is labelled with a fluorophor and a quencher, which allows for no detectable fluorescence as long as the two are in each other's vicinity. As soon as the Taq polymerase has cleaved the probe via its 5'-3' exonuclease activity in the course of copying the DNA, the fluorophor is released from its quencher and a fluorescent signal is emitted. This method therefore provides specificity because the fluorescent signal is only emitted, when the probe is bound to its specific target DNA. QPCR has several advantages compared to conventional PCR: first of all, no post-processing of samples is necessary. The samples are identified directly by the presence of an additional probe. Second, the method is even more specific because the increasing fluorescence is proportional to the presence of target DNA within a sample. Third, qPCR makes quantification of the sample in "real-time" possible.

1.4 The analytical chain

Molecular biological methods like PCR, which are often used for the detection and identification of target pathogens, are highly sensitive and very accurate but allow for no direct analysis of the food sample. It is therefore necessary to transform as heterogeneous samples as food into one qPCR suitable and standardised output. Consequently, the detection of food-borne pathogens cannot be a single step application like the microbiological methods, which simply rely on the growing of microorganisms. Rather, the sample has to undergo a standardised process made up of samples preparation, DNA isolation/purification and the core detection method, all of which make up the analytical chain or detection chain [25, 121, 119]. Figure 1.1 summarises the principle of the analytical chain.

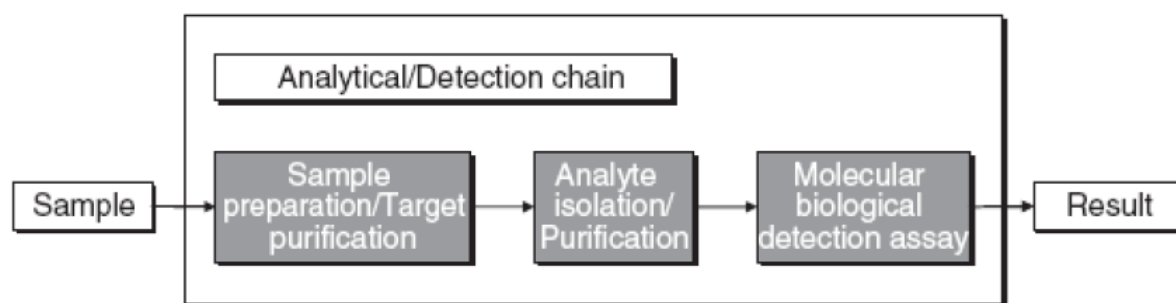


Fig. 1.1: The analytical chain for the detection of pathogens from food [114].

QPCR would have potential to detect target organisms down to a single DNA molecule present in the sample. Of course the underlying amplification mechanism itself has to be thoroughly validated [120], but qPCR is only the last event in the detection chain. Therefore, even the most accurate detection method is of no use if the other parts of the chain are not optimised as well. Consequently, each step of the detection chain will be discussed.

1.4.1 Sample preparation in general

The goal of pre-analytical sample preparation is to prepare the original sample for subsequent methods, being as DNA isolation and purification and finally qPCR [25, 115, 131].

Sample preparation depends on separation of the target organisms from the surrounding material. It can be based on different principles, being physical, adsorptive, biochemical, chemical, physicochemical or combined approaches [25, 131]. Table 1.2 summarises the advantages and disadvantages of the respective methods.

The ideal sample preparation would be universal and applicable to the widest range of matrices. It would have to accomplish the following [115]:

- Removal of inhibitory compounds within the sample that could affect the downstream detection method
- Preservation of the viability of the target organism
- Removal the sample matrix and concentration of the target organisms by reducing the sample volume
- Be non-complex, cost effective and time-saving
- Provide maximum recovery of the target organism as well as a low detection limit
- Production of a homogeneous output with a linear recovery of pathogens in every concentration
- Elimination of free target DNA and, if possible, dead cells to avoid false positive results

Table 1.2: *Diverse methods for separating target organisms from sample matrices*

Methods of Separation	Application Examples	Advantages	Disadvantages	References
Physical	Centrifugation; Filtration	Fast; inexpensive; simple; allows for large sample sizes	Non-specific; target cells may sediment with matrix; filter clogs easily	[25, 41, 55, 58, 66, 70, 131, 134, 142, 144, 146]
Adsorptive	Ion exchange resins; metal hydroxides	Fast; inexpensive; adaptable for complex matrices	Non-specific; sample pre-treatment needed	[18, 25, 53, 72, 90, 124, 131, 148]
Biochemical	Ligand-receptor interactions: Antibodies; Lectins; Mucins	Specific; high-affinity binding	Exclusivity/inclusivity; target cells may not express receptor	[13, 25, 61, 75, 87, 93, 95, 97, 100-103, 129, 131, 138]
Chemical	Aqueous two-phase partitioning	Fast; inexpensive; simple	Non-specific; composition of phases may affect viability	[25, 68, 73, 83, 104, 131, 132]
Physicochemical	Forces applied on cells in electric fields	Separation of cells by shape, size, charge	Small sample sizes	[25, 26, 62, 67, 122, 131]
Combined Approaches	Immunomagnetic separation; electropositive/negative	Fast; highly specific Fast; inexpensive; simple	Expensive; sample pre-treatment; filter clogs easily; small sample sizes	[22, 25, 29, 37, 105, 109, 111, 130, 131, 136, 139, 149]

All these prerequisites are even more difficult to meet when applied to as broad a category as food, which is commonly subdivided in the following sections: vegetables; fruit; dairy products; eggs; meat; fish and seafood; edible fat and oil; cereals and cereal products; pulses; sugar, alcohol and honey; spices and coffee; vinegar, salt and water [16]. Additionally, food can be categorised according to its chemical composition: water content; amino acids, peptides and protein; lipids; carbohydrates; food additives; pH- value [16]. Each of these factors is able to influence the reactions involved in sample preparation, e.g., the pH value. *Rossmann et al.* demonstrated that a food sample can affect the pH value, which is problematic insofar as most sample preparation procedures ask for a stable pH [118, 131].

Furthermore, the same food product can be raw or treated in different ways, leading to change in the physical or chemical properties: cooking, maturing, fermentation, smoking, salting, drying, freezing or mixing it with other foodstuffs, e.g., ready-to-eat meals [115].

All the above-mentioned parameters illustrate the difficulty of devising a sample preparation method applicable to all classes of food.

1.4.2 Sample Preparation: Matrix-Lysis

Matrix-Lysis was first introduced in 2007 [118] and has been further developed since. It presents a concept for separating target cells from the surrounding matrices by solubilising the food sample and following centrifugation steps. An initial sample volume of 6-12g is reduced to approximately 200 µl of mainly bacterial content, which can be further processed with commercial DNA isolation kits and then analysed by qPCR.

Compared to other sample preparation methods Matrix-Lysis has many advantages:

- it can process relatively large sample volumes
- there is no enrichment step necessary and hence, the data obtained is quantitative
- the procedure removes fats, carbohydrates proteins
- since recently [80], eukaryotic cells get disintegrated while leaving bacterial cells viable; this allows plating of the samples and direct comparison to the gold standard

- the method has become applicable to a broad range of foods, including dairy products, eggs, meat and fish; only starch and cellulose-rich compounds cannot be processed so far

The method is based on buffer systems containing a chaotrope and a detergent. The first approach used 8M urea and 1% SDS, leading to solubilisation of the food matrices but damaging cell walls so badly that no gram-negative bacteria could be analysed [118]. Therefore, the second approach looked to include a milder detergent, assuring the integrity of both gram-positive and gram-negative microorganisms: Lutensol AO-07 is a non-ionic detergent and is therefore unlikely to interact with polar membranes. Both attempts, however, isolated the target organisms intact but not viable (see Fig 1.1). On the other hand, up to five log scales of free target DNA is removed during the protocol [76] and consequently does not interfere with quantitative detection.

Mester *et al.* [80] adapted the protocol to permit the extraction of viable cells (see Fig. 1.1): they introduced ionic liquids (ILs), a group of compounds that have not been used in food analysis before. Ionic liquids are organic salts that possess some unique properties: they consist of ions, they are liquid at room temperature, and they are non flammable, non volatile, chemically and thermically stable and extremely easy to recycle [33, 60, 143]. The IL used by Mester *et al.* [80] belonged to the group of the 1-alkyl-3-methylimidazolium cation $[C_n\text{mim}]^+$, namely 1-Ethyl-3-methylimidazolium thiocyanate ($[C_2\text{mim}]\text{SCN}$). ILs belonging to this group have received much attention from biochemistry because the amphiphilic character of the cation lets the IL behave like a surfactant [71]. Also, the anion acts as chaotrope and is suspected to ease solubilisation of matrices [65]. $[C_2\text{mim}]\text{SCN}$ was shown to solubilise matrices as diverse as yogurt, cheese, poultry, fish and eggs while leaving the target organisms uncompromised [80]. All in all, this approach has mostly advantages with only one main disadvantage, being that ILs are very expensive.

Therefore, it seems reasonable to look for other substances with similar characteristics. MgCl_2 presents a cheap alternative (Mester *et al.*, Patent application, EPA09007959.1: Method for isolating viable cells). MgCl_2 displays a unique behaviour and its properties do not resemble either other Mg salts or chlorides. According to the Hofmeister series, which categorises ions according to their ability to salt in or salt out proteins, MgCl_2 should not be particularly good at either. Nonetheless, MgCl_2 has been shown to effectively solubilise proteins by a mechanism not depending on protein denaturation contrary to the other Matrix-Lysis buffer systems [11, 12].

Usually, protein solubilisation relies on protein denaturation leading to cell death [46, 65] but MgCl_2 leaves cells uncompromised (see Figure 1.1). Protein solubilisation is due to the affinity of the Mg^{2+} ion to the proteins, which in turn depends on the surrounding pH: extreme pH in any direction will influence the charge of the protein and hence its binding properties [11].

Therefore, to apply MgCl_2 to the Matrix-Lysis protocol, a stable pH of 7 had to be provided.

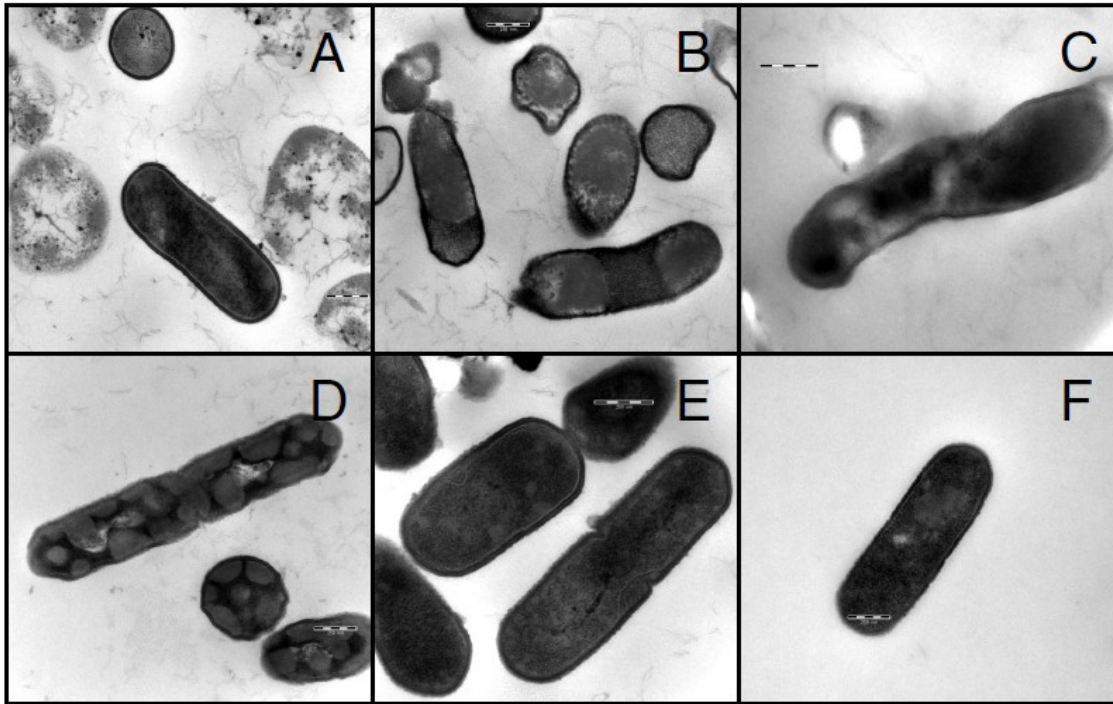


Fig 1.1: Transmission electron microscope pictures of *L. monocytogenes* after the Matrix-Lysis protocol. (A) Control group: overnight culture of *L. monocytogenes* cells, including storage for 4 weeks at 4°C to demonstrate the natural degradation of the cells. (B) A penicillin-G treated culture for comparison of cell wall damage. (C) Treatment with 8M urea and 1% SDS. The cells' appearances reflect the stress due to the chemicals used in the Matrix-lysis protocol. (D) Treatment with 8M urea and 1% Lutensol™. The cells cell walls remain intact. (E) Exposure to 1M MgCl_2 . The cells remain seemingly unaffected by the treatment. (F) Treatment with 7.5% $[\text{C}_2\text{mim}]\text{SCN}$. The cells are unaffected and explain the high recovery rate obtained by the sample preparation using this reagent [115].

The list established by Norman Good *et al.* encloses physiological buffers with their buffer capacities around pH 7 [42]. A buffer system composed of 0.5 - 1M MgCl_2 together with 1xTRIS was able to solubilise complex matrices such as fish, cheese and other dairy products, eggs and meat, the latter with the previous sucrose incubation step described by Mayrl *et al.* [76]. The target organisms remain alive and can cope with the issue that only a living organism poses a

threat (see Fig. 1.1). The MgCl_2 buffer was not able to solubilise fibre or carbohydrate-rich foods but embedded in the Matrix-Lysis protocol it offers nevertheless a cheap possibility of analysing a broad spectrum of foods accurately by qPCR as well as the cultivation of the target organisms.

1.5 Analyte extraction

Following sample preparation, analyte extraction is necessary to gain access to the respective target DNA. Its goals are on the one hand getting rid of the cell wall and inhibitory compounds within the cell as well as the remaining debris from the food sample [115]. On the other hand, the target DNA has to be purified to make it suitable for the following detection method, e.g., qPCR [36]. Figure 1.2 summarises the possible methods for the disruption of cell walls, being physical, chemical or biological.

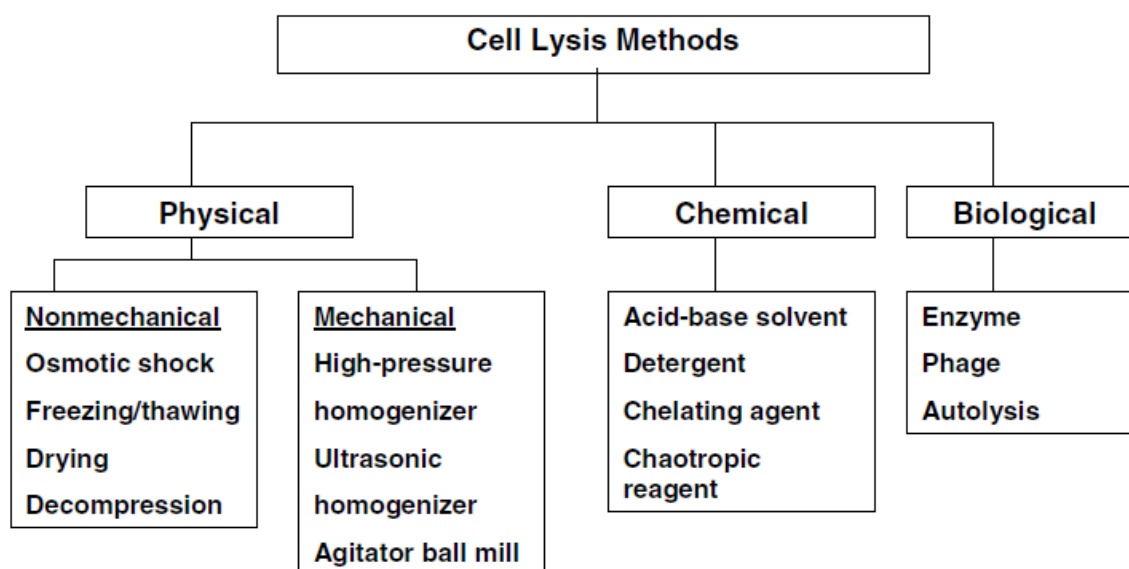


Fig 1.2: Schematic overview over sample preparation methods that can be used to disrupt the cellular of target bacteria in food samples [115]

For the subsequent isolation and purification of the DNA there are also several methods in use. The ones convenient as preparation for qPCR are solid-phase extraction and silica matrices [108, 137].

Solid phase extraction is based on different phases: typically, the matrix is non-polar, whereas the stationary phase is polar, just like the analyte. Under normal conditions the analyte is held

back in the stationary phase due to interactions like hydrogen bonding or dipole-dipole interactions. To elute the analyte, the stationary phase has to be replaced with a solvent that is more polar than the sample's original matrix. This system has become the basis for several commercial DNA isolation kits.

Silica matrices bind DNA tightly due to the positive charge of the silica particles and the strong negative charge of DNA. This tight boundary allows for washing away all possible contaminations until final elution of the DNA under low ionic conditions. Several commercial DNA isolation kits rely on this mechanism.

1.6 Internal sample process control

Internal amplification controls (IAC) for qPCR and conventional PCRs have become common practice. They provide the means to calculate the efficiency of any given reaction [51, 110, 112]. However, when it comes to processing food samples and isolating target organisms within, the analytical chain consists of many steps before the final analysis.

IACs are DNA based and can therefore not be applied to the analytical chain because obviously free target DNA will behave different than a microorganism. Therefore, the aim must be to develop sample process controls consisting of whole cells, mimicking the behaviour of the target organism as close as possible but without influencing the detection at the same time [119]. Several authors have described the requirements for IACs, which can consequently be applied to internal sample process controls (ISPC): they should bind to the same primers as the target DNA but differ in length and their emitted fluorescent signal. Their amplification should compete but not interfere with the amplification of the target and finally, their concentrations should be as low as possible to guarantee detection of even low amounts of target DNA [5, 51, 52].

Two studies picked that concept up: Murphy *et al.* [86] designed an internal sample process control (ISPC) using a recombinant *E.coli* strain: DNA sequences of *L. monocytogenes* and *Salmonella enterica* were cloned into the *E.coli* genome. The bacterial construct did co-amplify but the question remains whether *E.coli* actually represents the behaviour of the target organisms, especially that of gram-positive *Listeria*. Frühwirth *et al.* [39] designed an ISPC for *L. monocytogenes* resembling the pathogen regarding all relevant aspects. They deleted the target for the validated qPCR assay for *L. monocytogenes*, namely the *prfA* gene [39]. Both ISPC and target organism have been shown to perform equally in qPCR [116], using the same pair of

primers but different fluorescent probes. Therefore, if the ISPC is added in a known concentration at the onset of sample preparation, its recovery provides a reference for the performances of the individual samples. Also, its detection serves as an indicator for the presence of compounds inhibiting the PCR reaction, except when all of the ISPC is lost during the analytical chain (see Fig. 1.3).

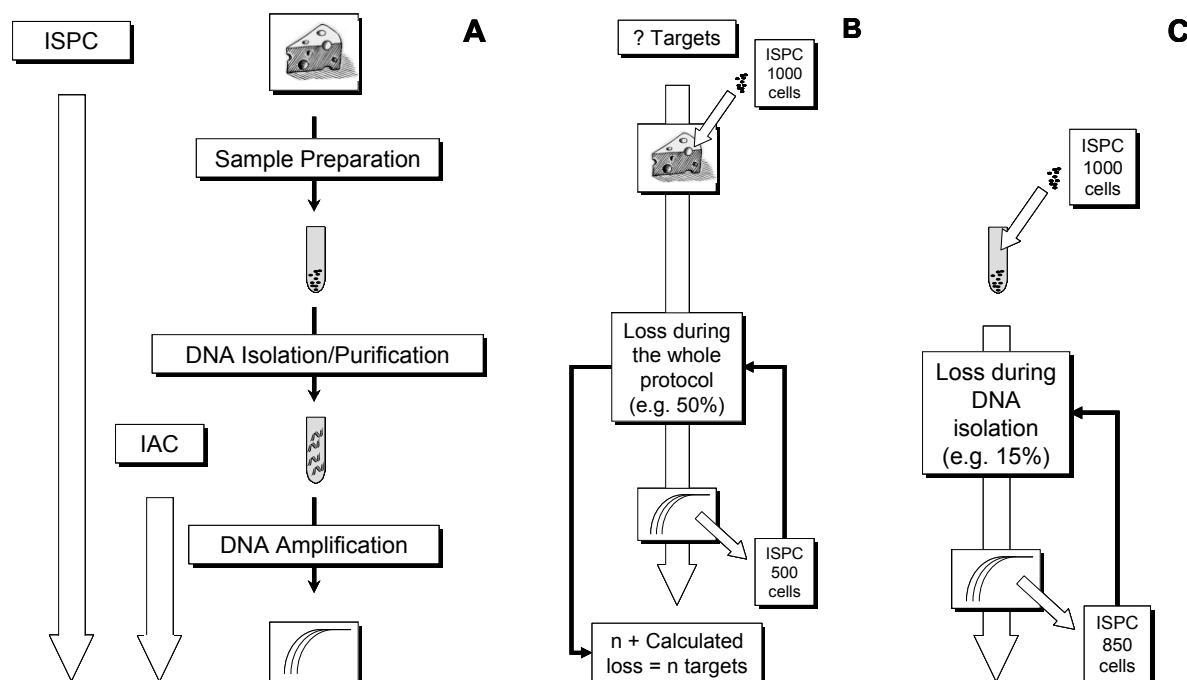


Fig 1.3: Schematic illustration of an internal sample process controls (ISPC). (A) Application of internal amplification controls (IACs) and ISPCs to the detection chain. (B) Calculation of target loss for the whole analytical chain and (C) the methodical step of DNA isolation [117]

1.7 Validating alternative methods

For validating any new method in food microbiology the alternative method, e.g., Matrix-Lysis, has to be compared to the reference method according to ISO 16140, in this case ISO 11290-1 and -2 [8]. The evaluation is carried out by means of the Chi-square test (see Fig. 1.4).

Method to be validated		+	-	Σ
Referencemethod	+	a	b	a+b
	-	c	d	c+d
	Σ	a+c	b+d	a+b+c+d = n

+ : positive confirmation

- : negative confirmation

a: positive results from both methods

b: false negative results relative to the reference method

c: false positive results relative to the reference method

d: negative results from both methods

n: all analytical results

Fig. 1.4: The chi square test for comparing qualitative methods [128]

- Relative accuracy (AC): describes the level of concordance of results obtained by the reference method and the alternative method for the same sample. Two types of deviations are possible: (i) Positive deviation (PD) occurs if the alternative method shows a positive result whereas the reference method shows a negative result. A positive deviation is called false positive if the real result can be shown to be negative, it is called positive if the real result can be shown to be positive. (ii) Negative deviation (ND) occurs if the alternative method produces a negative result whereas the reference method produces a positive result. A negative deviation is considered a wrong result if the real result can be shown to be positive.

Relative accuracy is calculated with the following formula:

PA ... positive concordance

$$AC = (PA + NA)/N \times 100\%$$

NA ... negative concordance

N ... number of samples (NA + PA + PD + ND)

- Relative sensitivity (SE): ability of the method for detecting the analyte, in case it is detectable with the reference method. It is calculated by

$$SE = PA/N^+ \times 100\%$$

N⁺ ... number of positive results with the reference method (PA + ND)

- Relative specificity (SP): ability of the method for not detecting the analyte, in case it is not detectable with the reference method either. SP is calculated as follows:

$$SP = NA/N^- \times 100\%$$

N⁻ ... number of negative results with the reference method (NA + PD)

1.8 Aim of this work

1. Matrix-Lysis has been demonstrated to be a sample preparation protocol targeting a broad range of foods. A few complications remain to be solved, though, for single food types and the MgCl₂ system:

- acid curd cheese contains NaHCO₃, which precipitates MgCl₂
- curd cheese destabilizes the pH of the reaction; a constant pH has been shown to be a requirement [118]
- MgCl₂ in combination with the previously used detergent Lutensol AO-07 has a toxic effect on *S. Typhimurium*
- TRIS, the buffer used in combination with the MgCl₂ system, leads to a massive growth reduction of *S. Typhimurium* in combination with *Salmonella*-specific Xylose-Lysine-Desoxycholate Agar (XLD)

The first goal of this work is therefore to optimise the Matrix-Lysis protocol: On the one hand problematic foods like acid curd cheese or curd cheese have to be made processable for the Matrix-Lysis protocol. On the other hand, the MgCl₂ system has to be combined with a buffer and a detergent that do not influence the viability of the target organisms.

2. An internal sample process control (ISPC) as basis for the validation of the analytical chain and quantitative detection will be introduced into the Matrix-Lysis protocol. The IAC⁺ *ΔprfA* clone of *L. monocytogenes* developed by Frühwirth *et al.* [39] will be used to analyse naturally contaminated samples and provide a basis for calculating the efficiency of the detection procedure for every single sample and every Matrix-Lysis buffer system.
3. Matrix-Lysis has not been evaluated according to international standards as of yet. Therefore, its validation according to ISO 16140 compared to the gold standard is the main goal of this work. Naturally contaminated samples are provided due to the recent listeriosis outbreak affecting acrid curd cheese [38].
4. Presumptive naturally contaminated samples that have been stored at -20°C yield different results when treated with Matrix-Lysis and qPCR rather than the microbiological reference method. The problem of long term storage and whether it affects pathogen's integrity and/or viability will hence be addressed by artificially contaminating milk with *L. monocytogenes* and subsequent freezing of the samples at -20°C. The samples will be analysed after 7, 14, 28 and 56 days of freezing.

2. Material and Methods

2.1 Microbiological methods

2.1.1 Bacterial strains and culture conditions

Listeria monocytogenes EGDe (serotype 1/2a, internal no. 2964) served as representative for gram-positive bacteria, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (NCTC12023) for gram-negative bacteria. Both bacterial strains belong to the stock of the Institute of Milk Hygiene, Milk Technology and Food Science (University of Veterinary Medicine, Vienna, Austria). *L. monocytogenes* EGDe (1/2 a) belongs to the stock at the Department of Microbiology, Theodor Boveri Institute (University of Veterinary Medicine, Vienna, Austria). All bacterial strains were kept at -80°C according to MicroBank technology (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). The respective strains were cultured over night in Tryptone Soy Bouillon + Yeast (Oxoid, Basingstoke, UK) at 37°C.

2.1.2 Media and growth conditions

2.1.2.1 Unselective media

The unselective media Tryptone Soy Agar + Yeast (TSA + Y; Oxoid) and Tryptone Soy Bouillon + Yeast (TSB + Y; Oxoid) consist of the following ingredients:

Table 2.1: Composition of the TSA + Y and TSB + Y unselective media

TSA + Y		TSB + Y	
	g/l		g/l
Casein peptone (pancreatic)	15.00	Casein peptone (pancreatic)	17.00
Soya peptone (papainic)	5.00	Soya peptone (papain digest,)	3.00
Sodium chloride	5.00	Sodium chloride	5.00
Agar	15.00	Dipotassium hydrogen phosphate	2.50
		Glucose	2.50

30g of the particular ingredients were dissolved in 1 litre of ddH₂O and the pH was adjusted to 7. The media were sterilized at 121°C for 15 minutes and then filled in 13.5ml dishes (TSA) and 9ml falcon tubes (TSB), respectively.

2.1.2.2 Selective media

2.1.2.2.1 OCLA

Oxoid Chromogenic *Listeria* Agar (Oxoid; Hampshire, UK) was developed by Ottaviani and Agosti [99] for the selective identification of *Listeria* spp. species. Selectivity of this medium is provided by Lithium chloride, Ceftazidime, Polymyxin B, Nalidixic acid and Amphotericin. The antibiotics and the antimyotics suppress growth of the gram positive and the gram negative secondary flora as well as fungi and moulds.



Fig. 2.1: *L. monocytogenes* on OCLA after 72h of incubation at 37°C, showing the characteristic blue colour and white halo formation [39]

The specific detection uses a chromogenic substance, X-glucoside, which is cleaved by β -galactosidase, an enzyme found in all *Listeria* species. This reaction will turn the agar blue. To further differentiate the pathogenic strains *L. monocytogenes* and *L. ivanovii* their ability to produce phosphatidylcholine phospholipase C (PCPLC) is tested. These enzymes utilise phosphatidylinositol or lecithin present in the medium, leaving a milky white halo encircling the respective colonies.

The ingredients for the basic formula were mixed according to Table 2.2 in ddH₂O and the pH was adjusted to 7.2 with NaOH. The medium was autoclaved at 121°C for 15 min and left to cool to app. 48°C. Then, the antimicrobial supplements were added and the mixture filled in 13.5 ml dishes.

Table 2.2: Composition of the OCLA basis medium and the antimicrobial and differential supplements

OCLA Basic Formula		OCLA Selective Supplement	
	g/l		g/l
Enzymatic digest of animal tissues	18.0	Nalidixic acid	0.02
Enzymatic digest of casein	6.0	Polymyxin B	76.7 IU
Sodium pyruvate	2.0	Ceftazidim	0.02
Glucose	2.0	Amphotericin	0.01
Magnesium glycerophosphate	1.0		
Magnesium sulphate (anhydrous)	0.5	OCLA Differential Supplement	
Sodium chloride	5.0		
Yeast extract	10.0	L- α -phosphatidylinositol solution	0.04
Lithium chloride	10.0		
Disodium hydrogen phosphate (anhydrous)	2.5		
X-glucoside chromogenic mix	0.05		
Agar	12.0		

2.1.2.2.2 PALCAM

PALCAM *Listeria* Agar (Solabia Biocar Diagnostics; Pantin Cedex, France) was developed specifically for the detection of *Listeria* spp. from foods [140] but it is also used for clinical samples [21, 45]. Its selectivity is based on Lithium chloride, Polymyxin-B-Sulphate, Acriflavin-HCl and Ceftazidim, suppressing growth of the gram positive and gram negative secondary flora. The detection mechanism works in two ways: on one hand the medium supplies the pathogens with Esculin, which all *Listeria* strains can hydrolyse. The product of this reaction, Eskuletin (=6,7-Dihydroxycoumarin) forms a brownish complex with Fe^{2+} ions contained in the medium.

On the other hand the medium contains Mannitol, which *Listeria* spp., unlike other frequent microorganisms like Staphylococci, cannot exploit. The presence of these microorganisms is displayed by a colour change of the medium from red to yellow, due to the acids produced by the microorganisms, which in turn interact with the pH indicator phenol red.

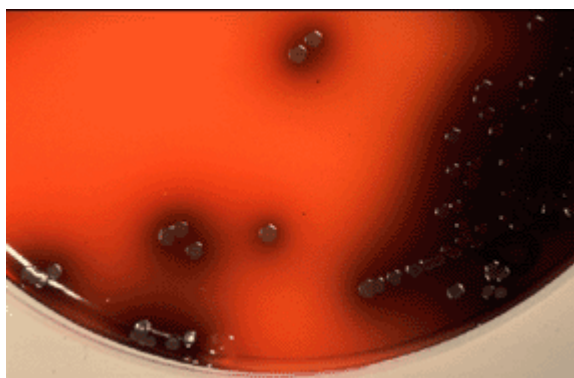


Fig. 2.2: *L. monocytogenes* on PALCAM after 48h of incubation at 37°C, forming the characteristic brown complex

The ingredients for the basic formula were mixed according to Table 2.3 in ddH₂O and the pH was adjusted to 7.2 with NaOH. The medium was autoclaved at 121°C for 15 min and left to cool to app. 48°C. Then, the antimicrobial supplements were added and the mixture filled in sterile 13.5 ml dishes.

Table 2.3: *Composition of the PALCAM basis medium and the antimicrobial supplements*

PALCAM Basic Formula		PALCAM Antimicrobial Supplements	
	g/l		g/l
Columbia blood agar basis	39.0	Acriflavin-HCl	0.005
Mannitol	10.0	Polymyxin-B-Sulphate	0.01
Glucose	0.5	Ceftazidim	0.008
Eskulin	1.0		
Ammonium iron (III)-Citrate	0.5		
Lithium chloride	15.0		
Phenol red	0.08		
Agar	2.0		

2.1.2.2.3 Rapid' *L.mono*

The Rapid' *L.mono* agar (Bio-Rad laboratories GmbH, Munich, Germany) was validated by NORDVAL (ref nr. 2003-20-5408-00024), AFNOR (attestation # BRD 07/04- 09/98) and AOAC (certificate # 030406) as an alternative method for the detection of *Listeria* spp. from

foods. The agar allows for the distinction of *Listeria* spp. on one plate: it provides selectivity for *L. monocytogenes* by detecting its phosphatidylinositol phospholipase C (PIPLC) as well as its inability to metabolise xylose, resulting in a dark blue colony. Other *Listeria* species appear white, with or without a yellow halo depending on their ability to metabolise xylose, e.g., the IAC⁺, Δ -prfA *L. monocytogenes* EGDe strain used as Internal samples process control [39].

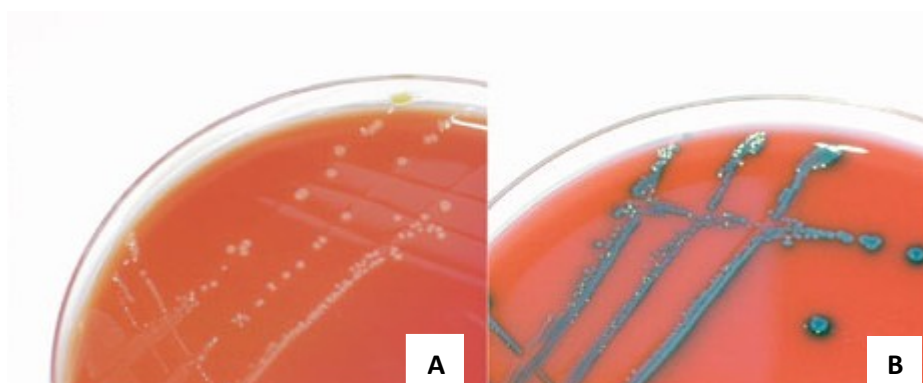


Fig 2.3: A: Δ -prfA *L. monocytogenes* EGDe on Rapid' L.mono, forming white colonies;
B: *L. monocytogenes* on Rapid' L.mono forming blue colonies without a halo [39]

The basic formula is given in table 2.4. The plates were purchased from Bio-Rad (Bio-Rad laboratories GmbH, Munich, Germany) and stored at -8°C.

Table 2.4: Composition of the RAPID' *L.mono* basis medium and the antimicrobial supplements

Rapid' <i>L.mono</i> formula	
	g/l
Peptones	30.0
Meat extract	5.0
Yeast extract	1.0
Lithium Chloride	9.0
Xylose	10.0
Phenol red	0.12
Agar	13.0
Chromogenic solution	1 ml
Selective Solution	20 ml

2.1.2.2.4 Luria Broth (LB) medium + Chloramphenicol

The original LB recipe was invented by Bertani while trying to improve plaque formation on a *Shigella* indicator strain [19]. Although the abbreviation “LB” can be read as “Luria broth”, “Lennox-Bertani” medium or “Lennox broth”, it originally stood for “Lysogeny broth” [20]. Even though this medium is mostly used for the cultivation of *Enterobacteriaceae* it provides a useful tool for recombinant DNA assays, e.g., if an antibiotic is added to select for a gene disruption via an antibiotic resistance cassette that replaced it [88]. The components of the medium were dissolved in 1 litre of ddH₂O according to table 2.5 and sterilised by autoclaving at 121°C for 15 minutes. After cooling, 25 µg Chloramphenicol was added to select for the Δ -*prfA* *L. monocytogenes* EGDe strain used as Internal samples process control. The colonies appear small and white without a halo.

Table 2.5: *Composition of the LB basis medium*

LB agar basic formula	
	g/l
Tryptone	10.00
Yeast Extract	5.00
NaCl	5.00
Agar	13.00

2.1.2.2.5. XLD agar

Xylose-Lysine-Desoxycholate Agar (Oxoid) was developed for the identification of enteric pathogens, like *Shigella* and *Salmonella* spp. [135]; both species can be distinguished on the same plate. The agar’s specificity is based on several components: Xylose can be rapidly fermented to acid by most *Enterobacteriaceae*, except for *Shigella*. This characteristic makes the differentiation of *Shigella* possible by a negative reaction. *Salmonella* spp. will ferment the xylose present and, other than non-pathogenic xylose fermenters, decarboxylate lysine also present in the medium, resulting in an alkaline pH. This reaction is also undertaken by *Shigella*, but, other than *Shigella*, *Salmonella* produces H₂S, the presence of which is indicated by sodium

thiosulfate and ferric ammonium-(III)-citrate present in the medium. This reaction leads to the characteristic blackening of *Salmonella* colonies, but only at alkaline pH. Non-pathogenic H₂S producers cannot decarboxylate Lysine and lysine-positive Coliforms will ferment lactose and sucrose, resulting in an acidic pH.

The ingredients for the basic formula were mixed according to Table 2.6 in 1 litre of ddH₂O and the pH was adjusted to 7.4.

Table 2.6: *Composition of the XLD basis medium*

XLD agar basic formula	
	g/l
Xylose	3.5
L-Lysine	5.0
Lactose	7.5
Saccharose	7.5
Sodium chloride	5.0
Yeast extract	3.0
Phenol red	0.08
Sodium deoxycholate	2.5
Sodium thiosulfate	6.8
Ferric ammonium -(III)-citrate	0.8
Agar	13.5

The medium was autoclaved at 121°C for 15 min and left to cool to app. 48°C. Then, the antimicrobial supplements were added and the mixture filled in sterile 13.5 ml dishes.

2.1.2.3 Over- night culture

9 ml of TSB + Y was inoculated with the respective strain and cultured over night at 37°C (*L. monocytogenes*) or at 42°C (*S. Typhimurium*). To establish standardised conditions, 1 ml of this over-night culture was transferred to another 9 ml of TSB + Y and let to grow for another 4 hours at 37°C.

2.1.3 Counting procedures

2.1.3.1 Direct counting procedure – BacLight™

The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Carlsbad, USA) allows for the distinction of alive and dead cells under a fluorescence microscope. The system uses the membrane integrity of microorganisms as criterion for bacterial viability. It uses two fluorescent dyes staining DNA: the green fluorescent, SYTO 9, is generally membrane-permeable. The red fluorescent, propidium iodide (PI), can also pass the membrane but is transported out of the cell again via active transport. If the membrane is damaged, active transport ceases and the cell will appear red as PI quenches the fluorescent signal emitted by SYTO 9 [133]. Hence, a red fluorescing cell is considered to be dead whereas a green fluorescing cell is assumed to be alive [17, 89]. This assumption has been shown to be problematic since an intact membrane does not necessarily imply a cell's proliferating activity [56].

A bacterial dilution series was established. 2 x 500 µl of the sample at the appropriate dilution factor were transferred to 1.5 ml Eppendorf tubes and 1 µl of each dye (components A and B of the LIVE/DEAD® BacLight™ Bacterial Viability Kit) were added respectively. After short vortexing the samples were left to incubate in the dark for 15 minutes. The samples were pressed onto a BacLight™ filter through a 5 ml syringe. This procedure was repeated to make sure that no liquid remained in the syringe. The filters were then fixed onto slides with mounting oil (component C) and covered with a cover slip. Subsequently, the slides were investigated by microscopic analysis.

2.1.3.2 Microscopic investigation

Microscopic slides were prepared as described in 2.1.3.1. The slides were examined on an inverse microscope Laborlux 8 fluorescence microscope (Leitz, Wetzlar, Germany) with a 470nm filter at a 1.000-fold magnification.

The quantitative analysis was performed as follows: the number of bacteria was established by counting 15 optical fields per filter and converting this number to bacteria/ml with the following formula:

$$\text{Bacteria per ml} = \text{average of counted bacteria} * 2 * 5230 * (1 / 10^{-n})$$

n ... dilution factor

2.1.3.3 Plate count method

The plate count method according to Koch is based on the assumption that a viable cell will form a colony on a nutritious agar plate. Since there is no way of telling whether the colony consists of one or several cells, the number is given in colony forming units (CFU) per ml.

Decimal dilution series of the samples were prepared and the appropriate dilution steps were spread out on TSA + Y agar plates with a drigalski spatula. The plates were incubated for 48 hours at 37°C for *L. monocytogenes* and 24 hours at 37°C for *S. Typhimurium*. The number of CFU was determined by multiplying the visually counted colonies with their respective dilution step.

2.1.4 ISO 11290

ISO 11290-1 determines solely the presence or absence of *L. monocytogenes* in a food sample or animal feeding stuff [6]. The procedure is depicted in Fig. 2.4.

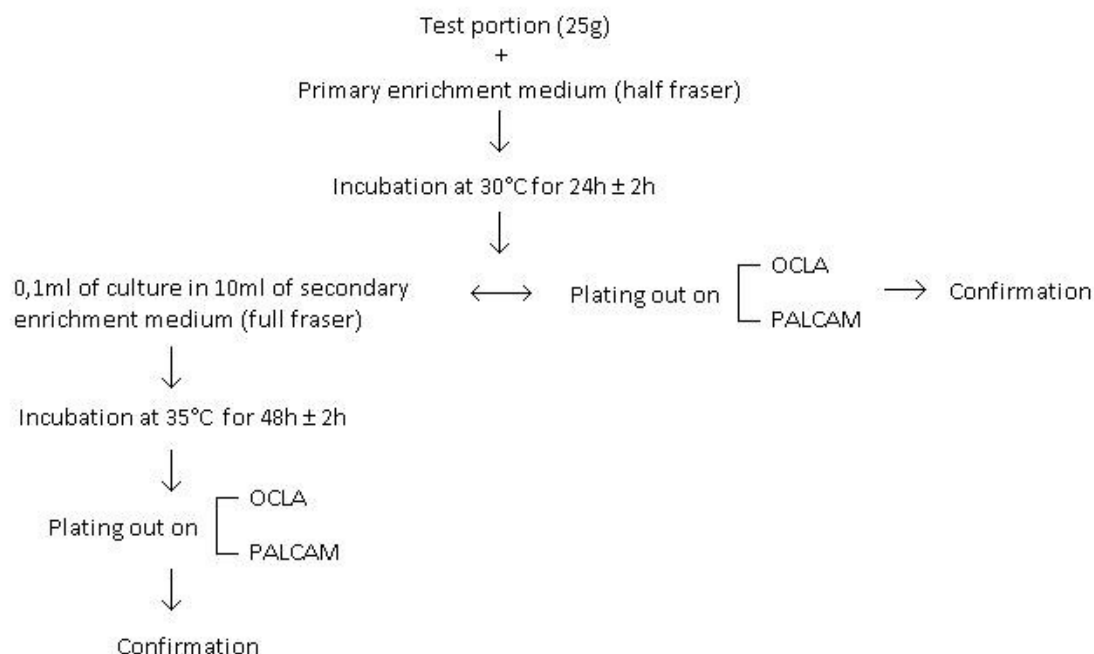


Fig.2.4: Workflow of ISO 11290-1, the qualitative detection method for *L. monocytogenes* [6]

25g of a sample are inoculated in the primary enrichment medium Half Fraser broth and incubated for 24h/30°C. Half Fraser broth suppresses the growth of other *Listeria* species or

other families and at the same time favours the proliferation of *L. monocytogenes*, which is necessary because samples may contain other *Listeria* species or *L. monocytogenes* in an injured state [6]. After 24 hours, 100 µl each are plated on selective agars OCLA and PALCAM and another 100 µl is put under further selective pressure in 10 ml full Fraser broth for another 48h/37°C. Then, 100 µl are plated on OCLA and PALCAM respectively. It needs another 48h/37°C to develop colonies on the plates, and presumptive *L. monocytogenes* colonies are confirmed biochemically [9].

Table 2.7 summarises the compositions of the primary and secondary enrichment media. The components of the respective media were dissolved in 1 litre of ddH₂O at 70°C and sterilised by autoclaving for 15min/121°C subsequently.

Table 2.7: Composition of the primary enrichment medium Half Fraser broth as well as the secondary enrichment medium Fraser broth [6]

Half Fraser broth		Fraser broth	
	g/l		g/l
Meat peptone	5.0	Meat peptone	5.0
Tryptone peptide digest of casein	5.0	Tryptone peptide digest of casein	5.0
Beef extract	5.0	Beef extract	5.0
Yeast extract	5.0	Yeast extract	5.0
Sodium chloride	20.0	Sodium chloride	20.0
Disodium hydrogen phosphate . H ₂ O	12.0	Disodium hydrogen phosphate . H ₂ O	12.0
Potassium dihydrogen phosphate	1.35	Potassium dihydrogen phosphate	1.35
Esculin	1.0	Aesculin	1.0
Lithium chloride	3.0	Lithium chloride	3.0
Sodium salt of nalidixic acid	0.01	Sodium salt of nalidixic acid	0.02

ISO 11290-2 is a detection method for *L. monocytogenes* but also allows for the enumeration of target organisms, albeit with a detection limit of 100 colonies/g [7]. 10 g sample is added to 90 ml of buffered peptone water and homogenised for 3 min each in a Stomacher 400 laboratory blender (Seward, London, UK). The sample is left to rest for 1h/20°C and 100 µl are plated on selective plates OCLA and PALCAM using decimal dilutions of the initial suspension in Ringer solution (Mayerhofer Pharmazeutika, Leonding, Austria). The plates get incubated for 48h/37°C and confirmed colonies of *L. monocytogenes* can be counted [9].

Table 2.8 lists the ingredients for buffered peptone water. The components were dissolved in 1 l of ddH₂O and the pH adjusted to 6.7. Then, the solution was sterilised by autoclaving for 15min/121°C.

Table 2.8: *Basic composition of buffered peptone water*

Buffered peptone water	
	g/l
Enzymatic digest of animal tissue	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate dodecahydrate	9.0
Potassium dihydrogen phosphate	1.5

2.2. Molecular biological methods

2.2.1 DNA isolation

For DNA extraction the NucleoSpin®[®] tissue kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's manual. Eluted DNA was either directly subjected to analysis via qPCR or stored at -20°C for later use.

To confirm the identity of colonies the Chelex method was used: suspected colonies on agar plates were suspended in 1 ml 0.01 M TRIS-HCl and centrifuged for 5min/5.000 x g. The supernatant was discarded and the pellet resuspended in 100 µl of 0.01M TRIS-HCl (pH 7.0) and 400 µl of lysis solution (0.25mM TRIS-HCl, pH 7.0; 2,5%, w/v, Chelex 100 resin (BioRad, Hercules, Ca, USA)). This mixture was incubated at 100°C/10min and centrifuged for 5 sec/14.000 x g. The supernatant was kept and the DNA amount determined. Afterwards, the DNA was adjusted to a concentration of 1ng/µl with ddH₂O.

2.2.2 Determination of DNA concentration

The DNA concentration was established by photometric measurement on an 8452A Diode Array Spectrophotometer (Hewlett Packard, Vienna, Austria). The absorbance was measured at 260nm and 280nm in a half micro quartz cuvette. 500 µl of ddH₂O were used as blank and samples in 1:10, 1:20 and 1:50 dilutions were measured subsequently. These dilutions corresponded to an

absorbance between 0.1 and 0.9. The ratio of 260/280 serves as an indicator for the purity of the DNA sample: as long as it is situated between 1.65 and 1.8 the DNA is considered to be pure.

The absorbance provides the basis for calculating the DNA concentration according to the following formula:

$$1 \text{ A}_{260\text{nm}} = 50\mu\text{g DNA/ ml solution} = 50\text{ng DNA/ }\mu\text{l solution}$$

and therefore:

$$\text{measured A} * \text{dilution of sample} * 50\text{ng / }\mu\text{l} = \text{DNA concentration (ng / }\mu\text{l)}$$

2.2.3 PCR confirmation of suspected colonies

Suspected *Listeria* species' colonies on OCLA or PALCAM agar were picked from the plates and the DNA isolated with a Chelex-based method, as described in section 2.2.1. The DNA was further analysed by two PCR reactions: one, developed by Border *et al.* targets the 16S rRNA gene, which is essential to all *Listeria* species, as well as the *hly* gene, which only *L. monocytogenes* possess [24]. The other one, according to Bubert *et al.* targets the *iap* gene, also common for *Listeria* spp., resulting in a fragment pattern characteristic for every species [28].

Tables 2.9 and 2.10 summarise the mastermixes of the respective PCR reactions. The mastermixes were always prepared as a multiple of the single reaction quanta and distributed to the tubes accordingly. Finally, 2 μl of the respective template DNAs were added. The PCR reactions were carried out in a Perkin– Elmer 2400 thermocycler (Applied Biosystems, Foster City, CA, USA).

The cycling conditions for the PCR reaction summarised in Table 2.9 were as follows:

- A single melting step of the ds template DNA for 2min/94°C
- 30 cycles of: dsDNA denaturation for 30 sec/94°C, primer annealing for 30 sec/50°C and elongation for 1min/72°C
- A final elongation step for 5 min/ 72°C
- Cooling at 4°C

Table 2.9: *PCR reaction after Border et al., with the final concentrations of all components in a single reaction tube [24]*

	Stock concentration	End concentration	µl/reaction
Aqua dest.			12.5
10x buffer		1x	2.5
MgCl ₂	50 mM	1.5 mM	0.75
L 11	18000 nM	1080 nM	1.5
U 1	18000 nM	1080 nM	1.5
LM 1	18000 nM	1080 nM	1.5
LM 2	18000 nM	1080 nM	1.5
dNTP's	5000 µM	200 µM each	1.0
Taq pol (Plat.)	5U/µl	1.25U	0.25
Volume mastermix			23.0
Template DNA			2.0
Final volume			25.0

The PCR reaction summarized in Table 2.10 was performed under the following cycling conditions:

- A single melting step of the ds template DNA for 2min/94°C
- 30 cycles of: dsDNA denaturation for 30 sec/94°C, primer annealing for 30 sec/56°C and template elongation for 30 sec/72°C
- A final elongation step for 5 min/ 72°C
- Cooling at 4°C

All PCR products were separated in 1.5% agarose gels at 90 V for 25 minutes. Afterwards, staining with 0.5 µg/ml ethidium bromide (Sigma-Aldrich GmbH, Steinheim, Germany) was undertaken. A standard GeneRuler 100 bp was used (MBI Fermentas, St. Leon-Rot, Germany).

Table 2.10: *PCR reaction after Bubert et al., with the final concentrations of all components in a single reaction tube [28]*

	Stock concentration	End concentration	µl/reaction
Aqua dest.			8.45
10x buffer		1x	2.5
MgCl ₂	50 mM	1.5mM	0.75
Siwi2	1600 nM	128 nM	2.0
Ino2	1600 nM	128 nM	2.0
MonoA	1600 nM	128 nM	2.0
MurgaI	1600 nM	128 nM	2.0
Lis1B	1600 nM	128 nM	2.0
dNTP's	5000 µM	200 µM each	1.0
Taq pol (Plat.)	5U/µl	1.5U	0.3
Volume mastermix			23.0
Template DNA			2.0
Final volume			25.0

2.2.4 Real-time PCR

2.2.4.1 Primers and probes for qPCR

An assay targeting the *L. monocytogenes prfA* gene that had been validated previously was employed [116]. Additionally, an internal sample process control that had been developed by the same group was used.

Lip probe 2 was purchased from Applied Biosystems (Foster City, CA, USA). The primers and the HEX-labelled probe pLucLm5 were purchased from MWG Biotech (Ebersberg, Germany).

Table 2.11 summarises the primers and probes used.

Table 2.11: *Primer and probe sequences from 5' -> 3'*

Oligonucleotide	Sequence 5' -> 3'	Reference/Source
Forward Primer LIP 1	GATACAGAAACATCGGTTGGC	[30]
Reverse Primer LIP 2	GTGTAATCTTGATGCCATCAGG	[30]
LIP Probe 2 – FAM	CAGGATTAAAAGTTGACCGCA	[116]
PLucLm 5 probe - HEX	TTCGAAATGTCCGTTTCGGTTGGC	[116]

2.2.4.2 PCR mastermix and cycling condition

Table 2.12 summarises the different components and their respective concentrations for a PCR reaction. The mastermix was always prepared as a multiple of the single reaction quanta and distributed to the tubes accordingly. Finally, 5µl of the respective template DNA were added. QPCR was performed in an Mx3000p thermocycler (Stratagene, La Jolla, CA, USA) according to the following cycling conditions:

- A single fusing step of the ds template DNA for 2min/94°C
- 45 cycles of: denaturation for 15 sec/94°C and elongation for 1min/64°C

All samples were present in duplicates and all values were depicted as bacterial cell equivalents (BCE). These were calculated based on the assumption that 1 ng DNA corresponds to 3.1×10^5 copies of the whole *L. monocytogenes* genome and that the *prfA* gene is a single copy gene [80, 91]. Regarding *S. Typhimurium*, 1.9×10^5 copies equalled 1ng DNA of the whole genome [77]. The calibration line was obtained from the qPCR results of a six-step dilution series of the DNA standard with 1ng/ml [80].

Table 2.12: *QPCR reaction batch with the final concentrations of all components in a single reaction tube*

	Stock concentration	End concentration	μl/reaction
Aqua dest.			6.95
10x buffer		1x	2.5
MgCl ₂	50 mM	3.5 mM	1.75
LIP 1	5 μM	0.5 μM	2.5
LIP 2	5 μM	0.5 μM	2.5
Lip Probe 2	5 μM	250 nM	1.25
p-Luc Lm 5	5 μM	250 nM	1.25
dNTPs	20 mM	200 μM each	1.0
Taq polymerase	5U	1.5 U	0.3
Volume mastermix			20.0
Template DNA			5.0
Final volume			25.0

2.2.4.4 DNA standards for qPCR quantification

The genomic DNA of *L. monocytogenes* was isolated by subjecting 1 ml of an overnight culture to the NucleoSpin[®] protocol. The DNA concentration was established by photometric measurement (8452 A Diode Array Spectrophotometer, Hewlett Packard, Palo Alto, CA). An optical density value of 1 equalled 50μg/ml DNA. A decimal dilution series of this DNA was set up at 1ng/μl to obtain a calibration line.

2.3 Artificially and naturally contaminated food samples

All food samples used as negative controls or for artificial contamination were bought at local supermarkets. The naturally contaminated acid curd cheese samples originated from a recent outbreak of Listeriosis in late 2009/early 2010 [38].

For artificial contamination of food samples fresh medium was inoculated with 1 ml of the respective overnight cultures, which were left to grow for 3h/37°C. The food samples were then spiked with 100 μl out of a suitable dilution step ($10^{-3} - 10^{-7}$) of the bacterial culture in 1x PBS and then processed according to the Matrix-Lysis protocol described below. To double-check the

number of spiked bacteria the concentration was also determined via the plate count method on the respective selective agars: OCLA and PALCAM for *L. monocytogenes* and XLD (Xylose-Lysine-Desoxycholate Agar) for *S. Typhimurium*. Also, two control samples from a suitable dilution step were subjected to the NucleoSpin[®] protocol and finally analysed by qPCR.

2.4 Matrix-Lysis

The original Matrix-Lysis protocol was developed in 2007 and has been further adjusted since [76, 80, 118]. All samples were processed accordingly.

12.5 ml of liquid or 6.25g of solid samples were mixed with 10 ml lysis buffer. The solution was homogenised twice for 3 minutes each in a Stomacher 400 laboratory blender (Seward, London, UK) and transferred to a 50 ml polypropylene tube (Corning, NY, USA). The respective lysis buffer was added until a final volume of 45 ml was reached. The lysis buffers were composed as follows:

- Lysis buffer I: 8M urea, 1% SDS, 1xPBS
- Lysis buffer II: 8M urea, 1% Lutensol AO-07, 1xPBS
- Lysis buffer III: 5% (vol/vol) aqueous solution of the ionic liquid [C₂mim]⁺ SCN⁻ (Merck, KGaA, Darmstadt, Germany) and 1x PBS on liquids, 7.5% (vol/vol) aqueous solution and 1x PBS on solid samples
- Lysis buffer IV: 1M MgCl₂; 1x Tricine; 1% Tween 20

The tubes were placed in water baths (35°C for buffer III and IV, 45°C for buffers I and II) with constant, horizontal shaking at 200 rpm for 30 minutes. The samples were then centrifuged at 3.220 x g for 30min/RT. After discarding the supernatant the pellet was resuspended in 40 ml of washing buffer (1% Lutensol AO-07, 1xPBS) and subjected again to the water bath and centrifugation under the conditions described before. The remaining pellets were then resuspended in 500 µl 1x PBS, transferred to 1.5 ml or 2 ml plastic tubes (Eppendorf, Hamburg, Germany) and washed twice with 1x PBS, with a centrifugation step at 5.000 x g/5min in between. The pellets were resuspended in 1.5 ml 1x PBS. Hard cheese samples containing many calcium phosphate remnants had to undergo a sedimentation step: after resuspending the pellet

by vortexing the remnants were allowed to settle for 2 minutes. The supernatant was transferred to a fresh tube and centrifuged at 8.000 x g/5min, the pellet resuspended again in 1.5 ml 1x PBS.

The following DNA isolation was accomplished with the NucleoSpin[®] tissue kit (Macherey-Nagel, Düren, Germany), finally eluting the DNA in 2x 50 µl ddH₂O. Lysis buffers III and IV allow for viable cell quantification, hence the bacteria remaining in the pellet after Matrix-Lysis were plated on TSA, OCLA, PALCAM, LB + Chloramphenicol and Rapid L. mono, which allowed for the distinction between *L. monocytogenes* and the Δ -*prfA* internal sample process control.

2.5 Chemicals

<u>Chemical</u>	<u>Source</u>
2-morpholino-ethansulfone monohydrate acid	Sigma (Munich, Germany)
2-Carbamoylmethylamino) ethanesulfonic acid	Sigma (Munich, Germany)
4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid	Gibco (Paisley, UK)
Agar-agar	Merck (Darmstadt, Germany)
AlCl ₃ .6 H ₂ O	Merck (Darmstadt, Germany)
Bacto [™] Tryptone	Becton, Dickinson & Co. (Le point de Claix, France)
Buffered peptone water	Oxoid (Basingstoke, UK)
Chelex 100 resin	BioRad (Hercules, Ca, USA)
Chloramphenicol	AppliChem (Darmstadt, Germany)
CaCl ₂	Merck (Darmstadt, Germany)
Ethylendiaminetetraacetic acid (EDTA)	Merck (Darmstadt, Germany)
Ethanol absolute	Sigma (Munich, Germany)
FeCl ₂ .6 H ₂ O	Merck (Darmstadt, Germany)
Fraser Broth (base)	Biokar diagnostics (Beauvais, France)
Fraser Broth (base II)	Biokar diagnostics (Beauvais, France)
KCl	Merck (Darmstadt, Germany)
KH ₂ PO ₄	Merck (Darmstadt, Germany)
LiCl ₂	Merck (Darmstadt, Germany)
Lutensol AO-07	BASF (Leverkusen, Germany)
Lysozyme	Sigma (München, Germany)
MgCl ₂	Merck (Darmstadt, Germany)
NaCl	Merck (Darmstadt, Germany)

Na ₂ HPO ₄	Merck (Darmstadt, Germany)
NaOH	Merck (Darmstadt, Germany)
Ocla Brilliance Listeria Agar Base	Oxoid (Basingstoke, UK)
Ocla differential supplement	Oxoid (Basingstoke, UK)
Ocla selective supplement	Oxoid (Basingstoke, UK)
Palcam Agar	Biokar diagnostics (Beauvais, France)
Palcam selective supplement	Biokar diagnostics (Beauvais, France)
Phosphate buffered saline	Oxoid (Basingstoke, UK)
Piperazine-1,4-bis(2-ethanesulfonic acid)	Sigma (Munich, Germany)
Protease	Novozymes (Bagsvaerd, Denmark)
Ringer solution	Mayerhofer Pharmazeutika (Leonding, Austria)
Saccharose	Merck (Darmstadt, Germany)
Sodiumdodecylsulfate	Thermo Fischer (Waltham, USA)
SrCl ₂	Sigma-Aldrich (Seelze, Germany)
Tricine	Sigma (Munich, Germany)
TRIS	Merck (Darmstadt, Germany)
TRIS HCl	Merck (Darmstadt, Germany)
Triton-X-100	Sigma (Munich, Germany)
Tryptic soy agar granulated	Merck (Darmstadt, Germany)
Tween 20	Merck (Darmstadt, Germany)
Tween 80	Merck (Darmstadt, Germany)
Urea	Merck (Darmstadt, Germany)
XLD agar	Oxoid (Basingstoke, UK)
Yeast extract	Merck (Darmstadt, Germany)
ZnCl ₂	Fisher Scientific (Leicestershire, UK)
Ionic Liquids:	
1-Ethyl-3-methylimidazolium thiocyanate	Merck, KGaA, (Darmstadt, Germany)
1-Butyl-3-methylimidazolium thiocyanate	Merck, KGaA, (Darmstadt, Germany)
1-Hexyl-3-methylimidazolium thiocyanate	Merck, KGaA, (Darmstadt, Germany)
1-Ethyl-3-methylimidazolium chloride	Merck, KGaA, (Darmstadt, Germany)
1-Hexyl-3-methylimidazolium chloride	Merck, KGaA, (Darmstadt, Germany)
1-Ethyl-3-methylimidazolium dicyanamide	Merck, KGaA, (Darmstadt, Germany)
1-Butyl-3-methylimidazolium dicyanamide	Merck, KGaA, (Darmstadt, Germany)

3. Results

3.1 Adjustments to the Matrix-Lysis protocol

MgCl₂ was previously shown to dissolve many food stuffs in the course of the Matrix-Lysis protocol but some remained problematic, e.g., acid curd cheese and curd cheese. This could be due to several reasons: one being that the pH was not stable. Since the MgCl₂-based solubilisation of foods is dependent on a pH around 7, an appropriate buffer had to be introduced.

Another reason could be that an additional detergent was needed for the solubilisation reaction. The first choice was Lutensol AO-07, which had been introduced into the Matrix-Lysis protocol because it did not affect the integrity of gram-negative bacteria [76, 118]. Previous experiments however had shown that Lutensol AO-07 in combination with MgCl₂ suppresses the growth of microorganisms. Therefore, an alternative detergent had to be looked for.

Hence, the experimental setup was twofold: on the one hand the reason for the lethal effect of Lutensol AO-07 with MgCl₂ was to be investigated and an alternative detergent to Lutensol AO-07 found. On the other hand a new buffer had to be optimised for the remaining problematic foods.

3.1.1 Toxicity tests with alternative detergents

All tests were performed with *S. Typhimurium* as model organism, since gram-negative bacteria react more sensitive to the used detergents and chemicals as well as physical stress than gram-positive bacteria [118]. All samples were treated according to the Matrix-Lysis protocol described in section 2.4.

First, two alternative detergents were considered, Tween 20 and Tween 80. They were compared to Lutensol in combination with 1M MgCl₂. To investigate the reason for the toxicity of Lutensol AO-07 and MgCl₂, two other chloride salts, NaCl and KCl, were included in the test, with and without Lutensol AO-07. The recovery was compared to that in 50 mM (1x) PBS. Neither NaCl nor KCl exerted a toxic effect on *S. Typhimurium* in combination with Lutensol AO-07. Tween 20 and Tween 80 do not influence the growth in combination with either salt, nor does either have an additional inhibitory effect to that of 1M MgCl₂, with the recovery rates around 40%.

1M KCl by itself seems to have a slight impact on the growth rate of *S. Typhimurium*, with an average recovery around 60% (see Fig.3.1).

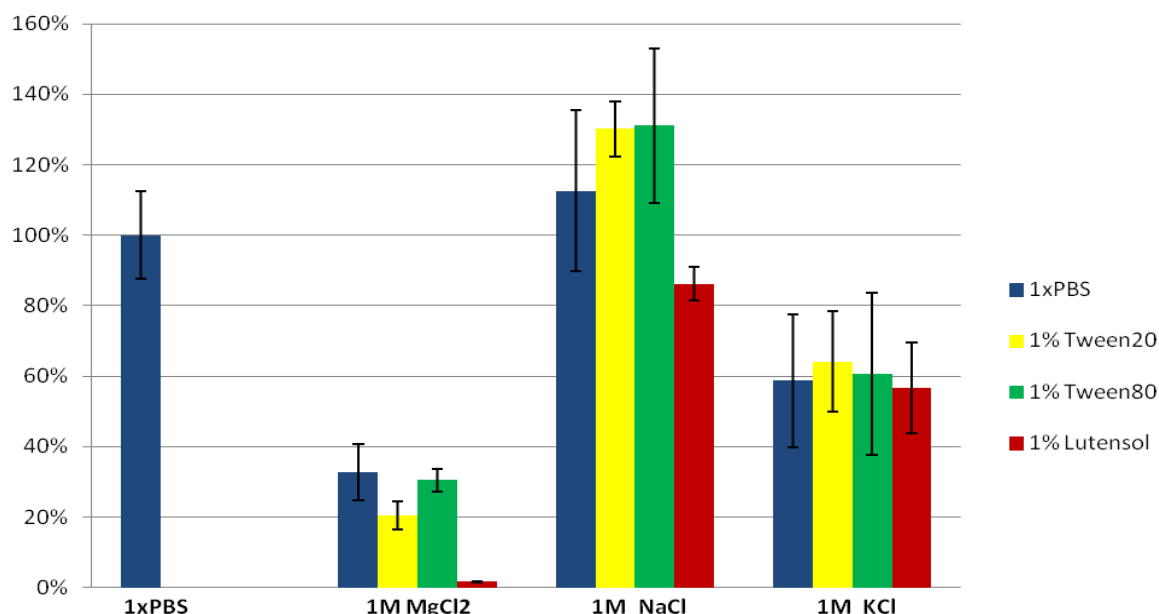


Fig. 3.1: Recovery rates of *Salmonella* Typhimurium in 1M of selected salts in 1x PBS or in combination with detergents Tween 20, Tween80 and Lutensol AO-07

Since MgCl₂ contains a divalent cation and NaCl and KCl both contain univalent cations, it was either possible that the toxic effect was related to the divalent cation-containing chloride salts or a reaction unique for MgCl₂. For further investigation, a broad range of chloride salts with either univalent or divalent cations (with the exception of AlCl₃) alone or in combination with Lutensol AO-07 were tested for their toxicity: FeCl₂, ZnCl₂, AlCl₃, MgCl₂, SrCl₂, CaCl₂, LiCl, KCl, and NaCl (see Fig.3.2).

The heavy metal containing compounds FeCl₂, SnCl₂, AlCl₃ completely inhibited cell growth and are therefore not displayed in the graph.

As can be seen, 1% Lutensol AO-07 by itself does not affect the target organisms ability to grow; neither do any of the salts, with the exception of MgCl₂ and KCl. In combination with Lutensol AO-07 however all divalent cation-containing compounds, being SrCl₂ and CaCl₂ as wells as MgCl₂, reduced the growth rate, e.g., from 77% to 2% for CaCl₂ (see Fig 3.2).

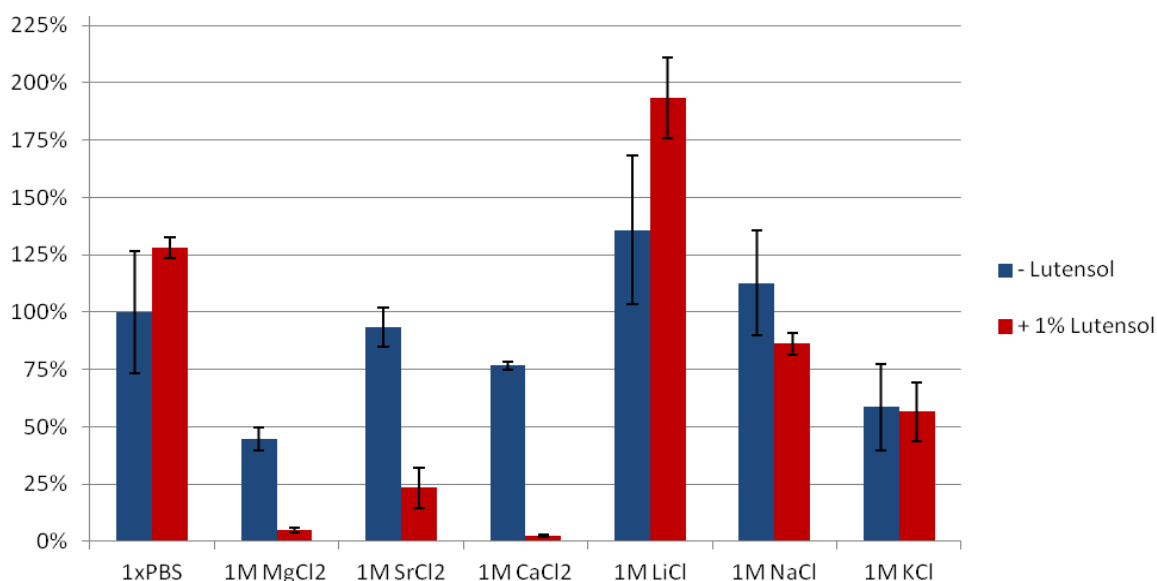


Fig. 3.2: Recovery rates of *Salmonella* Typhimurium in 1M of diverse salts and in combination with 1% Lutensol AO-07

Continuing the search for an alternative detergent, 1M MgCl₂ was combined with 1% of diverse detergents and ionic liquids (see Fig.3.3). The detergents Tween 20 and 80 as well as Lutensol were chosen because they are well known detergents of microbiology and had been used in Matrix-Lysis already [76, 118]. The ionic liquids were tested because they had previously been shown to act as mild detergents [81].

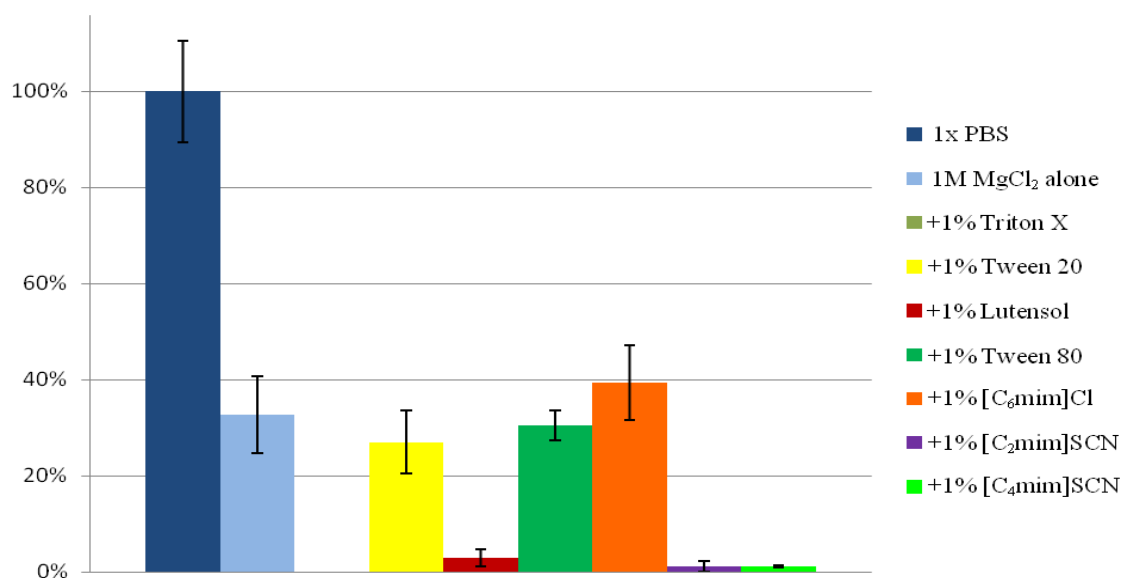


Fig. 3.3: Recovery rates of *S. Typhimurium* in 1M MgCl₂ alone or in combination with different detergents and ionic liquids

Three detergents turned out to increase the toxicity for *S. Typhimurium* in combination with MgCl_2 , being Triton X and the two ionic liquids $[\text{C}_2\text{mim}]\text{SCN}$ and $[\text{C}_4\text{mim}]\text{SCN}$ [80]. Tween 20 and 80 as well as the ionic liquid $[\text{C}_6\text{mim}]\text{Cl}$ all show recovery rates within the standard deviation of 1M MgCl_2 . Therefore, they do not add any toxicity to that of MgCl_2 itself and will be further investigated for their use on problematic foodstuffs.

3.1.2 Problematic food matrices

The two foods that could not be solubilised with 1M MgCl_2 alone were curd cheese and acid curd cheese. Therefore, it seemed reasonable to look for a detergent that would aid in the solubilisation of fat and protein-rich components. Also, both foods are very acidic and therefore it was suspected that the buffer capacity of the buffer used was not sufficient due to the pH dependency of MgCl_2 (see section 1.4.1.1).

3.1.2.1 Curd cheese

The buffer used was TRIS because of its pKa of 8.06 and its buffer capacity from pH 7 to 9. A series of 40 ml of 100 mM to 500 mM TRIS was mixed with 6.25g of curd cheese each and the pH analysed with an Orion 3 star pH benchtop (Thermo scientific, Singapore).

Table 3.1: Analysis of TRIS buffer capacities in varying concentrations with curd cheese samples

TRIS concentration	pH
100 mM	5.85
200 mM	6.81
300 mM	7.00
400 mM	7.00
500 mM	7.00

300 mM TRIS onward seems to provide a stable pH, and therefore 400mM TRIS was tested for its applicability to the Matrix-Lysis protocol. Nevertheless, the combination of 1M MgCl_2 and 400 mM TRIS alone did not reduce the pellet size and hence, the combinations with different detergents were tested, too. The eligible detergents were the ones found not toxic for the target

pathogens (see section 3.1.1), namely Tween 20, Tween 80 and the ionic liquid [C₆mim]Cl. They were combined with MgCl₂ in different concentrations and investigated for the ability to solubilise curd cheese, according to the Matrix-Lysis protocol. Table 3.2 depicts the results.

Table 3.2: *MgCl₂ in varying concentrations and 400 mM TRIS buffer were combined with various detergents and tested for their respective ability to reduce 6.25g curd cheese*

	no detergent	[Hmim] ⁺ Cl ⁻	Tween 20	Tween 80
1M MgCl₂ + 400 mM TRIS	n.l.	-	-	-
1.5M MgCl₂ + 400 mM TRIS	n.l.	n.l.	++	n.l.
2M MgCl₂ + 400 mM TRIS	n.l.	n.l.	n.l.	n.l.

n.l. indicates no lysis

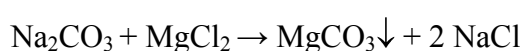
- indicates that this combination was not tested

++ indicates that the pellet size allowed for subsequent molecular methods following the Matrix-Lysis protocol

Neither Tween 80 nor [C₆mim]Cl enhanced the performance of the MgCl₂ system regarding solubilisation of the food matrix Tween 20 was the only detergent to profoundly reduce pellet size and it was therefore incorporated into the Matrix-Lysis protocol.

3.1.2.2 Acid curd cheese

Acid curd cheese, like curd cheese, is very acidic. Hence, an impact on the pH of the solubilisation reaction seemed likely and the buffer capacity of TRIS buffer had to be evaluated. 1M MgCl₂ and a series of TRIS buffer concentrations ranging from 50 mM to 400 mM were mixed with 6.25g of acid curd cheese and analysed with an Orion 3 star pH benchtop (Thermo scientific, Singapore). The pH with 50mM TRIS was at 7.94 and with 100 mM already at 8.16. Consequently, an unstable pH was excluded as a possibility. Then, acid curd cheese was analysed for its ingredients and Na₂CO₃ emerged as a prominent ingredient. This compound in combination with the MgCl₂ could lead to a precipitation reaction, according to the following formula:



The precipitated Mg^{2+} could not aid in the solubilisation of the foodstuff anymore and hence, the buffer system would be inhibited.

Since Na_2CO_3 is water soluble, acid curd cheese samples were washed with water once, twice or three times prior to Matrix-Lysis and the resulting pellets after the centrifugation step compared. Each washing step resulted in a substantial reduction of pellet size and therefore two washing steps were included into the Matrix-Lysis protocol.

3.1.3 Viability tests for an alternative buffer

In the course of toxicity tests a problematic reaction between the buffer TRIS (50 mM) and *Salmonella*-specific Xylose-Lysine-Desoxycholate Agar (XLD) was found, leading to a significant growth reduction of the pathogens. Therefore, an alternative buffer had to be found.

The starting point for an alternative buffer to TRIS was the list established by Norman Good *et al.* [42]. This list encloses physiological buffers with their buffer capacities around pH 7. Given that MgCl_2 already constituted a salty system, only buffers that are not salt based were considered. Fig.3.4 shows the recovery rates of *S. Typhimurium* for the respective buffers on both unselective and selective XLD agar. All buffers had been adjusted to a pH of 7 previously.

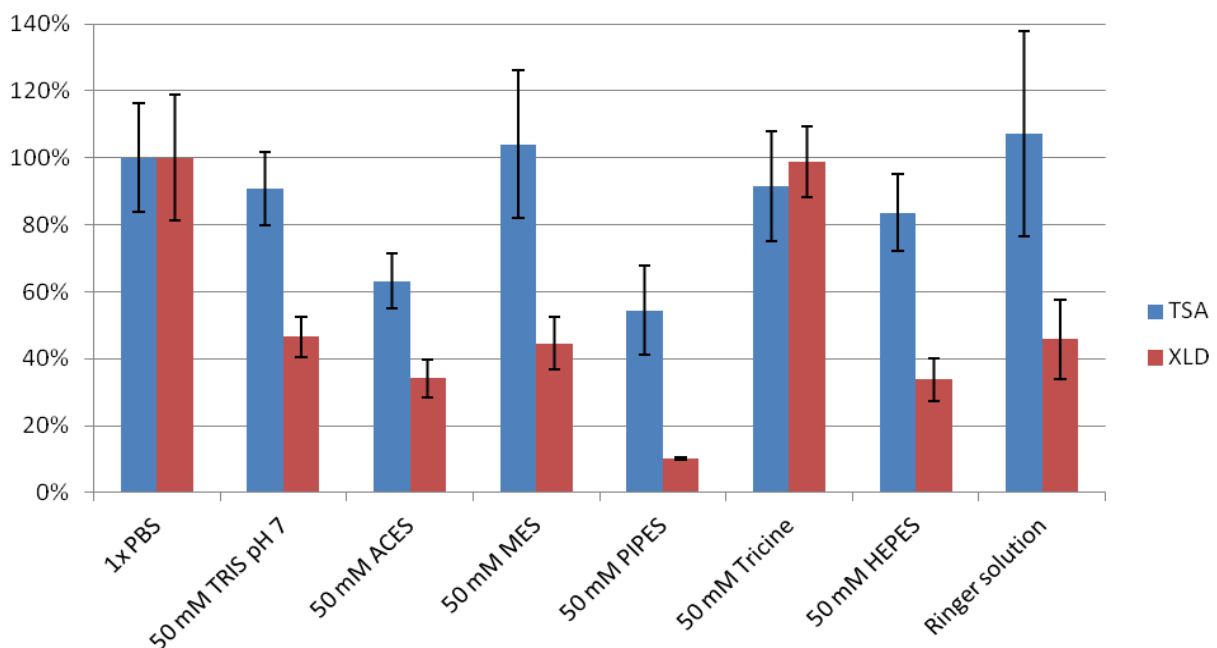


Fig. 3.4: Recovery rates of *S. Typhimurium* in different buffers on unselective (Tryptone soy agar, TSA+Y) as well as selective agar (XLD)

All tested buffers except for Tricine show a reduction in recovery in combination with the XLD agar plates. Therefore, Tricine was tested for its possible applicability in the Matrix-Lysis protocol, i.e., whether it affected target pathogen growth in varying concentrations and in combination with MgCl_2 . Recovery rates were compared to those in 50 mM PBS (see Fig.3.5).

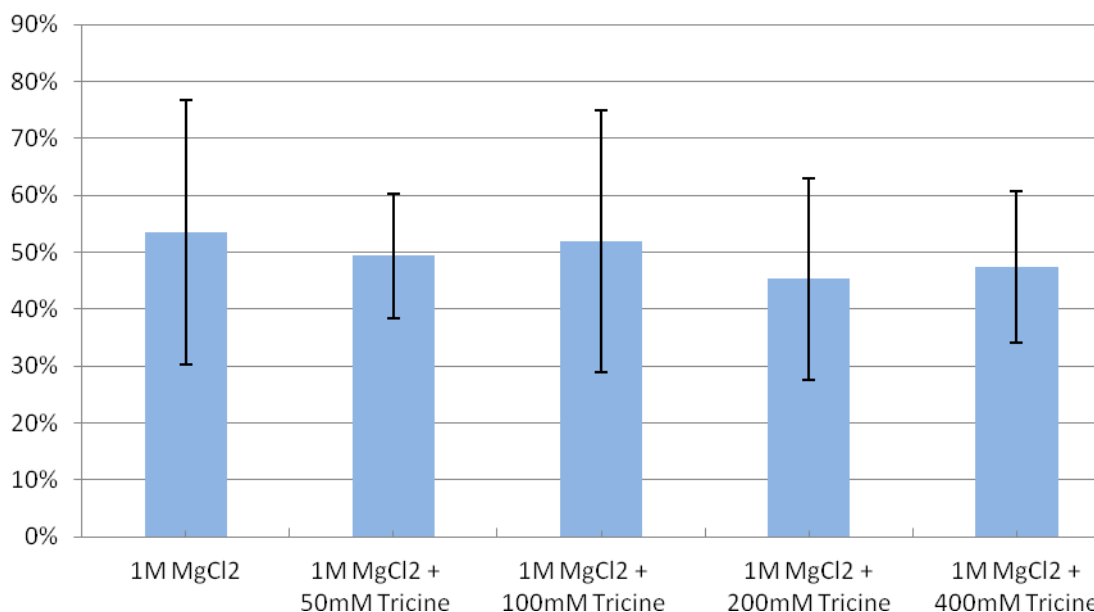


Fig 3.5: Recovery rates of *Salmonella* Typhimurium in 1M MgCl_2 with varying concentrations of the buffer Tricine

All recovery rates are around 45%, which are equal to those to be expected in 1M MgCl_2 alone, and within each other's standard deviations. They may appear high but in fact standard deviations within +/- 20% are normal for plate count method; they can only be counteracted by basing the experiment on more data. Therefore, the buffer Tricine was introduced into the Matrix-Lysis protocol.

3.2 ISPC as basis for validation of the analytical chain and quantitative detection

The idea of an internal sample process control (ISPC) is that it prevents false negative results on the one hand, and provides the basis for calculating the efficiency of the detection procedure for each sample on the other (see Fig 1.3). Fröhlich *et al.* developed a $\text{IAC}^+ \Delta\text{prfA}$ clone of *L. monocytogenes* [39], which was used as an ISPC since April 2011 on naturally contaminated frozen acid curd cheese samples from a previous outbreak in Austria [38].

The ISPC was specified before use on naturally contaminated samples by examining 72 (except buffer III: only 10 samples) acid curd cheese samples per Matrix-Lysis buffer system from retail markets, which were artificially contaminated with 10^4 IAC⁺ *ΔprfA* cells (see Fig 3.6). These samples were used as negative samples for the *L. monocytogenes* wild type during the validation of Matrix-Lysis according to ISO 16140 (see section 3.3, material and methods). Figure 3.7 depicts an exemplary result from this analysis. Results for buffer III are not shown because only 10 samples were analysed with Ionic liquids. The reason for this was that the IL was not available in greater quantities at the time of the analysis.

The concentration of an overnight culture of the ISPC was determined with the LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit and 10^4 bacteria added to every sample before Matrix-Lysis. The same amount of bacteria was subjected directly to the NucleSpin DNA isolation method and subsequent qPCR as well as unselective and selective plating on TSA+Y and LB + Chloramphenicol, respectively. Fig. 3.6 shows exemplary results for such an analysis, with the initial value obtained by BacLight and the recoveries after plating and qPCR. Both methods deliver stable recoveries around 1.2×10^4 bacteria.

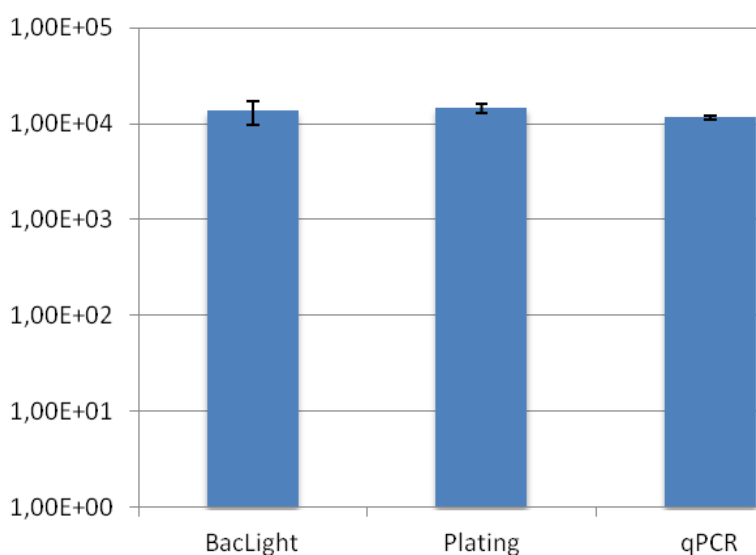


Fig. 3.6: Exemplary values from one experiment for the ISPC obtained by BacLight, plate counting and qPCR

The Matrix-Lysis samples were analysed with a multiplex qPCR, targeting both *L. monocytogenes* and the ISPC (see section 2.2.4.2, material and methods). Also, samples treated with buffer IV, the MgCl₂ system, were plated onto LB + Chloramphenicol plates. The qPCR and plate count results were then compared to the amount of ISPC for the respective method; this allowed estimation of the loss during the whole analytical chain, namely Matrix-Lysis and DNA isolation (see Fig. 3.7).

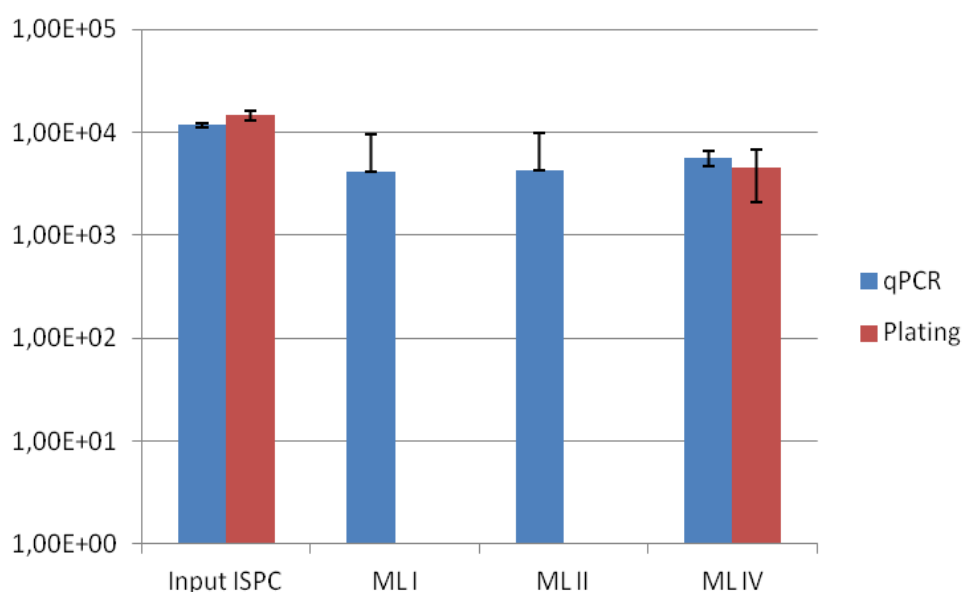


Fig 3.7: Exemplary recovery rates of the $\Delta prfA$ ISPC for the respective buffer systems after Matrix-Lysis and subsequent qPCR or plating. (Since only buffer IV leaves target cells uncompromised, it allows for plating after Matrix-Lysis, contrary to buffer systems I and II.)

For acid curd cheese samples naturally contaminated with *L. monocytogenes*, qPCR results of the ISPC were used to recalculate the initial level of contamination (see Fig. 3.8). Since the initial amount of ISPC added to every sample is known and the amount of ISPC remaining after Matrix-Lysis is detected via qPCR, a loss factor can be calculated for every individual sample. This factor allows recalculation of the original contamination level of the desired pathogen for every single sample.

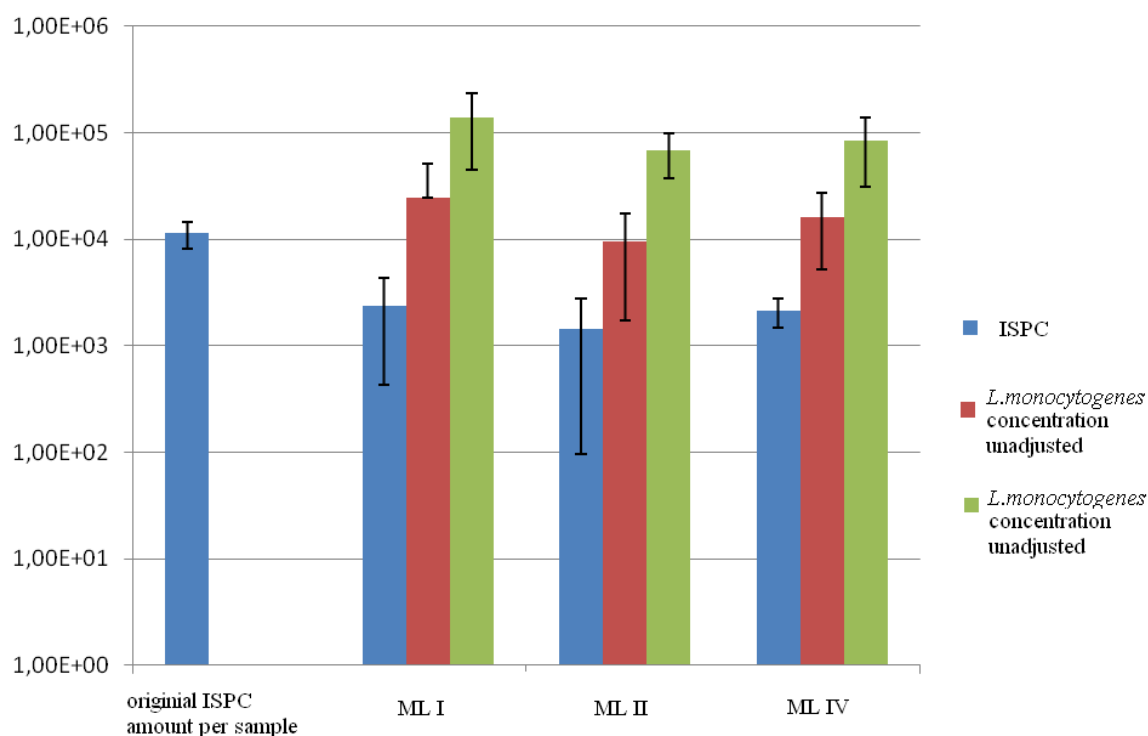


Fig 3.8: Exemplary recalculation of *L. monocytogenes* contamination in a acid curd cheese sample from the outbreak in Austria and Germany kept at -20°C on the basis of the ISPC qPCR

3.3 Validation of Matrix-Lysis according to ISO 16140

ISO 16140 defines the general principle and the technical protocol for the validation of alternative methods [8]. It states that the same amount of positive and negative samples have to be analysed both with the method to be established as well as the microbiological reference method. Here, Matrix-Lysis was compared to the standard methods ISO 11290-1 and 11290-2.

72 batches from 15 lots of naturally contaminated acid curd cheese were tested for the presence of *L. monocytogenes*. The samples originated from a recent outbreak in Germany and Austria and were analysed right after their recall from the markets [38]. All samples were processed according to the standard qualitative method ISO 11290-1 [6] and the quantitative method ISO 11290-2 [7]. Additionally, all samples were subjected to the Matrix-Lysis protocol, including all four lysis buffers. Treatment with buffer III (7.5% [C₂mim]SCN + 1x PBS) and IV (1M MgCl₂; 1x Tricine; 1% Tween 20) also allowed for the plating of samples after the Matrix-Lysis sample pre-treatment. The ISO 11290-2 was performed for all respective lots and batches.

Fig.3.9. summarises the experimental setup for this case: due to the possibly uneven distribution of target pathogens within a food sample, 5 batches (“a-e”) of each lot were pooled and thoroughly mixed in a stomacher. This pooled fraction was then investigated parallel to the several individual batches. This ensured that irregular distributions of target pathogens were made visible.

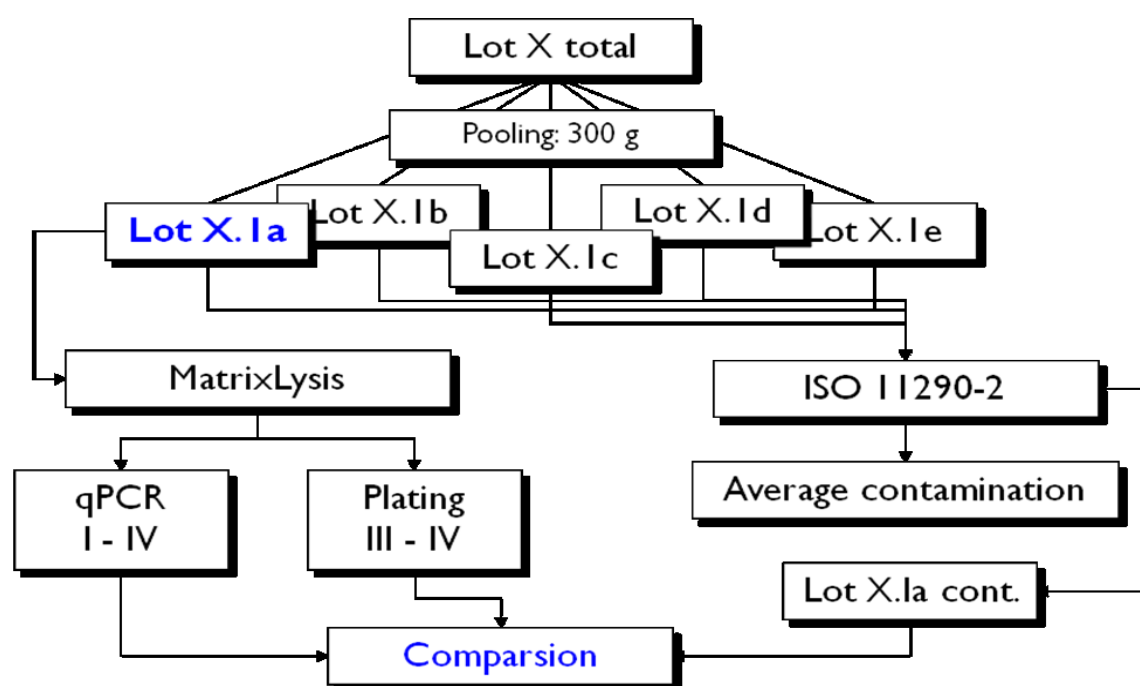


Fig. 3.9: Experimental setup for the validation of Matrix-Lysis, comparing it to the qualitative method ISO 11290-2. Batches a-e were pooled and analysed with ISO 11290-2 to give an average contamination of any respective lot, which could be compared to the ISO value of any single batch within that lot.

All batches were analysed with ISO 11290-1 and ISO 11290-2, batch “a” was additionally analysed with the Matrix-Lysis protocol. The ISO values of the respective batches were also averaged to give an overall contamination of a respective lot.

3.3.1 Quantitative Data

Initially, lots 2.1a - 10.1a were analysed (see Fig. 3.10). QPCR results for all Matrix-Lysis buffer systems are very incongruent and differ substantially (up to 3 log scales) from results gained by the reference method. This error could be traced back to the DNA isolation kit columns, which

have a maximum limit of DNA they can elute at once. This was overcome by splitting the samples prior to loading the NucleoSpin columns 1:10, 1:20 and 1:100. All results were then recalculated to display the initial concentration.

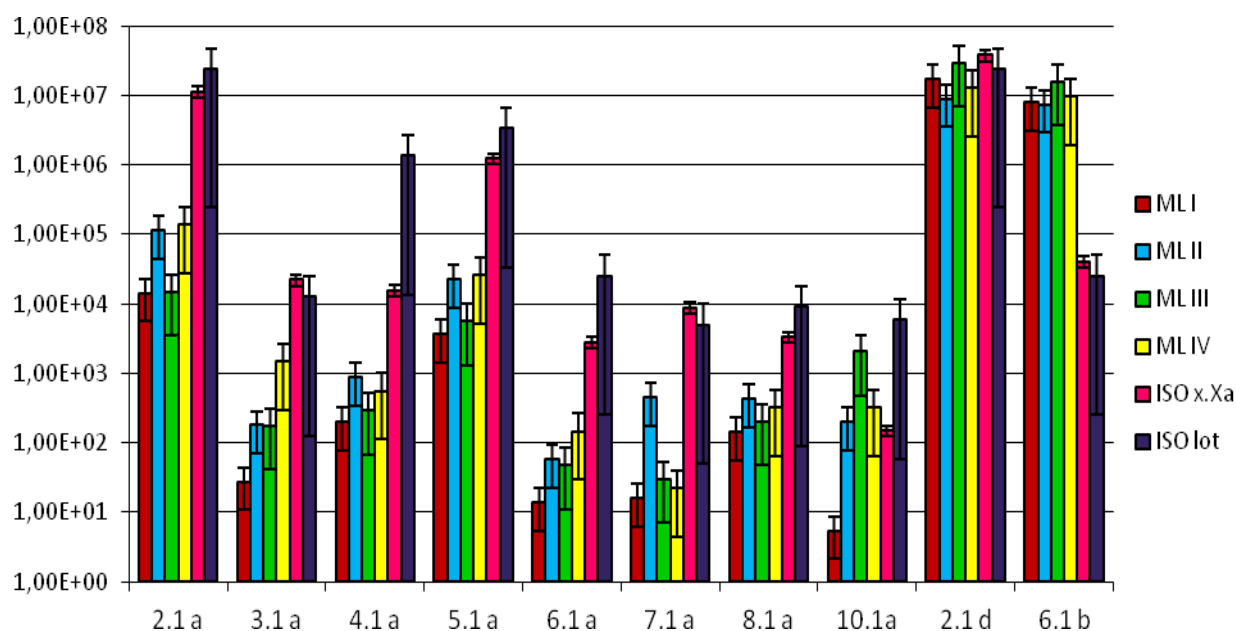


Fig 3.10: Results for lots 2.1a-10.1a analysed with Matrix-Lysis and ISO 11290-2 without splitting samples prior to NucleoSpin DNA isolation and re-examination of lots 2.1d and 6.1b with splitting samples

To show that splitting the samples solved the problem, lots 2.1d and 6.1b were re-examined with Matrix-Lysis (but not the ISO) after 4 weeks: for both batches, all four buffer systems display congruent results. The fact that the qPCR results for 6.1b do not match those gained with ISO 11290-2 can be readily explained: since the samples were analysed 4 weeks later, the target pathogens had had 4 more weeks to grow, but qPCR results are compared to the ISO results from 4 weeks earlier (see Fig. 3.10).

Consequently, all following acid curd cheese samples were analysed by splitting them prior to the Nucleospin analysis. Fig. 3.11 depicts the results for an exemplary lot, 13.1a.

All Matrix-Lysis buffer systems show results for qPCR analysis and plating within the same log scale and are consistent with ISO 11290-2 results for the respective lot 13.1a.

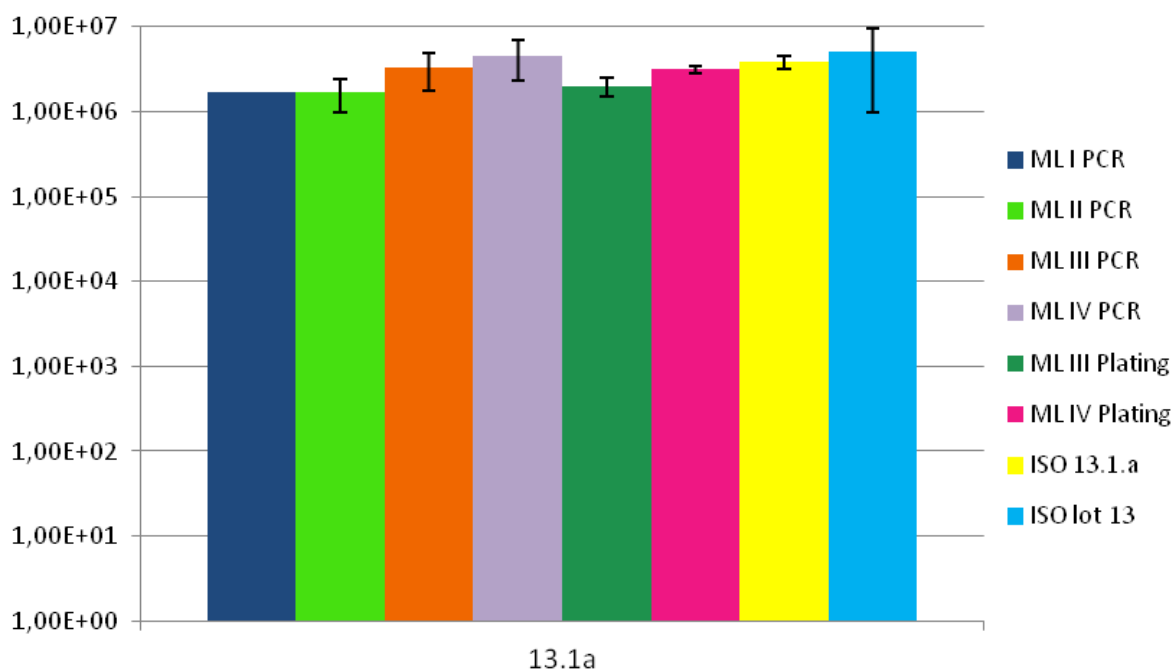


Fig 3.11: Representative results for batch a from lot 13; recoveries after qPCR and plating after Matrix-Lysis are compared to the ISO 11290-2 value of batch 13.1a and the ISO 11290-2 value of the whole lot 13.1

QPCR results for all lots, namely 12.1a – 18.1a, are summarised in figure 3.12.

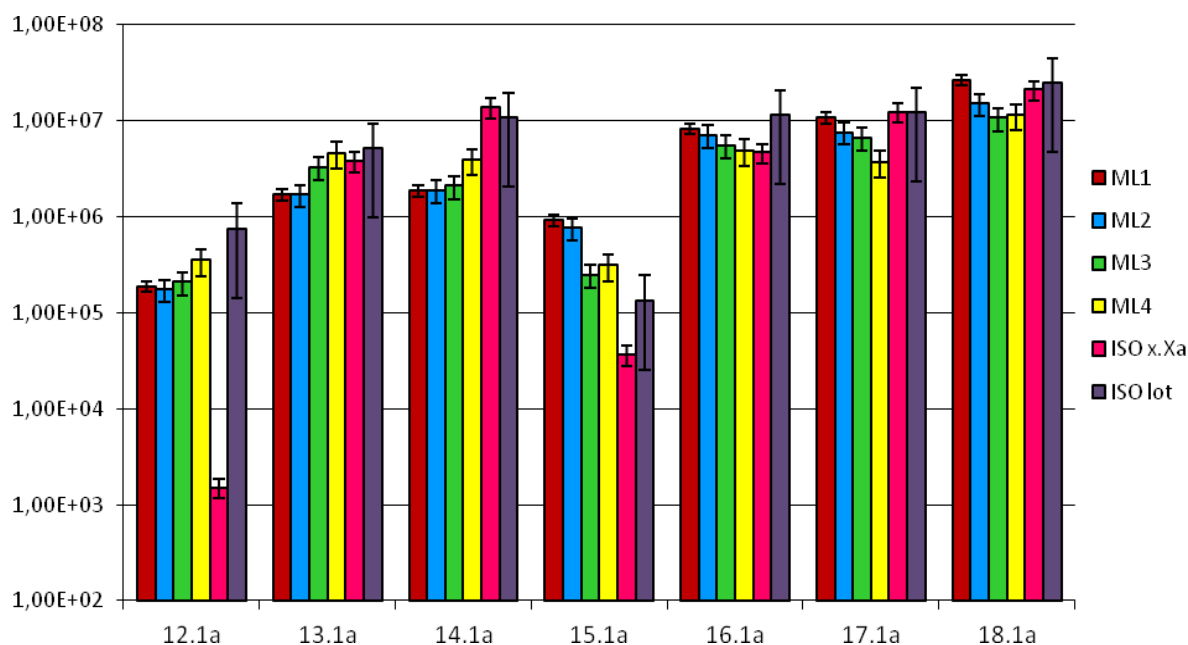


Fig. 3.12: qPCR Results for batches a from lots 12.1-18.1, treated with Matrix-Lysis buffer I-IV as well as the quantitative ISO 11290-2

Results from both methods, ISO and Matrix-Lysis, are consistent and within each other's standard deviations for all lots, with exception of lot 12.1a: the ISO value for batch 12.1a deviates from qPCR results and from the ISO value made up of pooled samples from batches “a-e” by two log scales.

Fig 3.13 depicts the plating results for lots 12.1a through 18.1a. All lots show consistent results for both methods, again with the exception of 12.1a.

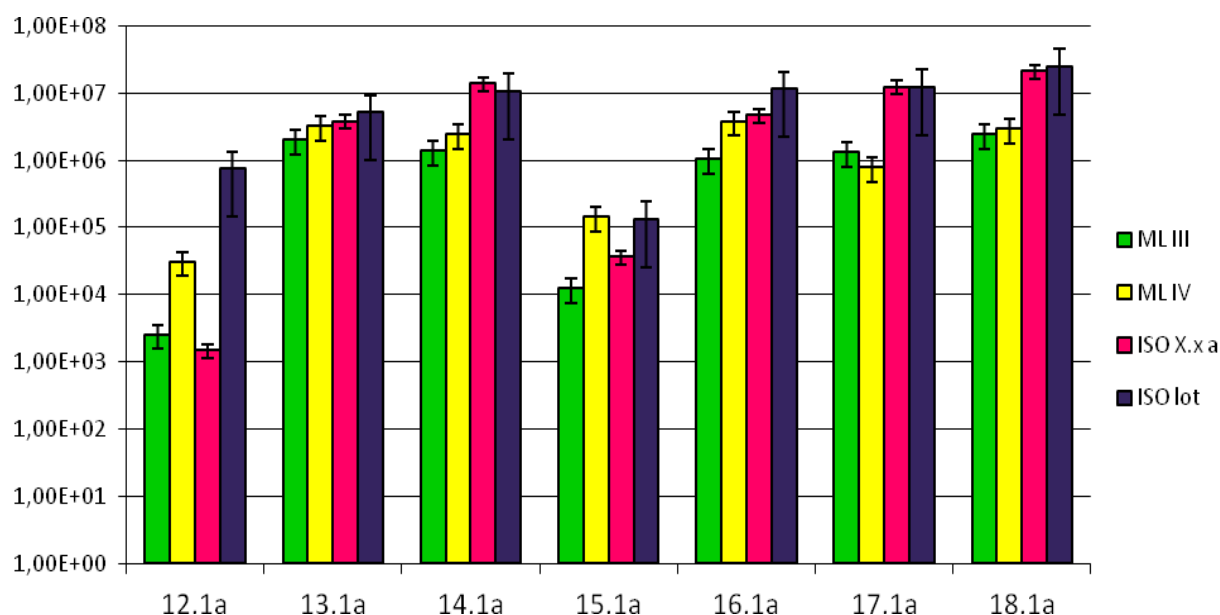


Fig. 3.13: Recoveries after plating in ML buffers III and IV after the Matrix-Lysis protocol, compared to the ISO of the respective batches and lots 12.1a – 18.1a

3.3.2 Qualitative Validation of Matrix-Lysis according to ISO 16140 against ISO 11290-1

To validate the Matrix-Lysis sample preparation protocol, 144 acid curd cheese samples were analysed with all buffer systems and compared to the reference method ISO 11290-1 according to ISO 16140. Table 3.1 summarises the results.

72 positive samples were analysed with all buffer systems. 72 negative samples were each analysed with buffer systems I, II and IV but only 10 samples with buffer III, because the ionic liquids needed were not available at the time of the analysis (see section 3.2). Comparison of the results from Matrix-Lysis as well as ISO 11290-1 according to ISO 16140 resulted in 100% relative accuracy, 100% relative specificity and 100% relative sensitivity.

Table 3.1: Results of the validation of Matrix-Lysis according to ISO 16140

	ISO 11290-1	ML I	ML II	ML III	ML IV
Total No. of samples	144	144	144	82	144
No. of positives	72	72	72	72	72
No. of negatives	72	72	72	10	72
False negatives	0	0	0	0	0
False positives	0	0	0	0	0
Relative accuracy (%)	100	100	100	100	100
Relative specificity (%)	100	100	100	100	100
Relative sensitivity (%)	100	100	100	100	100

3.4 Freezing test

To investigate the influence of long term freezing on bacteria, a target pathogen was added to a food matrix and its ability to grow monitored over two months.

UHT milk was artificially contaminated with *L. monocytogenes* and portioned for every experiment so that not one pooled sample would have to be thawed prior to every analysis.

Every single sample was artificially contaminated with 10^4 target pathogens (*L. monocytogenes*). The initial concentration of bacteria was determined with three methods: using BacLight™ viability kit, plating on TSA+Y and qPCR. Except for the samples for day 1, which were analysed immediately, all samples were stored at -20°C. All samples were analysed by Matrix-Lysis and subsequent qPCR, quantitative ISO 11290-2 and qualitative ISO 11290-1, to verify the identity of the target pathogens. The ISPC was added to every sample analysed by qPCR on the respective day, as described in section 3.2. The samples were analysed after 7, 14, 28 and 56 days of freezing.

Fig. 3.14 depicts the results for this experiment. Whereas Matrix-Lysis buffers I through IV show stable recoveries of the target pathogen *L. monocytogenes*, the recoveries for the

quantitative ISO, which is based on growth, decrease steadily over time- all in all over half a log scale.

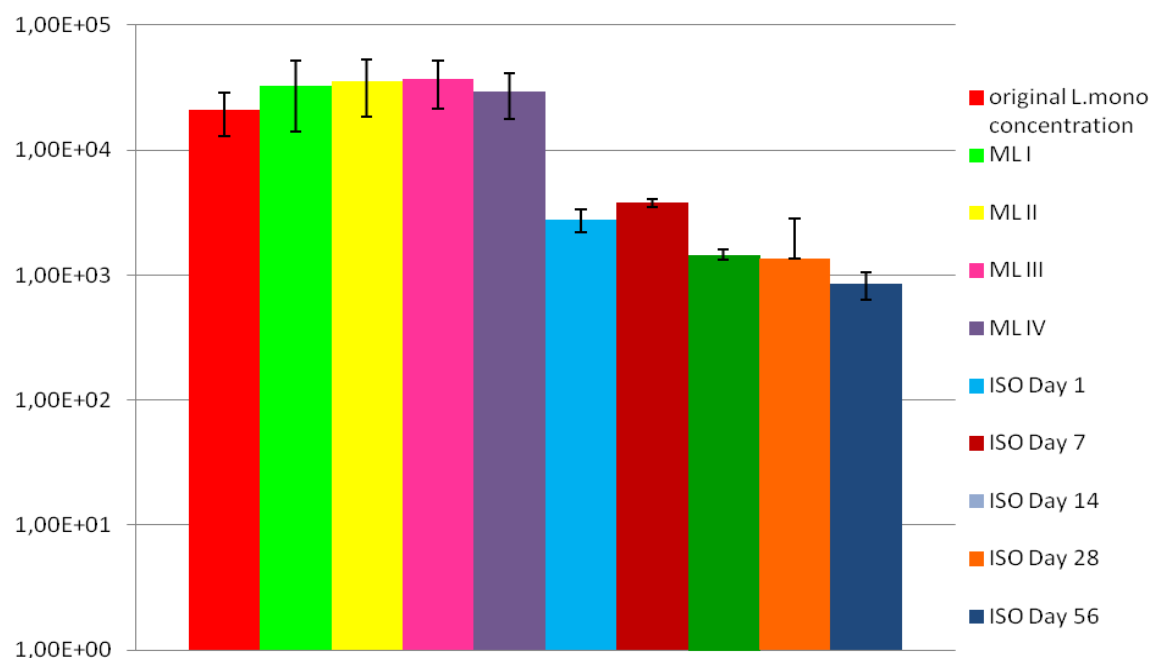


Fig. 3.14: QPCR results of *L. monocytogenes* for ML buffers I-IV, averaged for all 6 experimental days, and results for the ISO 11290-2 for days 1 through 56. All PCR results were readjusted by means of the ISPC (see section 3.2).

4. Discussion

Today, food is traded on a global scale, which has increased the speed at which foods are produced, transported and distributed. Consumers have lost touch with the provenance of their foods, being accustomed to having access to any kind of food at any time. The downside of this is that foods are produced in huge amounts. That together with their often elusive history makes the regular surveillance of food qualities complicated and, above all, very expensive. This in turn poses a challenge for the eradication of food-borne pathogens, which can spread rapidly and are a severe threat to public health. The EU met this challenge and made controls for every processing step of foods an obligation [1].

Foods are traditionally analysed with microbiological methods [6, 7]. This entails several problems:

- Microbiological methods depend on a microorganism's ability to grow and therefore do not address the problem of viable but not culturable, i.e., stressed cells, that could nevertheless still pose a threat to human health
- Even quantitative microbiological methods rely on enrichment of the pathogen in nutritional media and therefore give no account of the original concentration of a respective pathogen within a sample
- Foods can contain all sorts of background flora that may suppress growth of target pathogens
- The methods are time consuming, taking up to a week for the confirmation of results

The pre-analytical sample preparation method Matrix-Lysis presents a non-complex, cost-effective and fast way of transforming different kinds of food into the same homogenous output, that can be further analysed with as sensitive a method as qPCR. The protocol includes four buffer systems that are capable of solubilising a broad variety of foodstuffs, with few exceptions like carbohydrate-rich components. The first three buffers are well established within the protocol [76, 80], whereas buffer IV, the $MgCl_2$ system, still needed minor adjustments (Mester *et al.*, Patent application, EPA09007959.1: Method for isolating viable cells).

Also, Matrix-Lysis was to be validated according to ISO 16140, comparing it to the standard microbiological methods ISO 11290-1 and 11290-2. The aim was to establish Matrix-Lysis as a reliable, fast, accurate and cost-efficient method for the detection of food-borne pathogens.

4.1 Adjustments to the Matrix-Lysis protocol

In the course of analysing the underlying mechanism of the toxic effect of Lutensol AO-07 and MgCl_2 on *S. Typhimurium* a broad range of salts were investigated. The focus was thereby on the role that the anion played. In a first set of experiments, 1M KCl but not NaCl lowered the growth rate of *S. Typhimurium*. All further tested chloride salts with a univalent cation did not influence the growth, independent of the presence or absence of Lutensol AO-07. The divalent cation-containing compounds SrCl_2 , CaCl_2 and MgCl_2 , however, significantly reduced the growth rate of *S. Typhimurium* in combination with Lutensol AO-07. Consequently, the toxic effect of Lutensol AO-07 is connected to the divalent cation within a compound but the question was not further looked into, since the priority was to find an alternative detergent.

From different tested detergents, Tween 20 and 80 as well as the ionic liquid $[\text{C}_6\text{mim}]\text{Cl}$ did not add toxicity to that of 1M MgCl_2 on *S. Typhimurium*. They were used on previously problematic food matrices, namely acid curd cheese and curd cheese. Both these foods are very acidic and therefore it seemed reasonable to suspect their influence on the pH of the reaction to be obstructive to the lysis. After establishing an optimal concentration of the buffer TRIS, the combination with Tween 20 profoundly reduced the pellet size of curd cheese but not acid curd cheese. This could be traced back to Na_2CO_3 , which is an abundant compound of acid curd cheese. Acid curd cheese was made processable by including two washing steps into the Matrix-Lysis protocol. Tween 20 did not enhance the solubilisation of other foods.

Since TRIS lead to a significant growth reduction of *S. Typhimurium* on XLD agar, viability tests for an alternative buffer were undertaken. Tricine alone did not influence the growth of the pathogen, even when tested in concentrations up to 400mM.

To sum it up, the detergent Tween 20 and the buffer Tricine were successfully incorporated into the MgCl_2 buffer system.

4.2 The efficiency of qPCR can be calculated for every sample with an internal sample process control (ISPC)

Every fundamental step or significant parameter of an analytical chain has to be tested, validated and definitively determined. Therefore, the overall quality of the analytical chain is the sum of all optimised individual steps.

In that respect, the ISPC developed by Fröhwrth *et al.* [39] presents a useful tool in three ways: first, if added to any sample before the pre-analytical sample preparation method, it provides the basis for calculating the overall loss of targets over the whole procedure. Second, it provides a reference for the performance of each individual sample in the analytical method, e.g., qPCR. Third, it prevents false negative results.

Before the ISPC was used on naturally contaminated samples it was put to the test. Since the ISPC behaves like *L. monocytogenes*, it should yield identical recovery rates after the Matrix-Lysis protocol. This could be confirmed by analysing 72 acid curd cheese samples (see section 3.2, results).

The ISPC was then used on an exemplary matrix; the qPCR results for all buffer systems show a loss of about half a log scale. These confirming experiments for the ISPC were undertaken when the analysis of the naturally contaminated acid curd cheese samples from the recent outbreak [38] had already taken place, so naturally contaminated acid curd cheese samples were analysed in retrospect after having been kept at -20°C. This experiment clearly pointed out the benefit of the ISPC. The contamination levels of *L. monocytogenes* without the re-calculation on the basis of the ISPC are around 10^4 BCE/ml. The recalculated contamination values however are at 10^5 BCE/ml. The idea of losing one log scale of bacteria during the analytical process unnoticed is quite intolerable, especially since the EU recently defined clear limits for levels of contamination for *L. monocytogenes*, among many other pathogens [3]. The ISPC implemented provides a powerful tool for achieving that objective.

4.3 Validation of the Matrix-Lysis protocol according to ISO 16140 results in 100% relative accuracy, specificity and sensitivity

The ISO 16140 dictates that as many naturally contaminated as negative samples are to be analysed with the reference method, ISO 11290-1 and 11290-2 in this case, and the new method, Matrix-Lysis.

The 72 batches from the acid curd cheese contaminated with *L. monocytogenes* and 72 batches of acid curd cheese bought in local retail markets each yielded 100% relative accuracy, 100% relative specificity and 100% relative sensitivity.

The first round of erratic qPCR results for batches 2.1a - 10.1a due to overloading the NucleoSpin columns was overcome by splitting the samples. This was first shown exemplary for lots 2.1d and 6.1b, which were used because 2.1 and 6.1a had been depleted. The fact that the qPCR results for 6.1b do not match those gained with ISO 11290-2 can be readily explained: since the samples were analysed 4 weeks later, the target pathogens had had 4 more weeks to grow, but qPCR results are compared to the ISO results from 4 weeks earlier (see section 3.3.1, results).

The following analysis of batches 12.1a - 18.1a show consistent results, except for lot 12.1a: the ISO value deviates from the qPCR results and from the ISO value made up of pooled samples from batches “a-e” by two log scales. This is due to the irregular distribution of target pathogens within a food matrix, as described in section 3.3. Therefore, the value of the pooled samples is compared to the qPCR results for analysis.

Together, these results establish Matrix-Lysis as valuable sample preparation method:

- the sample matrix is disposed of and the target organisms concentrated by reducing the sample volume
- relatively large sample volumes can be processed in a short time
- inhibitory compounds within the food matrix that could affect the downstream detection method are removed
- Buffer systems III and IV preserve the viability of the target organism; therefore, samples can be plated and directly compared to the microbiological standard method.
- it provides maximum recovery of the target organism as well as a low detection limit

- a homogeneous output with a linear recovery of pathogens in every concentration is provided
- free target DNA as well as eukaryotic cells are eliminated and therefore do not interfere with the detection
- the resulting data is quantitative
- it is non-complex, cost effective and provides results within 24 hours

Rather than microbiological methods, which only find cells able of proliferating molecular biological methods are able to detect viable cells, dead cells and VBNC's. . This, together with the above mentioned factors, should help to establish Matrix-Lysis as the method of choice for analysing foods.

4.4 Freezing leads to a decline of the viability of *L. monocytogenes*

It has been suspected that the long term storage of matrices at -20°C has an impact on the viability of microorganisms [54]. The experimental setup therefore aimed to present in detail the impact the storage had on the viability of bacterial cells. Two methods were compared: on the one hand Matrix-Lysis with qPCR as detection method, on the other hand the quantitative ISO 11290-2. One has to keep in mind that the two methods rely on different methods for counting their targets: Matrix-Lysis keeps target cells intact and eliminates free target DNA [76]. The microbiological method in the other hand counts colony forming units, i.e., only viable cells that are able to proliferate.

All four buffer systems show consistent results for all days. However, the microbiological method, which is based on the growth of the target organism, shows decreasing numbers of target pathogens. All in all, from day 1 to day 56, over half a log scale of bacteria gets lost. This suggests that freezing either kills bacteria or moves them into a viable but not culturable state (VBNC). However, the pathogen's integrity is not affected, since Matrix-Lysis eliminates free target DNA as shown by Mayrl *et al.* [76]. On the contrary, the results for all four buffer systems are consistent for all days. Therefore, microbiological methods do not mirror the true contamination level. In conclusion, to prevent erratic results microbiological methods should only be used on fresh samples.

Conclusion

The Matrix-Lysis protocol was successfully adapted: the few problematic foodstuffs that could not be analysed before, namely acid curd cheese and curd cheese, were made processable. Also, an alternative buffer that could be used with the *Salmonella*-specific Xylose-Lysine-Desoxycholate Agar (XLD) and an alternative detergent to Lutensol AO-07 were incorporated into the Matrix-Lysis protocol.

The internal sample process control (ISPC) as basis for the validation of the analytical chain and quantitative detection was introduced. It was used to analyse naturally contaminated samples and provided the basis for assessing the efficiency of every Matrix-Lysis buffer system and re-calculating contamination levels.

Matrix-Lysis was validated according to ISO 16140. The analysis of naturally contaminated acid curd cheese samples with Matrix-Lysis and the standard methods ISO 11290-1 and 11290-2 resulted in 100% relative accuracy, 100% relative specificity and 100% relative sensitivity.

Additionally, the effect of long term storage on the integrity and viability of *L. monocytogenes* was addressed. Even though the integrity of target pathogens seems unaffected, the viability steadily decreases over time. Microbiological methods should therefore not be used for the analysis of frozen samples.

All in all, Matrix-Lysis could be established as a reliable sample preparation for the subsequent use of sensitive molecular biological detection methods. Every step of the analytical chain was thoroughly validated due to the incorporation of the ISPC. This enabled the correct identification and quantification of target pathogens from foods. As the data from this work shows, this is possible with microbiological and molecular biological detection methods. Hopefully, this work will therefore help to establish Matrix-Lysis and molecular biological detection methods as the analysis methods of choice: this could accelerate the detection process while making it more accurate and at the same time reduce financial as well as health risks.

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Zusammenfassung

Obwohl Lebensmittelsicherheit ein Thema von allgemeinem Interesse ist, sind die Analysemethoden für Lebensmittelpathogene nach wie vor mikrobiologische Methoden. Diese sind meist zeitaufwendig, nicht sehr genau und können außerdem nur wachstumsfähige Pathogene erfassen. Da Lebensmittel heutzutage global vertrieben werden, ist auch die Geschwindigkeit gestiegen, mit der potenzielle Bedrohungen in Lebensmitteln sich ausbreiten können. Die EU hat außerdem exakte Kontaminationslimits für diverse Pathogene definiert, daher sind schnellere und genauere Detektionsmethoden mehr als notwendig geworden.

Die kürzlich entwickelte Probenvorbereitungsmethode Matrix-Lysis kann Lebensmittelmatrizen jeder Art, mit wenigen Ausnahmen, auflösen. Dies hat zum Vorteil, dass das Volumen der zu analysierenden Proben signifikant reduziert wird und sie daher mit einer molekularen Methode, wie beispielsweise real-time PCR, analysiert werden können. Matrix-Lysis besteht aus vier Puffersystemen, von denen zwei die Pathogene intakt lassen und daher auch eine mikrobiologische Untersuchung erlauben.

Das Ziel dieser Arbeit war es das Matrix-Lysis System nach der ISO 16140 zu validieren. Im Zuge dessen wurde Matrix-Lysis mit den Standardmethoden ISO 11290-1 und ISO 11290-2 verglichen. Dies resultierte in 100% relativer Genauigkeit, 100% relativer Spezifität und 100% relativer Sensitivität.

Außerdem wurde eine internal sample process control (ISPC) als Basis für die Validierung der Analyseketten und der quantitativen Detektion in das Matrix-Lysis Protokoll inkorporiert. Die ISPC ermöglicht es, die Effizienz der Detektionsmethode für jede einzelne Probe sowie jedes Puffersystem zu berechnen. Bei der Analyse von natürlich kontaminierten Quargelproben war die so rekalkulierte, anfängliche *L.monocytogenes* Kontamination um eine Log-Stufe höher als sie ohne die ISPC berechnet worden wäre.

Natürlich kontaminierte Proben, die zuvor bei -20°C gelagert worden waren, führten mit Matrix-Lysis und mikrobiologischen Untersuchungen zu sehr unterschiedlichen Ergebnissen. Daher wurde untersucht, inwieweit eine Langzeitlagerung bei dieser Temperatur die Lebensfähigkeit des Pathogens beeinflusst. Dafür wurde Milch künstlich mit *L.monocytogenes* kontaminiert und über einen Zeitraum von 56 Tagen sowohl mit Matrix-Lysis als auch der ISO 11290-2 analysiert. Alle vier Puffersysteme zeigen konstante Ergebnisse, die mikrobiologische Methode allerdings weist über den experimentellen Zeitraum einen Verlust von einer halben Log-Stufe auf.

Abstract

Traditional methods for analysing foods are microbiological methods. These methods rely on growth of a respective pathogen, they are time consuming and also not very accurate. Today, foods are distributed globally and consumed within a few days. Consequently, the speed at which potential hazards are spread has increased, too. The EU has defined exact limits for respective pathogens. To meet these challenges, faster and more accurate detection methods are necessary.

The recently developed sample-preparation method MatrixLysis is capable of solubilising many foodstuffs, with the exception of carbohydrate-rich compounds. Disposing of the food matrix and possibly inhibitory compounds allows the analysis of the food sample with a subsequent molecular method like real-time PCR. The MatrixLysis protocol includes four buffer systems, two of which leave the pathogens intact and enable their subsequent microbiological analysis.

One aim of this study was to validate the MatrixLysis system according to ISO 16140, comparing it to a standard microbiological method. *L.monocytogenes* served as model organism, and therefore, MatrixLysis was compared to ISO 11290-2. Comparing the results of both methods yielded 100% relative accuracy, 100% relative specificity and 100% relative sensitivity.

Additionally, an internal sample process control (ISPC) as basis for the validation of the analytical chain and quantitative detection will be introduced into the MatrixLysis protocol. It provided a basis for calculating the efficiency of the detection procedure for each sample and every MatrixLysis buffer system. Naturally contaminated quargel samples were analysed and the re-calculated contamination was up to 1 log scale higher than without the addition of the ISPC.

Presumptive naturally contaminated samples that had been stored at -20°C yielded different results when treated with MatrixLysis and qPCR rather than the microbiological reference method. Hence, the problem of long term storage and whether it affected a pathogen's integrity and/or viability was addressed. Milk was artificially contaminated with *L.monocytogenes* and analysed over a period of 56 days with MatrixLysis as well as ISO 11290-2. All four MatrixLysis buffer systems yielded consistent real-time PCR results, the microbiological method showed a decrease in growth of half a log scale over the experimental time period.