Sanna Taskila

IMPROVED ENRICHMENT CULTIVATION OF SELECTED FOOD-CONTAMINATING BACTERIA
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Academic dissertation to be presented, with the assent of the Faculty of Technology of the University of Oulu, for public defence in Auditorium IT115, Linnanmaa, on 26 November 2010, at 12 noon

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Abstract

The aim of this work was to assess and improve the enrichment cultivation of food-contaminating bacteria prior to detection by means of RNA-based sandwich hybridization assay (SHA). The examples of beer-spoiling lactic acid bacteria (LAB) and food-borne *Salmonella Typhimurium* were selected based on their relevance in Finnish food industry. Also universal challenges affecting on the selection of the enrichment cultivation procedure are discussed, including some potential possibilities for improved enrichment cultivation. The results of this study may therefore be used for the assessment of the efficiency of bacterial cultivation in other applications.

The evaluation of the enrichment cultivation procedures prior to SHA lead to following conclusions: i) the enrichment cultivation procedure is necessary prior to rRNA-based SHA, and it directly influences the accuracy of SHA; ii) the improvement of the enrichment cultivation may allow faster recovery and growth of bacteria; iii) the improved recovery of bacteria can be achieved by reducing environmental stress factors in the enrichment culture; and iv) the growth of bacteria may be accelerated by ensuring the selectivity of medium and allowing accessibility to growth factors. Several growth factors were studied by means of full factorial design and response surface modeling. Measured cell densities, as well as predicted lag-times and maximum growth rates in the bacterial cultures were used as responses.

The results show that small shifts in the cultivation conditions extend the lag-time and decrease the growth rate of both LAB and *Salmonella*. Besides adjusting the temperature and pH, the growth of LAB was facilitated by reducing osmotic and oxidative stresses in the enrichment medium. In this study, a novel enzyme controlled glucose delivery system was used for the first time in the enrichment cultivation of food-contaminating bacteria. The glucose delivery system improved the growth of LAB in single strain cultures and in actual brewing process samples. The recovery of injured *Salmonella* was also enhanced by using the glucose delivery system together with selective siderophore ferrioxamine E, both in terms of reduced lag-times and increased growth rates. Based on the SHA, the adjusted BPW broth enhanced the molecular detection of heat-injured *Salmonella* in meat.

*Keywords*: beer, enrichment cultivation, food microbiology, lactic acid bacteria, *Lactobacillus*, meat, microbiological methods, *Pediococcus*, *Salmonella*, sandwich hybridization, 16S rRNA, 23S rRNA
Acknowledgements

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Oulu, October 14, 2010

Sanna Taskila
### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABD</td>
<td>Advanced Beer Detection broth</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BPW</td>
<td>Buffered Peptone Water broth</td>
</tr>
<tr>
<td>B-MRS</td>
<td>De Man-Rogosa-Sharpe broth with 25% beer</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
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<td>FISH</td>
<td>Fluorescence <em>in situ</em>-hybridization</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
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<tr>
<td>L.</td>
<td><em>Lactobacillus</em></td>
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<td>LAB</td>
<td>Lactic acid bacteria</td>
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<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal amplification</td>
</tr>
<tr>
<td>L-cysteine-HCl</td>
<td>L-cysteine hydrochloride</td>
</tr>
<tr>
<td>MRS</td>
<td>De Man-Rogosa-Sharpe broth</td>
</tr>
<tr>
<td>MKTTn</td>
<td>Müller-Kauffman tetrathionate novobiocin broth</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic acid sequence–based amplification</td>
</tr>
<tr>
<td>NB</td>
<td>Nutrient broth</td>
</tr>
<tr>
<td>NBB</td>
<td>Nachweismedium für Biersäulichen Bacterien</td>
</tr>
<tr>
<td>NBB-C</td>
<td>Concentrated NBB</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OD$_{490}$</td>
<td>Optical density at 490 nm</td>
</tr>
<tr>
<td>p</td>
<td>Probability for regression according to F-test</td>
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<tr>
<td>P.</td>
<td><em>Pediococcus</em></td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RSM</td>
<td>Response surface modelling</td>
</tr>
<tr>
<td>RVS</td>
<td>Rappaport-Vassiliadis Soy broth</td>
</tr>
<tr>
<td>S.</td>
<td><em>Salmonella</em></td>
</tr>
<tr>
<td>SC</td>
<td>Selenite cystine broth</td>
</tr>
<tr>
<td>SHA</td>
<td>Sandwich hybridization assay</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td><em>S. enterica</em> subspecies <em>enterica</em> serovar Typhimurium</td>
</tr>
<tr>
<td>Sensitivity ratio</td>
<td>The proportion of true positives from all positive results.</td>
</tr>
<tr>
<td>Specificity ratio</td>
<td>The proportion of true negatives from all negative results.</td>
</tr>
<tr>
<td>TT</td>
<td>Tetrathionate broth</td>
</tr>
<tr>
<td>U</td>
<td>Enzyme activity unit</td>
</tr>
<tr>
<td>UPB</td>
<td>Universal preenrichment broth</td>
</tr>
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</table>
List of original articles

This thesis is based on the following original articles which are referred to in the text by their Roman numerals. In addition, some unpublished data are included.

Articles in peer-reviewed journals:


Supplementary material:


The author’s contribution

I S. Taskila (nee Huhtamella) was responsible for the tests at the brewery. She wrote the article in cooperation with the other authors.

II–IV S. Taskila wrote the article, is the corresponding author and interpreted the results. She was responsible for all experimental work, except for the detection of actual samples at the breweries.

V S. Taskila was responsible for the RSM and the enrichment cultivations. She wrote the article in cooperation with the other authors.
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1 Introduction

1.1 Background

Food safety is one of the most important issues with regard to the sustainable development in global scale. It has been estimated that the growth of global food production and trade will result in an increase in the burden of food-borne disease in the next few decades. In order to assure safety, the food industry puts significant effort into the microbiological testing of foods. Good hygiene in the process can also directly contribute to the profitability by increasing the fermentation rates or avoiding recalls of spoiled product batches. In the past, the detection of microbial contaminants in foods was done mainly by cultivation, followed by microbiological and biochemical analyses. The contaminating microbes were identified by means of microscopy, enzymatic tests, and Gram-staining. These cultivation based assays are still widely in use, but in many cases together with molecular diagnostics.

The cultivation based detection methods have advantages, such as possibility for quantification of viable cells and further studies using the isolated strains, and the generally accepted protocols. The drawbacks of the cultivation based detection include lengthy procedures, and poor accuracy and selectivity. These methods are also operator-dependent and therefore, the results can be subjective. Due to growing efficiency of production and logistics, the management of food safety in the future relies on rapid microbial analysis methods (von Blankenfeld-Enkvist & Brännback 2002), also referred to as alternative methods in comparison to reference methods. Although, novel rapid detection methods and more effective sample processing techniques are frequently reported, the need for enrichment cultivation exists. This is mostly due to the low concentration of bacteria (Back 2005, Häge et al. 2005), and the presence of inhibitory components in the samples (Juvonen et al. 1999, Kanki et al. 2009). For example, in the European Union (EU), total absence of Salmonella is required in 25 g of minced meat (Anonymous 2005b). None of the currently available detection methods can meet the sensitivity demand for the Salmonella detection without enrichment cultivation.

The cultivation of bacteria in batch cultures, i.e. cultures with limited amount of nutrients, generally includes the following four main phases: i) lag phase (i.e. lag-time), during which bacteria adapt themselves to growth conditions; ii)
exponential growth phase, during which the cells double; iii) stationary phase, during which the growth rate slows down as a result of nutrient depletion and accumulation of toxic products; and iv) death phase, during which bacteria die. The enrichment cultivation generally comprises the most time-consuming part of the whole detection procedure of food-contaminating bacteria. The duration of the cultivation step mostly depends on the length of the bacterial lag-time, which can extend the detection procedure up to one week. Therefore, the word rapid usually refers only to the molecular detection method, and in order to speed up the detection procedure enhanced enrichment cultivation is needed.

The enrichment cultivation procedure depends on the detection methods used. In this study, the enrichment cultivation of food contaminating bacteria was evaluated and improved with respect to the rRNA targeting sandwich hybridization assay (SHA). The case examples of beer-spoiling lactic acid bacteria and food-borne Salmonella Typhimurium were selected for the study, based on their relevance in national and international food industries (Back 2005, Anonymous 2008).

The principle for the SHA-based detection has been described by Rautio and co-workers (2003). The SHA includes four main steps: the binding of the rRNA target to magnetic particles by means of specific oligonucleotide probes, referred to as capture probes; the binding of a second labeled oligonucleotide probe to the capture probe-RNA forming the sandwich; the signal generation via reaction between the label and corresponding enzyme; and the measurement of the signal intensity (fluorescent or electric read-out), or visual evaluation of the color change (colorimetric assay).

SHA has been applied in the detection of bacteria and their harmful metabolites in various samples, and in the quantification of genetic markers for microbial metabolism in bioreactors (Table 1). The most recent applications of SHA include the quantification of collagen prolyl 4-hydroxylase during recombinant production in different expression systems (Osmekhina et al. 2010) and the detection of staphylococcal virulence factor antigens on electric biosensors (Quiel et al. 2010).
Table 1. Applications of SHA in the detection of food contaminants and their metabolites, and measurement of transcriptional levels in cells.

<table>
<thead>
<tr>
<th>Application</th>
<th>Target molecules</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection and identification of microbes or their metabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food-poisoning <em>Bacillus cereus</em></td>
<td>marker DNAs</td>
<td>(Gabig-Ciminska et al. 2005, Liu et al. 2007)</td>
</tr>
<tr>
<td><em>Legionella</em> in water</td>
<td>16S rRNA</td>
<td>(Leskelä et al. 2005)</td>
</tr>
<tr>
<td>Viruses in different samples</td>
<td>capsid proteins</td>
<td>(Los et al. 2005)</td>
</tr>
<tr>
<td><em>E. coli</em>, <em>Pseudomonas aeruginosa</em>, <em>Enterococcus faecalis</em>, <em>Staphylococcus aureus</em>, <em>Staphylococcus epidermidis</em></td>
<td>16S rRNA</td>
<td>(Elsholz et al. 2006)</td>
</tr>
<tr>
<td>Mycobacteria in environmental samples</td>
<td>16S rRNA</td>
<td>(Nieminen et al. 2006, Pakarinen et al. 2007)</td>
</tr>
<tr>
<td>LAB in brewery samples</td>
<td>16S rRNA</td>
<td>This study</td>
</tr>
<tr>
<td><em>Salmonella</em> in minced meat</td>
<td>23S rRNA</td>
<td>This study</td>
</tr>
<tr>
<td>Staphylococcal virulence factors</td>
<td>virulence factor antigens</td>
<td>(Quiel et al. 2010)</td>
</tr>
<tr>
<td>Quantification of changes in metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>different mRNAs</td>
<td>(Pioch et al. 2008)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>different mRNAs</td>
<td>(Jürgen et al. 2005)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>different mRNAs</td>
<td>(Soini et al. 2005, Kemmer &amp; Neubauer 2006, Thieme et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>prolyl 4-hydroxylase</td>
<td>(Osmekhina et al. 2010)</td>
</tr>
<tr>
<td><em>Pichia pastoris</em></td>
<td>different mRNAs</td>
<td>(Resina et al. 2007)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>18S rRNA, SUC mRNA</td>
<td>(Rautio et al. 2003)</td>
</tr>
</tbody>
</table>

Based on the literature, the use of rRNA instead of other nucleic acids as target for the detection is advantageous due to several reasons (Amann & Ludwig 2000). RNA molecules are abundant in growing bacterial cells (Oerther et al. 2000), which contributes to the sensitivity of detection. It has also been shown that in non-growing cells precursor rRNA degrades rapidly and therefore the use of rRNA assures that only viable cells are detected. Additionally, the use of rRNA allows the differentiation between bacteria in species and genus level (Berthier & Ehrlich 1998).
1.2 Aims and outline of the study

The present study aimed to assess the enrichment cultivation of food contaminating bacteria prior to RNA-based SHA and to address bottlenecks in the enrichment cultivation with regard to the accuracy of detection. The final aim of this study was to improve the enrichment cultivation of beer-spoiling LAB and food-borne Salmonella Typhimurium in order to improve the applicability of rRNA SHA and also other alternative detection methods in the microbial quality control.

During the study rRNA-based SHA was applied in the detection of beer-spoiling LAB in brewing process samples, and S. Typhimurium in minced meat in order to assess the enrichment cultivation procedure. The effects of the cultivation conditions and media supplements on the growth of these bacteria in commonly used enrichment broths were studied by means of variable analysis and response surface modeling (RSM). Finally, the relevance of the effects was assessed with respect to the SHA. The work was done in international and national research projects and in close cooperation with the Finnish industry.
2 Literature review

2.1 Detection and enrichment cultivation of beer-spoiling LAB

The heterogenic group of LAB consists of Gram-positive, aerobic to facultative anaerobic, non-sporulating rods and cocci. They are oxidase and benzidine negative, lack cytochromes, and do not reduce nitrates to nitrite (Carr et al. 2002). LAB are divided into homofermentative and heterofermentative, based on their products of sugar fermentation. Homofermentative LAB ferment glucose with lactic acid as the primary product, while heterofermentative LAB produce also acetic acid and carbon dioxide. Most LAB are anaerobic, but some of them can shift to oxygen-dependent metabolism in aerobic conditions (Murphy & Condon 1984, Sedewitz et al. 1984).

LAB consist of a number of genera, from which *Lactobacillus* (*L.*) and *Pediococcus* (*P.*) are the most relevant with regard to the brewing industry (Priest 2003). Among the LAB, *L. brevis*, *L. lindneri* and *P. damnosus* are the most commonly encountered beer-spoilers (Hollerova & Kubizniakova 2001, Priest 2003, Thelen et al. 2006, Suzuki et al. 2008a, Menz et al. 2010). These three species show strong beer-spoiling ability and high tolerance to hop acids, and are often found in the brewing process samples. *L. brevis* grows relatively well in many culture media compared to other generally hard-to-cultivate species of beer-spoiling LAB, which may partially explain its predominance in incident reports for microbiologically spoiled beer products (Suzuki et al. 2008a). *L. lindneri* has been reported to be heat resistant and may survive through sub-optimal pasteurization process (Suzuki et al. 2006a). Novel LAB species occasionally emerge in brewing environments, most recently described including *L. backi* (Bohak et al. 2006), *L. paracollinoides* (Suzuki et al. 2004a), *L. paucivorans* (Ehrmann et al. 2009), *L. rossiae* (Corsetti et al. 2005) and *P. clausenii* (Chaban et al. 2002). At least the first two mentioned species are regarded as beer spoilers (Suzuki et al. 2008a).

The beer-spoiling LAB cause significant losses at the breweries by retarding the fermentation (Narendranath et al. 1997) and spoiling the final products that have a relatively long shelf-life (Back 2005). The ability of bacteria to spoil beer is known to be genetic. Several studies have shown that the hop resistance genes, such as *horA*, are essential for LAB to grow in beer (Sakamoto et al. 2001, Suzuki et al. 2002, Haakensen & Ziola 2008). Besides the hop resistance, the
beer-spoiling bacteria need to tolerate low pH, high concentrations of ethanol and carbon dioxide, and low oxygen tension (Jespersen & Jakobsen 1996).

2.1.1 Detection of beer-spoiling LAB

The main emphasis of microbiological quality assurance at the breweries is on preventive measures (Back 2005). These include process steps in which the raw materials or intermediate products are sterilized by means of heating or filtration, maintenance of hygiene in the process, and increasing the awareness of personnel on the topic. The filling stage has been recognized as a major place of bacterial contamination for beer, mostly due to presence of airborne bacteria (Henriksson & Haikara 1991) and bacterial biofilms (Timke et al. 2005).

In microbiological quality control, breweries mainly rely on classical cultivation methods, followed by phenotypic characterization of isolates. The detection of microbial contaminants in beer usually involves collecting cells from the samples by membrane filtration followed by plating on selective or non-selective media. The shelf-life test samples are incubated for up to six weeks, while the use of concentrated media at an elevated temperature (27–30°C) allows shortening of the incubation time to two weeks. Afterwards, confirmatory tests are carried out for suspected positive samples.

The major limitation of cultivation of beer-spoiling LAB is the long incubation time. The results are rarely available early enough to make decisions about the re-use of the pitching yeast or the release of product. Furthermore, the analysis of naturally turbid process samples on agar media can be difficult. Another drawback of the cultivation-based methods is the possibility of bacteria to enter into a viable but non-culturable state, which may result to false negative results in standard media (Amann et al. 1995, Suzuki et al. 2006a). Despite these disadvantages, cultivation-based methods are widely in use due to their simplicity and sensitivity, their possibility to distinguish between viable and dead cells, and isolation of bacteria for further analyses.

In order to speed up the detection of LAB, various alternative methods have been developed and utilized for use at brewery quality control. Due to low concentrations of LAB in the brewery samples, all of these methods require 24–48 hours of enrichment cultivation prior to detection. The alternative methods are mostly based on nucleic acid targets and antigens. The reported nucleic acid–based methods include hybridization assays, PCR, and other amplification techniques.
Fluorescence in situ hybridization (FISH) has been used for the quantitative detection of beer-spoiling LAB in several studies. FISH is based on the direct detection of bacteria with a species-specific fluorescent probe targeted to rRNA. DNA extraction is not needed since the FISH probes penetrate the target cells, which reduces the hand-on time and duration of the procedure. Thelen and co-workers (2002) showed the applicability of FISH for the detection of LAB in filtered and non-filtered brewery process samples, and for the evaluation of the prevalence of brewery associated LAB (Thelen et al. 2006). The detection limit for the assay is approximately 1,000 cells per mL. Asano and co-workers (2009) combined FISH with microcolony cultivation. Compared to the cultivation of traditional bacterial colonies, the microcolony method could detect considerably smaller colonies, and therefore the duration of the cultivation could be reduced.

The nucleic acid amplification techniques applied for the detection of beer-spoiling LAB include various different PCR-based methods and loop-mediated isothermal amplification (LAMP). The amplification techniques can be linked to other molecular methods in order to increase the sensitivity of the detection. For example, the use of PCR in combination with colorimetric hybridization (Satokari et al. 1998) and electric biosensors (Elsholz et al. 2009) may improve the detection of bacteria.

The majority of the PCR methods for the detection of beer-spoiling LAB rely on either specific marker genes for the spoiling ability (Suzuki et al. 2006b, Haakensen et al. 2007) or on highly conserved 16S rRNA sequences within species (Nakagawa et al. 1994, Suzuki et al. 2004b, Haakensen et al. 2008a, Juvonen et al. 2010). Selected examples of the used marker genes are presented in Table 2. Nowadays traditional end-point PCR detection has been mostly substituted by faster or more accurate techniques. These include fluorescent real-time PCR, such as LightCycler® (Roche Diagnostics, Mannheim, Germany), based on real-time fluorescent quantification of PCR products; and reverse transcriptase PCR (RT-PCR) which targets RNA instead of DNA and therefore allows the distinction between viable and dead cells (Juvonen et al. 2010). Loop-mediated isothermal amplification (LAMP), in which the amplification reaction takes place in isothermal conditions, has been applied for the detection and identification of beer spoilage Lactobacillus and Pediococcus (Tsuchiya et al. 2007).
### Table 2. Selected marker genes for the beer-spoiling ability of LAB.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
<th>References</th>
</tr>
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<tr>
<td>bsrA, bsrB</td>
<td>Beer-spoiling related genes in <em>Pediococcus</em> sp. Function not yet known.</td>
<td>(Haakensen et al. 2009a)</td>
</tr>
<tr>
<td>gyrB</td>
<td>Gene related to isohumulone resistance.</td>
<td>(Nakakita et al. 2003)</td>
</tr>
<tr>
<td>hitA</td>
<td>Gene encoding hop-inducible cation transporter. Putative divalent cation transporter that counteracts the hop-induced depletion of intracellular manganese.</td>
<td>(Back 1980, Hayashi et al. 2001)</td>
</tr>
<tr>
<td>ORFB1-B5</td>
<td>Open reading frame regions surrounding horA gene. Generally conserved in beer-spoilage <em>Lactobacillus</em> strains.</td>
<td>(Suzuki et al. 2004c)</td>
</tr>
</tbody>
</table>

Imunoassays for the detection of beer-spoiling LAB utilize specific antibodies that bind to marker antigens on bacterial cell surfaces and combination of them with for example fluorescent or luminescent labels (Whiting et al. 1992, Yasui & Yoda 1997, Ziola et al. 2000, March et al. 2005). The current trends in the field of immunoassays include the use of multiple monoclonal antibodies to marker antigens related with beer-spoiling ability for simultaneous detection of several beer-spoiling bacteria (Tsuchiya et al. 2000, Nakakita et al. 2002).

#### 2.1.2 Enrichment media for beer-spoiling LAB

The enrichment cultivation of LAB at breweries commonly takes place at the temperature of 29 ± 1 °C under anaerobic conditions. The enrichment media include various solid and broth media (Jespersen & Jakobsen 1996, Carr et al. 2002, Suzuki et al. 2008a), from which only a few are nowadays used in combination with molecular detection methods.

The most frequently used media for enrichment cultivation of beer-spoiling LAB include De Man-Rogosa-Sharpe broth (MRS)(de Man et al. 1960) and Nachweismedium für Bierschädliche Bakterien (NBB)(Dachs 1981, Back et al. 1984, Hammes et al. 1992, Holzapfel 1992). Their compositions are presented in Table 3. Both media have been used in combination with various detection methods, such as PCR and FISH (Juvonen et al. 1999, Takahashi et al. 2000,
The common ingredients in MRS and NBB include i) beef extract, yeast extract and glucose as sources for vitamins, nitrogen, amino acids, and carbon; ii) polysorbate 80 as an emulsifier and iii) the inclusion of salts for buffering and growth factors. Sodium acetate is used in MRS as a growth factor for LAB (de Man et al. 1960). The pH of MRS is 6.5±0.2. NBB contains casein digest and maltose as additional sources for energy. It also contains L-cysteine as an antioxidant, L-malic acid as a growth factor for LAB, and chlorophenol red as a pH indicator. The pH of NBB is 5.8±0.2. Concentrated NBB (NBB-C) and MRS broths are recommended for the forcing test in EBC Analytica Microbiologica (Hage et al. 2005).

The popularity of MRS is based on its ability to support the growth of a large variety of LAB, and its use in several generally accepted standard protocols (Crumplen et al. 1991, Brewery Convention of Japan 1999, Hage et al. 2005). The supplementation of MRS with beer (Beer-MRS, B-MRS) has been shown to benefit the enrichment of brewery strains (Holzapfel 1992, Storgårds et al. 1998, Juvonen et al. 1999). In B-MRS the concentration of beer is 20–25% and the pH is approximately 5.5.

In order to further improve the growth of beer-spoiling LAB, MRS has been modified by adding fructose (Juvonen et al. 1999), L-cysteine-HCl (Arroyo et al. 1994), and mevalonic acid (Hammes et al. 1992, Difco Laboratories 1995), substituting glucose with mannose cellobiose or salicin (Simpson & Taguchi 1995), and decreasing pH (Garvie 1984, Raccach 1987, Holzapfel 1992). Advanced Beer Detection -medium (ABD)(Suzuki et al. 2008b) is based on the adjustment of the concentrations of components and decreasing the pH of B-MRS broth. ABD has been successfully used in combination with FISH (Asano et al. 2009).
Table 3. Composition of MRS and NBB broths (per L).

<table>
<thead>
<tr>
<th>Component</th>
<th>MRS</th>
<th>NBB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>8 g</td>
<td>Beef extract 2 g</td>
</tr>
<tr>
<td>Casein peptone</td>
<td>10 g</td>
<td>Pancreatic digest of casein 5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4 g</td>
<td>Yeast extract 5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g</td>
<td>Glucose 15 g</td>
</tr>
<tr>
<td>Polysorbate 80 1 g</td>
<td></td>
<td>Polysorbate 80 0.5 g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5 g</td>
<td>Potassium acetate, 6 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>2 g</td>
<td>Disodium phosphate 2 g</td>
</tr>
<tr>
<td>Diammonium hydrogen citrate</td>
<td>2 g</td>
<td>Chlorphenol red 0.07 g</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.2 g</td>
<td>L-cysteine hydrochloride 0.2 g</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>0.05 g</td>
<td>L-malic acid 0.5 g</td>
</tr>
<tr>
<td>Final pH</td>
<td>6.2±0.2 (25 °C)</td>
<td>Final pH 5.8±0.2 (25 °C)</td>
</tr>
</tbody>
</table>

2.2 Detection and enrichment cultivation of food-borne Salmonella

Salmonella is a genus of rod-shaped, Gram-negative, non-spore forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5 µm, lengths from 2 to 5 µm, with peritrichous flagella (Madigan et al. 2009). They obtain their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Also, most species produce hydrogen sulfide. The optimum temperature for growth of Salmonella is between 35 to 37 °C, but growth has been observed at temperatures ranging from 5 °C to 47 °C. The optimum pH is near 7.0, but growth may occur between pH 4.0 and pH 9.0.

The genus Salmonella consists of two species, S. bongori and S. enterica, which currently include some 2,500 serotypes (Popoff et al. 2001). Among Salmonella, only S. enterica subspp. enterica is considered clinically significant for humans. The virulence of Salmonella requires the expression of numerous genes and it has been shown that serotypes that do not possess the invABC genes are unable to invade mammalian cells (Galan & Curtiss 1991). In Finland, the majority of salmonellosis incidents are caused by S. enterica serotypes S. Enteritidis and S. Typhimurium, from which the latter is the most common cause for the domestic infections (Anonymous 2008). Food borne salmonellosis is an important public health problem worldwide. The annual cost of salmonellosis in the USA has been estimated to be over $2,600 million (Economic Research Service 2010) and accordingly, Salmonella has been considered as a major public
health risk (Voetsch et al. 2004). The major sources of salmonellosis outbreaks include eggs, meats and dairy products (EFSA 2008).

2.2.1 Detection of food-borne Salmonella

The detection of Salmonella in foods faces with several challenges, such as the low concentration of target bacteria in foods, sub-lethally injured bacteria, and the high amount of other bacteria and inhibitory food components in samples. Furthermore, the legislative demand, the absence of Salmonella cells in 10–25 g of food, sets strict performance requirements for methods of analysis (Anonymous 2005a). The important characteristics of the detection method include the speed of analysis, ease of use, the analytical performance, possibility for automation, and reduction of total cost. In addition, other factors such as robustness, reliability, through-put, overall convenience, and the level of validation and standardization affect the applicability of the method.

The traditional confirmation and identification procedures for Salmonella spp. are usually based on preliminary identification based on the colony appearance on chromogenic and other selective agar media, followed by confirmation using classical biochemical and serological testing. The biochemical tests include fermentation of glucose, negative urease reaction, lysine decarboxylase activity, H₂S production, and fermentation of dulcitol. Serological confirmation tests typically use antisera for detection of flagellar (H) and somatic (O) antigens. Isolates with a typical biochemical profile, which agglutinate with both H and O antisera are identified as Salmonella spp. Positive isolates are often sent for further serotyping and identification using techniques such a phage typing, antibiotic susceptibility and pulsed-field gel electrophoresis (PFGE).

In the EU, the reference detection methods are published by the International Organization for Standardization (ISO). The reference procedure for the detection of food borne Salmonella includes non-selective enrichment cultivation in buffered peptone water (BPW) broth for 16–20 hours, selective enrichment cultivation in two selective media and incubation on two different selective agar plates for isolation of colonies (Anonymous 2002a). The colonies are then identified by means of biochemical tests. This procedure takes at least 72 hours to complete. In the United States (US), the government publishes the Bacteriological Analytical Manual (BAM) which includes a collection of recommended procedures for the detection of pathogens and toxins in foods. The BAM procedure for the detection of food-borne Salmonella includes non-selective
enrichment cultivation in nutrient broth (NB) for 16 hours and selective enrichment cultivation in either Rappaport–Vassiliadis (RV) or tetrathionate brilliant green (TBG) broth for an additional 16 hours (Andrews & Hammack 2007). The colonies are isolated by using selective agar plates and identified biochemically.

Also, various rapid confirmation methods have been reported for *Salmonella*, and a large number of them are commercially available. *Salmonella* rapid test and screening kits range from complex procedures including sophisticated techniques, such as thin agar underlay method or immunomagnetic separation, to simple lateral flow assays incorporating immunochromatographic technology. To date over 30 alternative *Salmonella* detection methods have been validated by the European validation and certification bodies such as Association française de Normalisation (AFNOR), the Nordic system for validation of alternative microbiological methods (NordVal), and MicroVal. The majority of these tests are based on either immunoassays or PCR (Anonymous 2010a, Anonymous 2010b, Anonymous 2010c). The Association of Official Analytical Chemists (AOAC) is a globally recognized, non-profit association that provides tools for development of the voluntary consensus standards. Since 2009, MicroVal and AOAC have worked together in order to combine their requirements for the certification and performance testing.

The immunoassays for *Salmonella* are mainly based on the heterogeneous non-competitive ELISA format where the target cells are bound between antibodies directed against H or O antigens (Cudjoe *et al.* 1995, Valdivieso-Garcia *et al.* 2001, Magliulo *et al.* 2007). The detection limits of *Salmonella* in foods with immunoassays range between $10^4$ and $10^6$ cells per assay. Immunoassays are generally robust and do not require extensive sample preparation. However, antibodies may cross-react with antigens in closely related bacteria, while showing low reactivity with some *Salmonella* serotypes. The commercially available immunoassays for the detection of *Salmonella* include both manual lateral flow devices (such as DuPont Lateral Flow System by Qualicon, US) and fully automated immunoanalyzers (such as VIDAS by bioMérieux, France).

More than 30 *Salmonella* specific genes have been utilized for different PCR applications (Levin 2009). These target genes include rRNA genes, genes coding for toxins and enzymes, and repetitive elements. Especially rRNA genes are prominent targets because the widely available information on rRNA sequences, and the possibility for differentiation at for example species or strain levels. Also,
the use of virulence genes, such as invA, allows also the confirmation of suspected Salmonella isolates. PCR together with enrichment cultivation has been applied for the detection of Salmonella in various food matrices, such as eggs (Malorny et al. 2007, Chen et al. 2010) and different meats (O'Regan et al. 2008, McGuinness et al. 2009). However, in many cases the methods have not been validated properly against the reference method, and the performances of the assays can therefore have significant variations. Most of the PCR methods which are in routine use provide results in approximately 24 hours, and the detection limits are in the range of $10^3$–$10^4$ cells per mL. A current trend in PCR diagnostics is the use of multiplex assays for simultaneous detection of several specific targets.

Other nucleic acid–based detection methods for food-borne Salmonella include the isothermal nucleic acid sequence-based amplification (NASBA), LAMP, and different hybridization assays. Probably the most widely used of these methods is fluorescence in situ hybridization (FISH) that has been reported to allow the detection within less than 24 hours in various food matrices (Fang et al. 2003, Almeida et al. 2010). The combination of hybridization with PCR can increase the sensitivity of detection (Feder et al. 2001, Thompson et al. 2006).

The bacteriophage assays for detection of Salmonella utilize either the gene transfer between the bacteriophage and the host cells or the lysis of bacteria by the bacteriophages. The transfer of luminescent or fluorescent genes from the bacteriophage to the host cell allows the measurement of appropriate signal intensity (Chen & Griffiths 1996, Mosier-Boss et al. 2003). The detection using lytic bacteriophages includes the infection of the host cells and the measurement of the level of the lysis (Favrin et al. 2001). Bacteriophages can also be used as probes coupled with biosensors. For example, Olsen and coworkers developed an acoustic wave biosensor with bacteriophages as probes. This biosensor is claimed to detect as low as 100 Salmonella cells per mL of sample (Olsen et al. 2006). However, the use of bacteriophages may also cause false negative results due to the exclusion of Salmonella serotypes.

### 2.2.2 Enrichment media for Salmonella

The enrichment media for food-borne Salmonella can be divided into non-selective and selective media. Since the molecular detection is usually done after cultivation in broth media, the solid media are not described here. The most frequently used non-selective enrichment broth is buffered peptone water (BPW).
A universal pre-enrichment broth (UPB) and nutrient broth (NB) are also widely used, although they are not common in the validated alternative methods. The use of these listed media is based on the maximal recovery of small amounts of sub-lethally injured cells (de Boer 1998). The drawback of the non-selective broths is that they also support the growth of various other microbes. The non-selective broths typically contain only peptones as sources of nitrogen, carbon, vitamin, and minerals. BPW and UPB also contain sodium chloride for maintaining the osmotic balance and phosphates for buffering. In addition to those, UPB contains salts as ion sources and glucose as an energy source. The pH of the non-selective media is initially adjusted near to 7.0.

A variety of enrichment media have been developed and evaluated for the selective isolation of *Salmonella*. These include the Rappaport-Vassiliadis soy broth (RVS) (Rappaport et al. 1956, van Schothorst & Renaud 1983, van Schothorst & Renaud 1985), selenite cystine broth (SC) (Leifson 1939), tetrathionate broth (TT)(Müller 1923), tetrathionate brilliant green broth (TGB), and the Muller-Kauffmann tetrathionate novobiocin broth (MKTTn)(Müller 1923, Kauffmann 1930, Kauffmann 1935). Summary of selectivity agents used for the cultivation of *Salmonella* is presented in Table 4.

RVS has been shown to be superior in the selective enrichment cultivation of *Salmonella* in several studies (van Schothorst & Renaud 1983, Rhodes et al. 1985, Maijala et al. 1992, June et al. 1995, Schönenbrucher et al. 2008) and it is included in the ISO standard method (Anonymous 2002a). The selectivity of RVS is based on the ability of *Salmonella* to tolerate relatively high osmotic pressure and low pH, as well as high concentrations of malachite green and magnesium chloride. However, other enteric bacteria are still able to grow in RVS (Krascsenicsova et al. 2006). The enrichment media used in some examples of alternative methods are summarized in Table 5.

The reported performances of *Salmonella*-selective media are controversial. However, following conclusions are obtained in the majority of studies: i) none of the developed media is 100% selective for *Salmonella*; ii) the selectivity of medium depends on the characteristics and concentration of background microbes (Beckers et al. 1987, Chen et al. 1994); iii) a certain minimum concentration of cells is required for *Salmonella* to survive in the selective conditions (Chen et al. 1993). Currently, a major trend is to develop enrichment media for the simultaneous isolation of several pathogenic bacteria; for example selective enrichment broths SEL and SSL may be used for detection of both *Salmonella* and *Listeria* (Kim & Bhunia 2008, Yu et al. 2010). However, simultaneous
detection of pathogens may constitute a risk of overgrowth of certain bacteria (Besse et al. 2010) which can in the worst case lead to false negative results.

Table 4. Summary of the selectivity agents used in the enrichment cultivation of food-borne Salmonella and media in which they are used.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Media</th>
<th>Target microbes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acriflavine</td>
<td>SEL</td>
<td>Several bacteria</td>
<td>(Kim &amp; Bhunia 2008)</td>
</tr>
<tr>
<td>Bile salts</td>
<td>MKTTn, RVS, TGB, TT</td>
<td>Gram-positive bacteria</td>
<td>(Müller 1923, Kauffmann 1930, Kauffmann 1935)</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>MKTTn, RVS, TGB</td>
<td>Gram-positive bacteria, Gram-negative bacilli</td>
<td>(Kauffmann 1930, Kauffmann 1935)</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>SEL</td>
<td>Eukaryotic cells</td>
<td>(Kim &amp; Bhunia 2008)</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>SEL</td>
<td>Several bacteria</td>
<td>(Kim &amp; Bhunia 2008)</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>SSL</td>
<td>Several bacteria</td>
<td>(Yu et al. 2010)</td>
</tr>
<tr>
<td>Malachite green</td>
<td>RVS</td>
<td>Several bacteria</td>
<td>(Rappaport et al. 1956)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>MKTTn</td>
<td>Other Enterobacteria, some Gram-negative bacteria</td>
<td>(Kim &amp; Bhunia 2008, Yu et al. 2010)</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>MKTTn</td>
<td>Proteus</td>
<td>(Kauffmann 1930, Kauffmann 1935, Restaino et al. 1977)</td>
</tr>
<tr>
<td>Potassium tellurite</td>
<td>SSL</td>
<td>Coliforms and intestine bacteria</td>
<td>(Yu et al. 2010)</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>SC</td>
<td>Gram-positive bacteria, coliforms</td>
<td>(Leifson 1939)</td>
</tr>
<tr>
<td>Tetrathionate</td>
<td>MKTTn, TGB, TT</td>
<td>Coliforms and intestine bacteria</td>
<td>(Müller 1923)</td>
</tr>
</tbody>
</table>
Table 5. Summary of the enrichment cultivation broths included in examples of validated alternative methods for the detection of food-borne *Salmonella*. In case that the enrichment procedure depends on the food type, the procedures for meat/meat products are presented. A – AFNOR, N – NordVal, BPW – buffered peptone water, RVS – Rappaport Vassiliadis Soy broth, BHI – brain heart infusion broth, TGB – tetrathionate brilliant green broth.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-selective</td>
<td>Selective</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoassays</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxoid Salmonella Rapid Test (OXOID Thermofisher Scientific)</td>
<td>BPW 18 35-38</td>
<td>SRTEM 1 24 40.5-41.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>RAPIDCHEK™ SELECT™ (Strategic Diganostics Inc.)</td>
<td>16-22 40.5-42.5</td>
<td>6-8 40.5-42.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>RayAI Salmonella assays (RayAI)</td>
<td>BPW 16-20 36-38</td>
<td>RVS 18-24 40.5-42.5</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>RIDASCREEN® Salmonella (R-Biopharm AG)</td>
<td>BPW 16-20 37</td>
<td>-</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>TAG 24 Salmonella (BioControl Systems)</td>
<td>BPW 14-20 36-38</td>
<td>BHI 4 4-5 40.5-42.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Tecra Salmonella Ultima/Unique (TECRA INTERNATIONAL Pty Ltd)</td>
<td>BPW 16-20 36-38</td>
<td>RVS 18-24 40.5-42.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>TRANSIA® Plate Salmonella Gold (BioControl Systems)</td>
<td>BPW 16-20 36-38</td>
<td>RVS 18-24 40.5-42.5</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>ViDAS Easy Salmonella (BioMerieux)</td>
<td>BPW 16-22 36-38</td>
<td>SX2 22-26 40.5-42.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>PCR-based methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADIAFOOD Salmonella (AES Chemunex)</td>
<td>BPW 18-24 36-38</td>
<td>BHI 2-4 35-38</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Assurence GDS Salmonella (BioControl Systems)</td>
<td>BPW 16-20 36-38</td>
<td>-</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>BAX Salmonella (DuPont Qualicon)</td>
<td>BPW 16-20 36-38</td>
<td>-</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>GeneDisc Salmonella spp. (Pall GeneSystems)</td>
<td>BPW 16-20 36-38</td>
<td>-</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>IQ-Check™ Salmonella II (BIO-RAD)</td>
<td>BPW 20-22 36-38</td>
<td>-</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>TAQMAN Salmonella (Applied Biosystems SA)</td>
<td>BPW 16-20 36-38</td>
<td>-</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Hybridization-based methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GeneQuence Salmonella (Neogen Corporation)</td>
<td>NB or BPW 18-24</td>
<td>RVS+TBG 22-26 42.5</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Lumiprobe 24 Salmonella (EUROPROBE SA)</td>
<td>RM 6-8 36-38</td>
<td>RVS 17-19 40.5-42.5</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

1 *Salmonella* Rapid Test Elective Medium, included in the test kit, 2 Primary and secondary enrichment broths utilizing *Salmonella*-specific bacteriophages are included in the test kit, 3 Also validated by MicroVal, 4 Specific TAG supplement included in the test kit is added, 5 Selective enrichment in parallel in RVS and TBG.
2.3 Important factors in the selection of the enrichment procedure

Although the standard media for the enrichment of food contaminating bacteria are widely accepted and in some cases also tightly regulated, it has been reported that the use of case-specific enrichment media could be beneficial (Besse et al. 2010). The factors that should be taken into consideration in selection of the media include the regulations laid by the authorities, the sample matrix, the concentration of the background microbiota in the sample, the concentration and the physiological condition of the target bacteria, and the applied detection method. Additionally, the applicability and the cost-efficiency of the selected enrichment procedure should be at an acceptable level.

2.3.1 Regulations

Beer-spoiling LAB are not known to be pathogenic to human, and therefore their detection is not regulated by the authorities. However, there are associations that give guidelines for the efficient microbial quality control at the breweries. These include the European Brewing Convention (EBC), American Society of Brewing Chemists (ASBC) and the Brewery Convention of Japan (BCOJ). Due to the long process time and the shelf-life of beer, it is generally regarded that even a low level of spoilage microbes constitutes a risk (Jespersen & Jakobsen 1996). Therefore, the microbiological guidelines suggest for unpasteurized beer a range between 0 and 50 cells in 100–250 mL sample volume (Jespersen & Jakobsen 1996, Back 2005). In pitching yeast and in process samples before beer filtration, a single spoilage organism in $10^6$–$10^8$ cultivation yeast cells should be detected.

The detection of food-borne *Salmonella* is essential for the safety of the consumers. Therefore, the use and validation of the monitoring procedures are regulated by the authorities. In the EU, the regulations are given by the European Commission (Anonymous 2005a). In Finland, the National *Salmonella* control program has been applied since 1995 to protect consumers against *Salmonella* infections spread through foods of animal origin (Anonymous 1994). Due to a low prevalence of *Salmonella* in Finland and Sweden, each batch of meat, minced meat and eggs imported to Finland and Sweden must be tested for *Salmonella* using either the ISO reference method (Anonymous 2002a) or an alternative method that has been compared against the ISO method, validated according to the ISO 16140 method and certified by a third party (Anonymous 2002b).
2.3.2 Sample matrix

The composition of the enrichment broth and the cultivation conditions should be selected based on the sample matrix. Firstly, food components can hinder or slow down the nutrient transfer in the enrichment culture, and thereby affect the growth of bacteria. Food particles may also challenge the pre-processing of the samples, or inhibit the growth of target bacteria. Additionally, the enrichment cultivation may be needed for the removal or dilution of food components that might hamper molecular detection.

Effect on the pre-processing methods

Pre-processing of food samples usually aims to increase the concentration of target bacteria prior to enrichment cultivation (pre-concentration) and change the structure of the sample (homogenization). The increase in the concentration of target cells may shorten the lag-time of the cells in the enrichment culture and fasten the detection, while the removal of food particles allows decreasing the cultivation volume. Pre-processing may also remove inhibitory components that could hamper the detection. In the food industry, common applications of pre-processing techniques include for example filtration of beer samples, removal of yeast cells by means of differential centrifugation (Whiting et al. 1992), and immunomagnetic separation (IMS) of Salmonella cells from enrichment cultures prior to detection (Cudjoe et al. 1995, Hagren et al. 2008, Moreira et al. 2008).

The use of pre-concentration methods is sometimes hampered due to sample components. Examples include clogging of membranes, co-flocculation of bacteria with sample components in differential centrifugation, or non-specific binding of immunomagnetic beads with other than target proteins (Payne & Kroll 1991, Stevens & Jaykus 2004a). According to Asano and co-workers (2007), the membrane filtration of brewery samples may cause false negative results in detection due to passing of bacteria that have changed their morphology during the adaptation in the environment.

When the use of pre-concentration methods is not possible, the concentration of bacteria in the sample remains low and the enrichment is more challenging. In such cases the structure of the sample material may be changed by homogenization. The attachment to the food particles has been shown to improve the survival of Salmonella under stressful conditions (Kinsella et al. 2007), and homogenization may be needed for the separation of attached cells from the food
particles. However, it may cause excessive elution of PCR inhibiting food components (Kanki et al. 2009).

**Inhibition of growth by food compounds**

Several food components are known to be antibacterial. These components can cause the induction of so called viable but not-culturable status of the target bacteria and thereby lead to failure in the enrichment cultivation. For example, the growth of *Salmonella* has been shown to be inhibited by site metabolites of LAB (Rubin et al. 1982, Alakomi et al. 2007). Muniesa and co-workers (2005) showed that the enrichment cultivation of contaminating bacteria can also fail due to bacteriophages in food samples. The inhibitory effects of certain components may vary in the sample and in the enrichment broth (McCann et al. 2005), which can lead into false negative results in the detection. The effect of inhibitory compounds can be reduced by sufficient dilution of samples for the enrichment cultivation (Ramnani et al. 2010).

**Inhibition of molecular detection**

Molecular detection methods may be inhibited by certain food components. Especially biosensor-based detection tools have been reported to have a low tolerance for interference with contaminating food particles (Gomez et al. 2001, Radke & Alocilja 2005, Terry et al. 2005). Also, several sources report the inhibition of PCR detection by food components (Rossen et al. 1992, Rådström et al. 2003, Chen et al. 2010). The use of internal amplification controls in PCR enables the recognizing of false negative results originating from inhibitory compounds (Juvonen & Haikara 2009, Chen et al. 2010).

**2.3.3 Background microbiota**

Enrichment cultivation might be needed in order to reduce background microbiota. Nonpathogenic microbes present in the foods can overgrow the target bacteria or hamper their detection. For example, meat products carry LAB that have been shown to inhibit the growth of enteric bacteria (Vold et al. 2000). Some *Listeria monocytogenes* strains have been reported to be outcompeted by other strains within the species (Besse et al. 2010) or even different lineages of one strain (Bruhn et al. 2005). Singer and co-workers (2009) showed that the cultivation
media can select preferentially for specific Salmonella strains and decrease the overall sensitivity of Salmonella detection. Furthermore, elevated initial concentrations of certain serotypes did not necessarily increase the probability for their detection. Brewery samples often include pitching yeast and wild yeast cells that may challenge the enrichment cultivation and detection of LAB (Priest 2003).

In order to suppress the growth of background microbiota, the selectivity of the medium can be increased by the addition of antibiotics or selective growth factors, or by adjusting the pH and the temperature. In the case of beer-spoiling LAB, antibiotics are usually added into the medium. Cycloheximide and 2-phenylethanol are commonly used for the inhibition of yeasts and gram-negative bacteria, respectively. Supplementing media with beer or its components supports the growth of beer-spoiling bacteria and suppresses the growth of other bacteria present (Holzapfel 1992, Suzuki et al. 2008b, Haakensen et al. 2009b). The bacteriocins produced by some LAB can increase the selectivity of the enrichment cultivation.

The enrichment cultivation of Salmonella is usually carried out in two steps, of which the latter takes place in selective media. The reports on the necessity of the selective cultivation are controversial. Several detection methods allow the detection of Salmonella directly after cultivation in non-selective media (Löfström et al. 2009, Tatavarthy et al. 2009, Matías et al. 2010, Chen et al. 2010). However, several case studies have shown that selective enrichment cultivation enhances the accuracy of molecular detection (Myint et al. 2006, Upadhyay et al. 2010). The performance of selective media varies depending on the target strain (Huang et al. 1999, Besse et al. 2010).

The use of selective media is not advisable if the concentration of the cells is low (Chen et al. 1993). Therefore, usually a non-selective enrichment cultivation period is preferred at the beginning of analysis. On the other hand, the introduction of some selectivity in the non-selective enrichment cultivation has been shown to prevent from the overgrowth of the background microbiota. Antibiotics, such as novobiocin or malachite green, might significantly reduce the amount of background bacteria and therefore, benefit the recovery of Salmonella (van Schothorst & Renaud 1985, Jensen et al. 2003). However, the addition of antibiotics at the early stage of enrichment cultivation also causes stress to Salmonella cells and can therefore risk their recovery (Chen et al. 1993). Test kit manufactured by Strategic Diagnostics Inc. (RAPIDCHEK™ SELECT™) utilizes selective bacteriophages for the suppression of the background microbiota.
and thereby improves enrichment cultivation of *Salmonella* (Stave & Teaney 2009).

Techniques that allow a release of selective supplements in the non-selective culture have been utilized for the cultivation of *Salmonella*. Sveun and Harman (1977) used wax-coated gelatin capsules for a gradual release of iodine and selenite into a non-selective broth. So called solid-repair methods have been used for the two-step recovery of injured bacteria (Kang 2002). Initially the recovery takes place inside or on top of the non-selective agar plate. The selective agents are then diffused to the non-selective agar, changing the cultivation conditions to selective. Disadvantages of solid-repair methods include formation of small colonies that are difficult to pick under the selective medium layer, and the negative effect of the melted selective overlay agar on the injured cells. Additionally, the direct pour-plating of the sample also incorporates inhibitors present in the food sample into the culture, which may negatively influence the enrichment procedure.

Oxoid Inc. markets a test that incorporates the use of special growth facilitators for an improved recovery of injured cells, followed by the timed release of selective agents into the recovery medium. The method has been shown to improve the rate of detection of low numbers of injured *Salmonella* cells after 24 hours of enrichment cultivation (Baylis *et al.* 2000a, Baylis *et al.* 2000b). Panula-Perälä and co-workers (2008) developed a method for the controlled release of glucose into the cultivation medium. The method is based on enzymatic degradation of glucose from polymeric materials, in either gel or soluble form. Commercial products based on the method (EnBase®, BioSilta Oy, Finland) have been used to enhance the protein production in *E. coli* (Krause *et al.* 2010, Glazyrina *et al.* 2010, Siurkus *et al.* 2010).

### 2.3.4 Concentration and condition of target cells

The concentration and physiological condition of the bacterial cells have been shown to affect their recovery in laboratory media. Harvey and Price (1975) showed that the performance of different medium brands can vary depending on the inoculation concentration of *Salmonella*. The effect of the inoculation concentration on the lag-time has been shown for example for *L. plantarum* (Smelt *et al.* 2002), *Salmonella* Typhimurium (Stephens *et al.* 1997) and *Listeria monocytogenes* (Besse *et al.* 2006).
Food-borne bacteria may be injured due to food processing and handling procedures, such as thermal treatment, refrigeration, freezing, drying, irradiation, from exposure to preservatives, acidity, and low water activity, or from being starved. The presence of injured cells needs to be considered when enrichment cultivation conditions are selected (Shintani 2006). Moreover, different stress factors can cause large variations in the lag-time between individual cells (Mackey & Derrick 1984). When the concentration of cells is low the effect of stress factors on the bacterial lag-time tends to increase (Shida et al. 1975b). It is known that sub-lethally injured cells become temporarily susceptible to many selective compounds in the media, and that they do not repair or multiply in the presence of the selective compounds (Busta 1976). Therefore, the primary recovery should take place in non-selective conditions.

The enrichment cultivation of *Salmonella* is usually challenging due to the poor physiological status of the cells. Sub-lethal injury of *Salmonella* cells has been reported to cause changes in the metabolism leading to for example degradation of ribosomal DNA and RNA (Tomlins & Ordal 1971, Gomez & Sinskey 1973, Gomez et al. 1976, Chambliss et al. 2006). This can lead in failure in recovery of the cells, especially on nutrient rich media (Clark & Ordal 1969, Gomez et al. 1973). The tolerance of *S. enterica* against different stress factors varies between serotypes (Sherry et al. 2004).

**Solutions for improved recovery**

The recovery of injured cells can be improved using repair methods, based on either solid or liquid media. These methods have been recently reviewed in detail (Wu 2008). A liquid-repair method for *Salmonella* cultivation reported by Kang and Siragusa (2001) includes a two-fold serial dilution of samples in a 96-well microtiter plate containing BPW, followed by incubation at 37 °C for 3 hours for resuscitation of sub-lethally injured cells. After that an equal volume of double strength selective broth is added to each well, and the cultures are further incubated for 13 hours at 37 °C in the dark. Solid-repair methods that are used in the release of selective agents in the non-selective culture (see previous chapters) have been reported to improve the recovery of injured bacteria, including *S. Typhimurium* (Kang & Fung 2000, Wu & Fung 2006).

The use of growth factors, such as iron supplements in the medium can improve the recovery of sub-lethally injured cells (Gast & Holt 1995). Siderophores, high-affinity iron chelating compounds secreted by bacteria, have
been shown to improve the recovery of injured bacteria in an enrichment cultivation. Ferrioxamines are selective siderophores that have been used for resuscitation of injured Salmonella (Reissbrodt et al. 1996, Reissbrodt et al. 2000). Some Salmonella cells may even be non-recoverable without the addition of such sole iron supplement (Thammasuvimol et al. 2006).

Since magnesium is necessary for the recovery of injured bacteria, the addition of magnesium sulfate may be beneficial in enrichment cultivation (Hurst 1977). Tween 80 is an emulsifier, that has been shown to increase the recovery of injured bacteria by repairing damaged cells membranes (Murthy & Gaur 1987). The use of antioxidants (see next chapter) may also contribute to the recovery of injured bacteria.

2.3.5 Stress factors in enrichment media

The effect of low concentration and poor physiological condition of the target cells on enrichment cultivations are further increased if the growth conditions in the sample and the media are different (Gomez & Sinskey 1973, Gomez et al. 1973, Zwietering et al. 1991, Zwietering et al. 1994, Suzuki et al. 2008b, Haakensen et al. 2009b). The recovery and the lag-time of especially injured bacteria depends largely on the cultivation conditions. Important factors include the osmolarity, i.e. the concentration of nutrients and salts (Clark & Ordal 1969, Shida et al. 1975a, Shida et al. 1975b, Chambliss et al. 2006); pH (Shida et al. 1975b, Chambliss et al. 2006); oxygen concentration (Lushchak 2001); and temperature (Shida et al. 1975b, Zwietering et al. 1994, Mattick et al. 2001). The stress responses of bacteria can vary between species and even within the species (van de Guchte et al. 2002, Alegria et al. 2004, Sherry et al. 2009).

Osmotic stress

Changes of nutrient and salt concentrations in the medium can cause osmotic stress to bacteria. Osmotic stress can affect both lag-time and growth rate in bacterial cultures (Shida et al. 1975a, Shida et al. 1975b). Viable Salmonella cells tolerate high osmolarity relatively well compared to other Enterobacteriaceae. Therefore, elevated salt concentration may be used to suppress the growth of competing bacteria in enrichment cultivation of food-borne Salmonella (van Schothorst & Renaud 1983). However, sub-lethal injury of cells reduces the tolerance to high osmolarity (Gomez & Sinskey 1973), and therefore the use of
media with low osmolarity is recommended for the recovery of *Salmonella* in foods (Clark & Ordal 1969, Gomez *et al.* 1973).

**Acidic and alkaline stresses**

The stress caused by a sudden decrease or increase in pH is called acidic or alkaline stress, respectively. Bacteria can tolerate acidic and alkaline stresses by applying various mechanisms (Humphrey *et al.* 1993, Flahaut *et al.* 1997), but in the case of enrichment cultivation pH shifts can extend the lag-time of the culture (Cheroutre-Vialette *et al.* 1998), and even cause the death of individual cells. The appropriate pH of the enrichment broth depends therefore on the pH of the sample; in case of the beer-spoiling LAB the inoculation from beer to broth with neutral pH causes alkaline stress. Besides during inoculation, pH may shift also during the enrichment cultivation; the metabolites produced by bacteria often decrease the pH in the bacterial cultures. Therefore, the buffering capacity of enrichment medium should be sufficient to slow down the acidification.

**Oxidative stress**

Oxidative stress is a condition in which the production of reactive oxygen species results in adverse effects on bacteria. Formation of reactive oxygen radicals in the broth media has been shown to affect the recovery of injured bacteria (Mizunoe *et al.* 1999). The major enzymes protecting the bacterial cells against reactive oxygen species are superoxide dismutase, catalase, and peroxidases (Morris 1977). Although the majority of LAB are catalase negative, some strains are able to degrade hydrogen peroxide through the action of specific heme-dependent catalases (An *et al.* 2010b) or superoxide dismutases (Götz *et al.* 1980a). The activity of enzymes involved in degradation of reactive oxygen species in bacteria can diminish due to sub-lethal injury, which may explain the toxic effects of relatively low peroxide levels in enrichment media (Andrews & Martin 1979, Kobayashi *et al.* 2005).

The addition of antioxidants into the enrichment medium has been shown to improve the growth of both LAB and *Salmonella*. The recovery of beer-spoiling LAB may be facilitated by addition of L-cysteine-HCl (Nishikawa & Kohgo 1985, Taguchi *et al.* 1990, Anonymous 2007) or sodium bicarbonate (Thamaraj & Shah 2003). Antioxidants used in the cultivation of *Salmonella* include catalase
(Rayman et al. 1978), sodium pyruvate (Rayman et al. 1978), and Oxyrase® (Niroomand & Fung 1992, Reissbrodt et al. 2002).

Thermal stress

Thermal stress is caused by a shift in the cultivation temperature. The temperature has been shown to influence both lag-time and growth rate of bacteria (Shida et al. 1975b). The cold and heat tolerance of LAB, as well as their ability to adapt after sudden temperature shifts, varies between species (De Angelis & Gobbetti 2004). According to the literature, the effect of the cultivation temperature on the lag-times and growth rates of Salmonella does not significantly vary between serotypes (Oscar 2000). The recovery from thermal stress may relate to other stress responses. According to Kobayashi and co-workers (Kobayashi et al. 2005), the recovery of Salmonella induces the expression of both heat-inducible and oxidative stress related genes. Therefore, the selection of enrichment medium requires holistic analysis of possible stress factors and their possible interactions.

2.3.6 Detection methods

The performance of enrichment broths is linked to the selected detection method in several ways. The medium components can directly interfere with the detection and therefore the optimal media for the recovery of bacteria might not always be the optimal choice with respect to following analytical steps. PCR is known to be relatively sensitive to interference, and inhibition by medium components, such as agar or antibiotics, has been reported (Gibb & Wong 1998, Faraq et al. 2010). Enrichment medium may also affect the detection by decreasing the concentration of marker molecules in the cells. For example, the cultivation of sub-lethally injured Salmonella in unsuitable medium can cause denaturation of certain antigens (Hahm & Bhunia 2006) and repression of virulence marker genes (Cochrane & O’Connor 2002).

The duration and the number of the enrichment cultivation steps depend on the selectivity and the specificity of the detection method. However, due to the need for detecting low levels of food-borne Salmonella and the non-equal distribution of the cells in the samples almost all rapid test protocols include at least one enrichment cultivation step. For example, PCR methods generally require 8–24 hours of non-selective enrichment cultivation. Some bacteriophage assays are claimed to allow the detection already after six hours of cultivation in
BPW. In case of some foods even the PCR detection directly from the samples may be possible (Stevens & Jaykus 2004b). However, nearly all of the alternative methods validated by AFNOR or NordVal require enrichment cultivation, and take at least 24 hours to complete (Anonymous 2010a, Anonymous 2010c). Despite enrichment cultivation steps, the majority of the alternative detection methods can be completed in less than 48 hours.

In summary, the length of the total detection procedure can be affected by decreasing the enrichment cultivation periods by allowing faster and more efficient recovery, and accelerating the growth of the target cells while suppressing the growth of background microbiota. Various efforts have been made to validate shorter enrichment cultivation procedures and therefore allow faster detection of *Salmonella* in foods. Generally, the use of highly selective detection method may allow the use of less selective enrichment media. However, if the analyzed food carries high concentration of background microbes the use of only non-selective enrichment media creates a risk of outcompeting of the target bacteria.

Despite active reporting on novel enrichment cultivation media or techniques for detection of *Salmonella*, the majority of the reported studies focus on novel media for the second, selective or indicative enrichment cultivation, while the non-selective enrichment cultivation is still usually carried out in BPW (Table 5). This is probably because the use of commonly accepted enrichment procedure is safe and allows saving research and development resources.
3 Summary of the material and methods

An overview of the materials and methods used in this study is given below. Detailed descriptions are presented in the original articles according to Table 6.

3.1 Bacterial strains and preparation of injured cells

The type strains of LAB were obtained from the German Collection of Microorganisms (Braunschweig, Germany). Beer-spoiling LAB strains isolated from a Finnish brewery was purchased from VTT Technical Research Centre of Finland, Culture Collection (Espoo, Finland). *Salmonella* Typhimurium was a food isolate strain kindly provided by Dr. Antje Breitenstein (Scanbec GmbH, Halle, Germany). The beer-spoiling LAB were adapted to beer for a minimum of three days prior to the experiments. Injured *Salmonella* cells were prepared at temperatures of 55 °C (Feng et al. 2007), 4 °C (Yuk & Schneider 2006) or – 20 °C (Wu et al. 2001).

3.2 Enzyme controlled glucose delivery system

The glucose delivery system used for the cultivation of LAB was based on a two-phase gel and glucoamylase controlled release of glucose (Panula-Perälä et al. 2008). For cultivation of *Salmonella*, the soluble carbon source format of the same system was used (Krause et al. 2010). The reagents for both EnBase® applications were purchased from BioSilta Oy, Oulu, Finland.

3.3 Experimental design and variable analysis

The experimental design and variable analysis were done by means of RSM using MODDE 8 software for Design of Experiments and Optimization (UMETRICS AB, Umeå, Sweden)(Eriksson et al. 2000). A full factorial design was used for the screening experiments. The RSM was done with face centered central composite design (CCF) in case of only quantitative factors. D-optimal design was used for the RSM with both qualitative and quantitative factors. The data was fitted using multiple linear regressions (MLR). The analysis of variance (ANOVA) of the models was done using F-test.
3.4 Growth curves and definition of lag-times and growth rates

The growth curves of the cultures based on optical densities at 490 nm (\(\text{OD}_{490}\)) were fitted into the bacterial growth model described by Baranyi and Roberts (1994) using DMFit software (Baranyi & Tamplin 2004). The corresponding lag-times and maximum growth rates were calculated by the software.

3.5 Measurement of rRNA by means of SHA

Oligonucleotide probes targeting highly conserved 16S rRNA in LAB were designed using the ARB software package (Ludwig et al. 2004). The specificity of probes was checked in silico using the program “Probe Match” provided by the Ribosomal Database Project II (RDP II, http://rpd.cme.msu.edu/index.jsp) (Cole et al. 2003) and in the EMBL database using the NCBI Blast2 alignment tool (Altschul et al. 1997). *Salmonella enterica* 23S rRNA targeting oligonucleotide probes were designed using the CloneManager5 software (Scientific & Educational Software, Cary, NC) and their specificity was checked in silico using BLAST database (Altschul et al. 1990) and in SHA against *E. coli* cell extracts. The rRNA for the SHA was constructed from the corresponding PCR amplified rRNA genes by means of T7 RNA polymerase in vitro transcription using the MAXIscript™ T7-kit (Ambion, Austin, TX, USA). The rRNA concentrations were measured by means of SHA described by Rautio et al. (2003) using either fluorescent or electric signal detection.

3.6 Food and process samples

Brewer’s yeast slurries were aseptically collected from the bottom of fermentation tanks in 50 mL bottles. In standard cultivation-based detection 200 \(\mu\)L of each sample were incubated on Schwarz differential agar (SDA) and inoculated into MRS broth (Oxoid MRS Broth, Fisher Scientific International, Basingstoke, UK) containing 25% (v/v) of beer and 0.01 g per L of cycloheximide. The samples from the beer can seamer were taken either by swabbing, or by processing a can containing sterile Ringer solution through the seaming device. The beer samples were vacuum filtered through membrane discs with a pore size of 0.45 \(\mu\)m and a diameter of 47 mm (EZ-Pak Sterile Membrane Filters, Millipore Oy, Espoo, Finland). Minced meat samples were prepared using Finnish commercial minced meat and spiked with heat injured *Salmonella* cells.
Table 6. Summary of the methods used in this study. For more detailed information, see the original articles.

<table>
<thead>
<tr>
<th>Objective</th>
<th>Method</th>
<th>Study</th>
</tr>
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<tbody>
<tr>
<td>Cultivation of LAB</td>
<td>Microaerophilic cultivation in B-MRS</td>
<td>I-IV</td>
</tr>
<tr>
<td>Cultivation of <em>Salmonella</em></td>
<td>Aerobic cultivation in BPW or RVS</td>
<td>V</td>
</tr>
<tr>
<td>Monitoring of growth</td>
<td>OD$_{490}$ measurement</td>
<td>II-V</td>
</tr>
<tr>
<td>Calculation of cells per mL</td>
<td>Agar plating on selective media</td>
<td>II-V</td>
</tr>
<tr>
<td>Growth curve fits</td>
<td>DMFit software</td>
<td>IV-V</td>
</tr>
<tr>
<td>RSM</td>
<td>MODDE8 software</td>
<td>III-V</td>
</tr>
<tr>
<td>rRNA detection</td>
<td>SHA</td>
<td>I-V</td>
</tr>
</tbody>
</table>
4 Summary of results and discussion

4.1 Improved enrichment cultivation of beer-spoiling LAB (Studies I-IV)

4.1.1 The role of enrichment cultivation in 16S RNA SHA detection of beer-spoiling LAB

The first aim of this study was to assess the enrichment cultivation of beer-spoiling LAB with respect to detection by means of rRNA-based SHA. For this purpose, 16S rRNA-based SHA was applied for the detection of beer-spoiling LAB in actual pitching yeast and beer samples in a Finnish lager brewery (52 samples). The detection procedure of SHA included the filtration of beer samples, inoculation of samples into B-MRS broth, over-night incubation at 29±1 °C, harvesting of the cells by means of centrifugation, and SHA. The results of SHA were compared to simultaneous detection by cultivation (reference method). Group specific oligonucleotide probes targeting several Lactobacillus and Pediococcus strains were used in the SHA (Study I, Table 3). In comparison to the reference method, 10 false negative and 8 false positive results were detected in SHA (Study I, Fig. 4). The relative specificity and sensitivity of detection of SHA were 85 and 81%, respectively.

The false results of SHA may originate from any of the steps in the detection procedure, or from the failure in the reference method. False positive results in SHA may originate from the detection of slow-growing LAB that were not recovered during the cultivation (Storgårds et al. 1998, Suzuki et al. 2008b). Since the used oligonucleotide probes are specific to certain groups of Lactobacillus and Pediococcus, while several other LAB may be encountered at the breweries (O'Sullivan et al. 1999), it is possible that some false negative results were due to detection of other LAB in the reference method. Furthermore, some of the positive results in the reference method may originate from the poor selectivity and thereby detection of bacteria other than LAB (Storgårds et al. 1998).

It was also concluded that too short enrichment cultivation could be a major origin of false negative results. Therefore, 16 more beer samples were analyzed by means of SHA by using up to 144 hours of enrichment cultivation in B-MRS broth (unpublished data). The 16S rRNA concentrations in the cultures were
measured in 12 hour intervals using oligonucleotide probes targeting a large group of beer-spoiling LAB (Study III). After 144 hours of enrichment cultivation, 5 false positive results and no false negative results were detected in SHA in comparison to the reference method (Table 7). Accordingly, the specificity and the sensitivity of SHA were 29 and 100%. Enrichment cultivation intervals needed for positive detection in SHA ranged from 36 to 144 hours. The specificity of the SHA decreased and the sensitivity increased when the enrichment cultivation was prolonged (Fig. 1). However, this may be due to poor performance of the reference method. It is possible, that the prolonged enrichment cultivation prior to SHA allowed recovery and detection of fastidious LAB strains, that were not recovered in the cultivation-based detection (Jespersen & Jakobsen 1996, Storgårds et al. 1998, Suzuki et al. 2008a).

Table 7. The results of the detection of LAB in brewery samples by means of SHA and reference method (unpublished data). Additionally, the enrichment cultivation intervals needed for positive detection of LAB in brewery samples by means of 16S rRNA SHA are presented. Pos – positive result; Neg – negative result.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference method</th>
<th>SHA</th>
<th>Enrichment cultivation [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pos</td>
<td>Pos</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>Pos</td>
<td>Pos</td>
<td>144</td>
</tr>
<tr>
<td>3</td>
<td>Neg</td>
<td>Neg</td>
<td>144</td>
</tr>
<tr>
<td>4</td>
<td>Pos</td>
<td>Pos</td>
<td>120</td>
</tr>
<tr>
<td>5</td>
<td>Pos</td>
<td>Pos</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Pos</td>
<td>Pos</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>Neg</td>
<td>Pos</td>
<td>144</td>
</tr>
<tr>
<td>8</td>
<td>Neg</td>
<td>Pos</td>
<td>72</td>
</tr>
<tr>
<td>9</td>
<td>Pos</td>
<td>Pos</td>
<td>72</td>
</tr>
<tr>
<td>10</td>
<td>Pos</td>
<td>Pos</td>
<td>72</td>
</tr>
<tr>
<td>11</td>
<td>Neg</td>
<td>Pos</td>
<td>96</td>
</tr>
<tr>
<td>12</td>
<td>Neg</td>
<td>Pos</td>
<td>48</td>
</tr>
<tr>
<td>13</td>
<td>Pos</td>
<td>Pos</td>
<td>48</td>
</tr>
<tr>
<td>14</td>
<td>Pos</td>
<td>Pos</td>
<td>96</td>
</tr>
<tr>
<td>15</td>
<td>Neg</td>
<td>Pos</td>
<td>72</td>
</tr>
<tr>
<td>16</td>
<td>Neg</td>
<td>Neg</td>
<td>144</td>
</tr>
</tbody>
</table>
Based on the present results, the decision concerning the duration of enrichment cultivation is critical for the 16S rRNA-based detection of beer-spoiling LAB. Enrichment cultivation for 48 hours or less may be enough for the detection of LAB by means of bioluminescence measurement (Takahashi et al. 2000), immunoassay (Yasui & Yoda 1997), PCR (Juvonen et al. 1999) or FISH (Thelen et al. 2002). However, those methods are more sensitive than SHA; the detection limit of SHA is approximately $10^6$ cells per assay (Study I), while for example the detection limit of PCR can be as low as 10 cells per assay (Juvonen & Haikara 2009).

In order to shorten the enrichment cultivation period, the lag-time of LAB should be minimized, and the growth rate maximized. The concentration of 16S rRNA in LAB cells has been shown to depend on the growth phase (de Vries et al. 2004). Therefore, the accelerated growth might also increase the concentration of target molecules per cell. Based on the literature (see chapter 2.3), the possibilities to improve the recovery are to i) adjust the temperature in order to reduce thermal stress; ii) decrease the salt and sugar concentrations in order to reduce osmotic stress; iii) decrease the pH in order to reduce alkaline pH stress; and iv) add antioxidants in the medium in order to reduce oxidative stress.

Additionally, the growth rate of LAB may be increased by suppressing the growth of other bacteria in the enrichment culture, and improving the accessibility of LAB to growth factors (Enfors et al. 2001). In order to improve the accessibility to nutrients, enzyme controlled glucose delivery described by Panula-Perälä and co-workers may be useful (2008). Shaking of cultures allows equal distribution of growth factors in the culture, although it can also increase
the diffusion of oxygen to the media and therefore accelerate the production of reactive oxygen species. The effects of different stress factors on growth of beer-spoiling LAB, as well as possibilities to reduce them and improve the recovery and growth rate of beer-adapted cells are discussed in the following chapters.

4.1.2 Reduced thermal and alkaline pH stress

The thermal stress due to elevated cultivation temperature may extend the lag-time of LAB in enrichment cultures. Therefore, the effect of varying the cultivation temperature between 25 and 30 °C on the growth of beer-spoiling LAB was studied by means of RSM. The elevation of the temperature affected both lag-times and maximum growth rates in the cultures of beer-spoiling *P. damnosus* (Study IV, Fig. 2). The elevated temperature extended the lag-time with approximately 12 hours.

Besides thermal stress, the LAB transferred from brewery samples into B-MRS may experience alkaline pH stress due to the elevated pH. The effect of alkaline pH stress on the recovery of *P. damnosus* was therefore also studied by means of RSM. Similarly to the elevated temperature, the pH in B-MRS broth extended the lag-times in the cultures (Study IV, Fig. 2). The effect of pH on the lag-time of *P. damnosus* was some hours more compared to the effect of temperature. However, unlike elevated pH, the elevated temperature affected also the maximum growth rates in the cultures, and therefore the effect of cultivation temperature on the OD$_{490}$ was larger than that of the pH (Study IV, Fig. 1).

The enrichment cultivation of beer-spoiling LAB commonly takes place at 29±1 °C. However, the present results suggest that this temperature is too high for the efficient recovery of all the relevant strains. The cultivation temperature has been shown to affect the lag-time of bacteria especially in the low cell concentrations (Smelt & Haas 1978, Robinson *et al.* 2001). Therefore, the effect of the temperature on the recovery of beer-spoiling LAB is likely more prominent when actual samples with very low cell concentrations are used.

The present results suggest the use of pH 5.2 for the cultivation of *P. damnosus*. This is in agreement with the literature, indicating that the pH around 5.5 or even higher in the commonly used B-MRS broth may cause extensive lag-times of LAB in the enrichment cultivation and therefore affect their detection (Jespersen & Jakobsen 1996, Satokari *et al.* 2000, Carr *et al.* 2002, Suzuki *et al.* 2008b). Besides reducing the alkaline pH stress, the use of low pH suppresses the growth of bacteria not able to spoil beer, and thereby acts as a selection step in the
enrichment cultivation (Behr et al. 2006). In the future, the actual effect of the adjusted cultivation conditions will be tested with respect to the relevant microbiota and preferably using actual brewery samples.

4.1.3 Reduced oxidative stress

The oxidative stress of bacteria is caused by reactive oxygen species. Since most LAB are catalase-negative, their oxidative stress may be reduced by adding antioxidants in the enrichment medium. In this study, L-cysteine-HCl and sodium bicarbonate were used as antioxidants in the cultivation of beer-spoiling LAB. L-cysteine-HCl was selected, since it is widely used in the cultivation of LAB (Taguchi et al. 1990) as well as other bacteria (Stark et al. 1994). Sodium bicarbonate is used in the storage of probiotic LAB (Tharmaraj & Shah 2003), and it was therefore thought to improve the recovery of beer-spoiling LAB as well.

As could be expected, the addition of L-cysteine-HCl improved the growth of LAB (Study III, Fig. 2; Study IV, Fig. 4). The suggested concentration of L-cysteine-HCl was 0.25 g per L, which is close to those recommended in the literature (Taguchi et al. 1990, Anonymous 2007). The use of sodium bicarbonate was beneficial for the growth of L. backi and L. brevis, (Study III, Fig. 2–3) but did not affect the growth of P. damnosus (Fig. 2). The proposed optimal concentration of sodium bicarbonate within the studied range is 4 g per L.
Fig. 2. The effects of antioxidants on the growth of beer-spoiling *P. damnosus* (unpublished data). The scaled effect of each model term with 95% confidence level is presented. The effect is statistically significant if the confidence intervals do not cross the x-axis. X-axis: L-cys - L-cysteine-HCl; Na-c - sodium bicarbonate.

The addition of 0.25 g per L of L-cysteine-HCl and 4 g per L of sodium bicarbonate in B-MRS broth improved the growth of *L. backi* and *L. brevis* in single strain cultures (Study III, Fig. 3). The effect could be seen in the measured OD both after over-night incubation and at the end of cultivation. The use of antioxidants did not affect the lag-times of the cultures, but the growth rates were enhanced for both species (Fig. 3). The growth in the cultures supplemented with antioxidants continued for longer time than in cultures with plain B-MRS. This suggests that the benefit from the addition of antioxidants can continue for several days, and therefore also enhances the growth of slowly recovering strains.
Fig. 3. Effect of supplementing B-MRS with L-cysteine-HCl and sodium bicarbonate on lag-times (A) and maximum growth rates (B) of *L. backi* (grey columns) and *L. brevis* (white columns). The average values and standard deviations for three independent cultures are presented. X-axis: 1 – plain B-MRS; 2 – B-MRS supplemented with L-cysteine-HCl and sodium bicarbonate.

The applicability of B-MRS supplemented with antioxidants was further tested in the enrichment cultivation of LAB from the actual brewery samples prior to SHA-based detection (Study III, Fig. 4). In the majority of the samples, the measured concentrations of 16S rRNA were higher when plain B-MRS was used. However, for the samples with the highest cell densities, the use of antioxidants improved the growth. Considering the lag-times of LAB in the single strain cultures (Fig. 3), as well as the measured 16S rRNA concentrations (Study III, Fig. 4), the use of antioxidants may not necessarily speed up the adaptation of LAB in B-MRS broth. However, their use seems to increase the growth rate in cultures, which contributes to higher concentration of cells after for example 48 hours of enrichment cultivation in comparison to plain B-MRS. Therefore, also the accuracy of SHA could be improved by addition of antioxidants. Some LAB are also known to be able to utilize oxygen and degrade reactive oxygen species (Götz *et al.* 1980a, Götz *et al.* 1980b). The presence of such strains could explain the variability in the effect of antioxidants.

The beneficial effect of antioxidants onto the cultivation of bacteria has been reported in several studies. Besides L-cysteine-HCl and sodium bicarbonate, various other antioxidants may be used for the improved cultivation of beer-spoiling LAB. These include for example Oxyrase® (*Ordonez et al.* 2000), catalase (*An et al.* 2010a), ascorbic acid (*Elli et al.* 2002), and thiol compounds (*Elli et al.* 2002). Probably the optimal combination of the antioxidants depends
on the bacterial strain; therefore the screening of the most suitable ones with respect to specific application is advisable.

4.1.4 Reduced osmotic stress

The effect of reduced osmolarity on the recovery of beer-spoiling LAB was studied by adjusting the composition of enrichment broths. Firstly, the effect of initial glucose concentration was demonstrated in cultivation of *L. backi*, *L. brevis* and *L. lindneri* in B-MRS with 0.1-2.0% glucose (Fig. 4). Based on the growth curves, the common B-MRS that contains 2.0% glucose would be the most suitable for the cultivation of these strains. In average, the shortest lag-times were achieved in B-MRS with 1.0% glucose, and the highest growth rates in 2.0% glucose. The effects of glucose concentration on the lag-times or maximum growth rates in the cultures were not statistically significant (in both cases *p* > 0.1, unpublished data). However, earlier it has been shown that especially injured bacteria benefit from the use of medium with low osmolarity for the recovery (Gomez & Sinskey 1973, Gomez *et al.* 1973). Therefore, the decreased concentration of sugars and salts could allow faster recovery of stressed LAB present in the actual brewery samples (Suzuki *et al.* 2006a, Suzuki *et al.* 2008b).

![Fig. 4. The growth of beer-spoiling LAB in B-MRS broth containing 0.1–2.0% glucose (unpublished data). The average growth curves (A), lag-times (B) and maximum growth rates (C) for the three strains in B-MRS containing 0.1 (%) 0.5 (●), 1.0 (○) or 2.0 (★) % glucose are presented. The cultures were inoculated with ca. 100 cells of *L. backi*, *L. brevis* or *L. lindneri*. The error bars present the standard deviations between the three strains.](image)

In order to facilitate the growth of beer-spoiling LAB, the composition of B-MRS broth was adjusted by means of RSM, using beer-spoiling *P. damnosus* as the test
strain. The tested concentrations of beer and dehydrated MRS powder (Difco) were selected between the concentrations that are used in ABD (Suzuki et al. 2008b) and B-MRS (Holzapfel 1992). Accordingly, the concentrations of MRS powder from 2.6 to 55 g per L and beer from 25 to 100% were used. The concentration of dehydrated MRS powder (Difco) showed the largest effect on the measured OD after 96 hours of cultivation (Fig. 5). Based on the RSM, the optimal concentration of MRS powder is lower than what is recommended by the manufacturer (Study IV, Fig. 4). Varying the concentration of beer from 25% to 100% (v/v) did not affect the growth of P. damnosus.

![Fig. 5. The effects of concentrations of compounds in the B-MRS broth on the growth of beer-spoiling P. damnosus (unpublished data). The scaled effect of each model term with 95% confidence level is presented. The effect is statistically significant if the confidence intervals do not cross the x-axis. X-axis: MRS – Difco dehydrated MRS powder; L-Cys - L-cysteine-HCl.](image)

The adjusted B-MRS broth containing 25% of beer, and 40 g per L of Difco MRS powder was tested in the cultivation of P. damnosus (0). The difference between common and adjusted B-MRS broths was minor in terms of measured OD, lag-time and maximum growth rate.
Fig. 6. The growth of beer-spoiling *P. damnosus* in B-MRS broth containing different concentrations of beer and MRS powder (unpublished data). The average growth curves for three independent cultures incubated in adjusted (●) or common (○) B-MRS broth are presented in A. Average lag-times (grey columns) and maximum growth rates (white columns) for the three independent cultures are presented in B. The error bars present the standard deviations for three series. X-axis: 1 – common B-MRS; 2 – B-MRS with adjusted concentrations of beer and MRS powder.

The effect of reduced osmolarity on the enrichment cultivation of beer-spoiling LAB in actual brewery samples was demonstrated by comparing the efficiencies of B-MRS and ABD (Suzuki et al. 2008b) broths prior to 16S rRNA-based SHA. The use of ABD medium, that contains relatively low concentrations of salts and sugars, did not allow faster SHA detection of LAB in the actual brewery samples (Study III, Fig. 1). The specificities and sensitivities of SHA using both media were 80 and 100%, respectively (unpublished data). Based on the present results, the effect of osmolarity on the growth of beer-spoiling LAB varies between different strains. Although, direct effect on the detection procedure was not achieved, the reduced osmolarity might improve the detection of some fastidious LAB. The use of media with low osmolarity could therefore be beneficial in for example trouble-shooting cases, for tracking of certain slowly growing contaminants.

4.2 The effect of enzyme controlled glucose delivery

The accessibility to nutrients is one of the factors affecting the growth of bacteria. However, as was discussed earlier, the initially high concentration of nutrients
may suppress the recovery of stressed cells. In order to improve the accessibility to nutrients without increasing the osmolarity of the medium, enzyme controlled glucose delivery system developed by Panula-Perälä and co-workers (2008) was applied for the cultivation of beer-spoiling LAB in B-MRS broth.

The feasibility of the gel-based glucose delivery system was demonstrated in cultivation of L. brevis type strain in B-MRS (Study II, Fig. 1). The use of glucose delivery increased the measured OD after 24 hours of incubation, as well as at the end of cultivation (Study II, Fig. 3). However, the effects on lag-times and growth rates were not statistically significant (Fig. 7). These results support the findings of other studies, suggesting that the glucose supply allows increasing the final cell density by controlled growth rather than accelerates the growth rates in the bacterial cultures.

Fig. 7. The effect of glucose delivery on lag-times and growth rates of L. brevis type strain in B-MRS (unpublished data). Average lag-times (grey columns) and maximum growth rates (white columns) for three independent cultures are presented. The error bars present the standard deviations of three cultures. X-axis: 1 – B-MRS; 2 – B-MRS with gel-based glucose delivery and 1,000 units per L enzyme.

Due to the positive effect on growth of the L. brevis type strain, the glucose delivery system was also applied to cultivation of beer-spoiling LAB strains (Study II, Fig. 2). With the exception of slow-growing strain L. malefermentans, the growth of beer-spoiling Lactobacillus increased in proportion to the increased enzyme concentration. Similarly the growth of P. damnosus was improved by the use of glucose delivery (Fig. 8). The initial glucose concentration did not affect the final OD.
Fig. 8. The effect of glucose delivery on the growth of beer-spoiling *P. damnosus* (unpublished data). The scaled effect of each model term with 95% confidence level are presented. The effect is statistically significant if the confidence intervals do not cross the x-axis. X-axis: Enz – enzyme in glucose delivery system; Glu - glucose.

The glucose delivery system was applied at brewery quality control for cultivation of LAB in actual samples. The use of B-MRS with glucose delivery resulted in higher OD in the enrichment cultures (Study II, Fig. 4). In further tests, the use of glucose delivery also directly contributed to shorter total detection time by means of SHA (Study III, Fig. 5). The supplementing of the B-MRS broth with the glucose delivery system increased the sensitivity of SHA from 63 to 88%, and decreased the specificity from 100 to 67% (unpublished data). The increased sensitivity and specificity could be due to recovery of contaminants that were not recovered in plain B-MRS.

The use of the glucose delivery system is also likely to support the growth of bacteria other than LAB in the samples (Panula-Perälä et al. 2008). Therefore, the medium that is used with it should be selective enough to suppress the overgrowth of LAB by fast-growing background microbiota. The concentration of enzyme applied for the cultivation of LAB was several orders of magnitude higher than what is recommended for *E. coli* in the literature (Panula-Perälä et al. 2008). This may be explained by the different applications of the glucose delivery system; the reported uses for high cell density cultivations of *E. coli* are based on avoiding the anaerobic over-flow metabolism, which may lead to accumulation of side metabolites and decrease the pH of the medium (Panula-Perälä et al. 2008, Krause et al. 2010). However, especially beer-spoiling LAB are tolerant to low pH, which allows growth despite the accumulated acidic metabolites. For slow-growing strains, too high concentration of enzyme might result in the
accumulation of glucose in the culture during the lag-time, and even in the suppression of growth (Panula-Perälä et al. 2008). Therefore, the enzyme concentration in the system should be separately optimized with respect to the studied bacteria.

4.3 Improved enrichment cultivation of heat-injured Salmonella (Study V)

4.3.1 Enrichment cultivation in 23S rRNA SHA

In order to assess the enrichment cultivation of food-borne Salmonella, SHA with rRNA-targeting oligonucleotide probes was applied. Due to the similarity of 16S rRNA sequences within different enterobacteria (Lin & Tsen 1996, Christensen et al. 1998), the probes were targeted to S. enterica 23S rRNA (Study V, Table 1). The detection limit of the SHA was approximately $10^6$ cells per mL (unpublished data). Despite the relatively low sensitivity, SHA was chosen for the study due to its flexible detection system that allows various different applications with minor modifications (see chapter 1.1). Minced meat was used as sample matrix due to its high concentration of background microbiota. The SHA was not affected by the interference of sample matrix (Study V, Fig. 5).

Due to the low concentration of Salmonella in foods, and the relatively high detection limit of the SHA, the applied method would not allow the detection of Salmonella directly in food samples. Even if the cell concentration would be high enough, the partial degradation of ribosomal DNA and RNA in Salmonella cells due to sub-lethal heat injury could falsify the results of SHA (Deutscher 2009, Chen & Deutscher 2010). Therefore, the measurement of Salmonella 23S rRNA without enrichment cultivation is not feasible.

The detection of Salmonella 23S rRNA directly from the non-selective enrichment culture may be possible. However, the concentration of 23S rRNA in the Salmonella cells has been shown to decrease rapidly when the cells reach the stationary growth phase (Hsu et al. 1994). Since the growth rate of Salmonella cells depends on a variety of unknown factors, such as the lag-time of the cells or the growth of background microbiota, it is theoretically possible that Salmonella cells are already in stationary phase after over-night incubation. Therefore, the use of a selective enrichment cultivation step prior to SHA is advisable.
The duration of the non-selective cultivation step could possibly be shortened by several hours from the commonly used 18–24 hours by optimizing the growth conditions. However, the over-night incubation assures the sufficient recovery of *Salmonella* cells, and thereby allows them to survive in the selective conditions (Chen *et al.* 1993). Moreover, the adaptation time needed for the sufficient recovery of injured cells cannot be accurately predicted (Beckers *et al.* 1987), and therefore shortening of the non-selective enrichment cultivation may lead to a failure in the detection. Considering the workflow in the industrial laboratories, it is probably of minor relevance whether the enrichment cultivation takes 18 or 24 hours, since the analysis is not processed during the night. In the future, the use of automated detection systems that would proceed with the samples during the night could perhaps change this situation. Generally, the over-night cultivation is also practical due to its flexibility; the samples taken at different times during the day are basically ready for the analysis in the morning at the same time. Based on the discussed reasons, the non-selective over-night incubation followed by short incubation in selective broth may be the most practical choice for the enrichment cultivation procedure prior to SHA. However, the efficiency of the enrichment cultivation steps could possibly be improved by means of medium optimization.

The improved growth of *Salmonella* in non-selective medium can be achieved by either facilitating the recovery of injured cells, or by accelerating the growth after recovery. The addition of nutrients or other growth factors into non-selective medium might affect both recovery and growth of *Salmonella* (Bailey & Cox 1992). However, the selection of suitable initial glucose concentration is somewhat complex; although *Salmonella* tolerate high osmolarity better than many other bacteria, the provided glucose may actually end up accelerating the growth of fast-recovering background microbiota. Furthermore, the use of complex medium might suppress or extend the lag-times in the cultures (Clark & Ordal 1969, Gomez & Sinskey 1973, Gomez *et al.* 1973). Therefore, the slow release of nutrients into the culture could be beneficial. The enzyme controlled glucose delivery system (Panula-Perälä *et al.* 2008) that was earlier in this study successfully applied for the cultivation of LAB is a potential candidate for the nutrient supply.

Besides adding more nutrients to the medium, the growth may be improved by increasing the selectivity of conditions. Since the addition of selective agents into the recovery medium is not advisable due to possible inhibitory effect against stressed *Salmonella* cells, the suitable possibilities include the suppression of background microbes by means of inhibitory compounds, such as bacteriophages.
(Stave & Teaney 2009); the use of Salmonella-specific growth factors (Reissbrodt & Rabsch 1993); the adjustment of the cultivation temperature (Rhodes et al. 1985); and the increase of the buffering capacity.

4.3.2 Improved growth of Salmonella in BPW

In order to improve the growth of Salmonella, BPW broth was supplemented with glucose. Instead of adding the glucose batchwise, it was slowly released by means of enzyme controlled glucose delivery system. The feasibility of glucose delivery for Salmonella was tested in over-night cultivation of viable S. Typhimurium and Citrobacter freundii, which belongs to non-pathogenic background microbiota in meat. Cultures were inoculated with approximately $10^3$ cells of Salmonella or $10^7$ cells of Citrobacter. Gel-based glucose delivery and 3 U per L enzyme were used based on the literature (Panula-Perälä et al. 2008). As was expected, the use of glucose delivery improved the growth of both strains. The measured OD after over-night incubation was doubled when the glucose delivery was used instead of plain BPW (Fig. 9).

![Fig. 9. The growth in single strain cultures of S. Typhimurium and Citrobacter freundii in BPW with and without glucose delivery (unpublished data). The average OD and standard deviations for three independent cultures of Salmonella (grey columns) and Citrobacter (white columns) are presented. X-axis: 1 – plain BPW; 2 – BPW supplemented with gel-based glucose delivery system and 3 U per L enzyme.](image-url)
4.3.3 Improved recovery of injured Salmonella in single strain cultures

After showing the feasibility of glucose delivery in the cultivation of viable Salmonella, it was applied in the recovery of injured cells. Both gel-based and soluble carbon source based glucose delivery systems were tested. Injuring of Salmonella cells was carried out at temperatures of 4, -20 or 55 ºC. In case of cold-injured cells, the highest OD after 18 hours of cultivation was measured in cultures where soluble carbon source based glucose delivery was used (Fig. 10). Heat-injured cells were only recoverable in cultures where glucose delivery systems were used.

Fig. 10. The recovery of stressed S. Typhimurium cells in different BPW broths (unpublished data). White columns present the OD after 18 hours of cultivation. The grey columns present the OD after 24 hours (A and B) or after 48 hours of cultivation. Average values and standard deviations for three independent cultures are presented. The cells were injured at temperatures of 4 ºC (A), -20 ºC (B) or 55 ºC (C). X-axis: 1 – BPW; 2 – BPW + gel-based glucose delivery; 3 – BPW + soluble carbon source-based glucose delivery.

Due to its positive effect on the recovery of injured cells, the soluble carbon source –based glucose delivery system was used in cultivation of heat-injured Salmonella. Based on the literature and this study, the use of glucose delivery is likely to facilitate also the growth of background microbiota in minced meat samples. In order to specifically support the recovery of Salmonella, siderophore ferrioxamine (Reissbrodt et al. 2000) E was added to BPW. Additionally, elevated cultivation temperatures were tested. The use of glucose delivery improved the growth of heat-injured Salmonella in single strain cultures by decreasing the lag-times (Study V, Fig. 2&3) and increasing the maximum growth rates (Fig. 11). As
could be expected, the use of ferrioxamine E was also beneficial for the recovery of Salmonella (Study V, Fig. 2).

In order to minimize the lag-times, the proposed optimal levels of the tested compounds are 15 g per L of carbon source, 6 U per L enzyme, and 0.5 µg per L ferrioxamine E. The suggested cultivation temperature is 40 °C. Independent of the cultivation temperature, the use of BPW with proposed composition resulted in improved growth of heat-injured S. Typhimurium in single strain cultures (Study V, Fig. 4).

Fig. 11. The effect of soluble carbon source-based glucose delivery on growth of heat-injured S. Typhimurium in BPW (unpublished data). The scaled effects of each model term on the maximum growth rates with 95% confidence levels are presented. The effect is statistically significant if the confidence intervals do not cross the x-axis. X-axis: Enz - Enz*I'm, Sub – Soluble carbon source; Temp – temperature; FOE - ferrioxamine E.

The glucose delivery systems have not been used earlier for the cultivation of Salmonella. However, the reported effects on the growth of E. coli are similar to the effects on Salmonella presented here (Panula-Perälä et al. 2008, Krause et al. 2010, Osmekhina et al. 2010). The optimal concentration of the enzyme proposed in this study is also in agreement with earlier studies, suggesting that the use of concentration of 6 U per L would be suitable in order to facilitate the growth in the beginning of the cultivation. Although, the use of elevated temperature did not seem to have an effect in the cultivation of heat-injured Salmonella in single strain cultures (Study V, Fig. 4), it may increase the selectivity of cultivation in
the presence of background microbiota (Rhodes *et al.* 1985, Krascenicsova *et al.* 2006). However, it has to be noted that *Salmonella* serotypes may respond differently to the elevated cultivation temperature, and that the heat-tolerance can also depend on the sample matrix and enrichment medium (Quintavalla *et al.* 2001).

### 4.3.4 Improved recovery of heat-injured *Salmonella* in minced meat

In the previous chapters, the adjustment of BPW was shown to improve the growth of viable *Salmonella* cells, as well as to allow faster recovery of injured cells. Therefore, the effect of supplementing BPW broth with glucose delivery and ferrioxamine E on the SHA detection of *Salmonella* in minced meat was studied. Four different commercial minced meats were used as sample matrices. The samples were spiked with low concentrations of heat-injured *Salmonella* (Study V). Statistically significant difference in the measured 23S rRNA concentrations between plain and supplemented BPW started to show after six hours of cultivation (Study V, Fig. 6).

Although, the growth of *Salmonella* could be facilitated by the use of glucose delivery, the possibility for shorter enrichment cultivation was not studied. Based on the measured 23S rRNA concentrations, four hours of enrichment cultivation was not sufficient for the recovery of heat-injured cells (Study V, Fig. 6). Similar findings have been reported by Zhao and Doyle (2001), who claimed that an incubation time of less than 6 h would not assure sufficient recovery of injured *Salmonella* in foods.

The present results indicate that the recovery of heat-injured *Salmonella*, as well as its detection in food samples may be enhanced by addition of the tested glucose delivery system. The effect of glucose delivery in case of other *Salmonella* strains, or different sample matrices should be tested before the applicability of the system can be evaluated. Additionally, it is advisable to adjust the concentrations of soluble carbon source and enzyme separately for different detection systems. The applied glucose delivery system could also be beneficial in the selective cultivation of *Salmonella*, which possibility is in the scope of further studies.
5 Conclusions

This study has shown the effect of reducing different stress factors on the recovery and growth of food-contaminating bacteria with respect to rRNA-based detection. The results can be used in the evaluation of enrichment cultivation procedures in food diagnostics, and in the development of new cultivation media and techniques. The provided information may be useful in the assessment of bacterial cultivations also in other applications. The main findings of this thesis were as follows.

1. The used SHA is comparable to other alternative detection methods in terms of industrial applicability (i.e. cost-efficiency, hands-on time, and the duration of the assay). The sensitivity of SHA is relatively low compared to e.g. PCR. However, due to low concentration and slow growth of LAB in brewery samples, enrichment cultivation is required also for other alternative detection methods. Therefore, with respect to the total duration of the procedure, the difference in sensitivity is not fundamental.

2. The enrichment cultivation influences the rRNA-based SHA detection of food-contaminating bacteria in several ways. The duration of the cultivation is especially critical with respect to specificity and sensitivity of the detection. In practice, the SHA detection without enrichment cultivation is impossible in case of both beer-spoiling LAB and food-borne Salmonella.

3. In single strain cultures even minor adjustment of the cultivation conditions may reduce the lag-time and increase the growth rate of beer-spoiling LAB. However, similar effect is not necessarily seen in the cultivation of LAB in actual brewery samples. This may be due to complexity of mixed bacterial cultures. The only solution for actually improving the overall quality of detection might be to separately optimize the enrichment cultivation with respect to the relevant sample and bacterial species.

4. Slow release of glucose by means enzyme controlled glucose delivery system improves the growth of beer-spoiling LAB in single strain cultures, and in case of actual brewery samples. The duration of the detection procedure can be decreased by several hours by the use of the glucose delivery.

5. The recovery of injured Salmonella cells was enhanced by using the glucose delivery system together with selective siderophore ferrioxamine E. This effect was seen in reduced lag-times and increased growth rates. The adjusted BPW broth increased the 23S rRNA concentrations also in mixed cultures,
and it may therefore improve the rRNA-based detection of *Salmonella*. The glucose delivery system is one potential tool in the development of more efficient media for enrichment cultivation of food-contaminating bacteria.

6. In order to minimize the necessary resources, the possibility for miniaturization and automation of the enrichment cultivation should be considered. Other proposals for future studies include i) screening for components that would inactivate the growth inhibitors present in the cultures; ii) improved techniques for timed release of medium components by development of biodegradable capsule materials, or methods for monitoring the physiological status in the cultures; and iii) the development of basal media, into which for example selective components could be added in case of tracking specific contaminant sources at the processes.
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Original articles


Supplementary material:


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Original publications are not included in the electronic version of the dissertation.
358. Ylioinas, Jari (2010) Iterative detection, decoding, and channel estimation in MIMO-OFDM
359. Tervonen, Pekka (2010) Integrated ESSQ management. As a part of excellent operational and business management—a framework, integration and maturity
364. Väisänen, Mirja (2010) Communication in high technology product development projects: project personnel’s viewpoint for improvement
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IMPROVED ENRICHMENT CULTIVATION OF SELECTED FOOD-CONTAMINATING BACTERIA